Molecular Microbiology (2007) **64**(3), 719–737 doi:10.1111/j.1365-2958.2007.05684.x

A novel compartment, the 'subapical stem' of the aerial hyphae, is the location of a *sigN*-dependent, developmentally distinct transcription in *Streptomyces coelicolor*

Kate A. Dalton, Annabelle Thibessard,† James I. B. Hunter and Gabriella H. Kelemen*
University of East Anglia, School of Biological Sciences, Norwich NR4 7TJ, UK.

Summary

Streptomyces coelicolor **has nine SigB-like RNA polymerase sigma factors, several of them implicated in morphological differentiation and/or responses to different stresses. One of the nine, SigN, is the focus of this article. A constructed *sigN* null mutant was delayed in development and exhibited a bald phenotype when grown on minimal medium containing glucose as carbon source. One of two distinct *sigN* promoters, *sigNP1*, was active only during growth on solid medium, when its activation coincided with aerial hyphae formation. Transcription from *sigNP1* was readily detected in several *whi* mutants (interrupted in morphogenesis of aerial mycelium into spores), but was absent from all *bld* mutants tested, suggesting that *sigNP1* activity was restricted to the aerial hyphae. It also depended on *sigN*, thus *sigN* was autoregulated. Mutational and transcription studies revealed no functional significance to the location of *sigN* next to *sigF*, encoding another SigB-like sigma factor. We identified another potential SigN target, *nepA*, encoding a putative small secreted protein. Transcription of *nepA* originated from a single, aerial hyphae-specific and *sigN*-dependent promoter. While *in vitro* run-off transcription using purified SigN on the *Bacillus subtilis ctc* promoter confirmed that SigN is an RNA polymerase sigma factor, SigN failed to initiate transcription from *sigNP1* and from the *nepA* promoter *in vitro.* Additional *in vivo* data indicated that further *nepA* upstream sequences, which are likely to bind a potential activator, are required for successful transcription. Using a *nepA–egfp* transcriptional fusion we located *nepA* transcription to a novel compartment, the 'subapical stem' of the aerial hyphae. We suggest that this newly recognized compartment defines an interface between the aerial and vegetative parts of the *Streptomyces* colony and might also be involved in communication between these two compartments.**

Accepted 3 March, 2007. *For correspondence. E-mail g.kelemen@uea.ac.uk; Tel. (+44) 1603 593288; Fax (+44) 1603 592250. †Present address: University Henri Poincare, Laboratoire de Génétique et Microbiologie, Nancy, France.

Introduction

Morphological development in *Streptomyces coelicolor* is initiated by germination of the unigenomic spores and followed by the formation of long, multigenomic and branching hyphal filaments that generate a dense network of vegetative hyphae growing on the surface and into the solid medium. A major step in the progression of *Streptomyces* development is the formation of hyphal branches that emerge into the air and later develop into chains of unigenomic spores (Kelemen and Buttner, 1998; Chater and Horinouchi, 2003; Elliot and Talbot, 2004). Although the transition from vegetative to aerial growth is a widely studied topic, little is known in detail about what triggers this process. Nutrient limitation, encounters with stresses and intercellular signalling are all considered as cues that prompt development of the aerial hyphae. Mutants that fail to make aerial filaments lack the fluffy appearance of the wild-type *Streptomyces* colony and exhibit a 'bald' phenotype (Hopwood, 1967; Merrick, 1976). Many such *bld* genes have been cloned and characterized, and they include three main functional classes: regulatory genes (the major class); genes involved in signalling; and genes encoding structural elements of the aerial hyphae (Kelemen and Buttner, 1998; Chater, 2001).

An intriguing characteristic of several pairs of these *bld* mutants is that they exhibit the phenomenon called 'extracellular complementation' by restoring aerial development, in a unidirectional manner, when grown in close proximity on rich media (Willey *et al.*, 1991; 1993; Kelemen and Buttner, 1998). The cascade of *bldJ*→*bldK*→*bldA*/*bldH*→*bldG*→*bldC*→*bldD* has been established, where each mutant acts as a donor and restores aerial development of the mutants to its left. Because this 'complementation' did not require direct contact of the mutants, but reflected a 'co-synthesis' of the type originally described for mutants blocked in antibiotic

biosynthetic pathways (Delic *et al*., 1969), it was suggested that a cascade of at least five extracellular signals are transmitted to finally produce SapB, a morphogenetic surfactant (Willey *et al*., 1991; 2006). The signals themselves are uncharacterized, except for a small, 655 Da, serine- and glycine-containing oligopeptide shown to induce aerial development of *bldJ* in a *bldK*-dependent manner (Nodwell and Losick, 1998). Although the structure of this oligopeptide has not been determined, it was suggested that, because it is taken up by the BldK oligopeptide permease, it might in fact be signal 1 of the cascade shown above. Another signalling molecule with a different chemical structure, the γ-butyrolactone A-factor, plays a major role in both morphological differentiation and antibiotic production of *Streptomyces griseus* as reviewed in Chater and Horinouchi (2003). In *S. coelicolor*, the role, if any, of the family of γ-butyrolactones in aerial development is less clear. However, *adpA* of *S. coelicolor*, encoding a homologue of the A-factor-dependent central regulator of *S. griseus*, has been shown to be identical to *bldH*, a member of the *bld* signalling cascade (Chater and Horinouchi, 2003; Takano *et al*., 2003).

Not all *bld* mutants fit into the hierarchy of the signalling cascade. This applies not only to some of the original *bld* mutants, *bldB* and *bldF*, but also to mutants in the adenylate cyclase gene, *cya* (Susstrunk *et al*., 1998) and mutants in the recently reported *dasR*, encoding a GntR-type regulator involved in *N*-acetylglucosamine metabolism (Rigali *et al*., 2006), both of which exhibit bald phenotypes. In addition, carbon utilization of several of the *bld* mutants is de-regulated (Pope *et al*., 1996), suggesting that progress of the developmental programme is closely linked to the general physiology of the mycelium.

Recently, significant developments were made in our understanding of the physical requirements for the emergence of hyphal filaments into the air during colony development. The structure of SapB, exogenous application of which restored aerial development to all the *bld* mutants of the cascade (Willey *et al*., 1991; 1993), has been solved and revealed a lantibiotic-like modified oligopeptide (Kodani *et al*., 2004). In addition to SapB, two new classes of proteins, the rodlins and chaplins, were shown to be part of a hydrophobic sheath covering the aerial hyphae and hence the spores. Although mutations in the rodlins did not affect development of the *S. coelicolor* colony, multiple knockouts of the chaplin family resulted in a bald phenotype (Claessen *et al*., 2002; 2003; 2004; Elliot *et al*., 2003a; Elliot and Talbot, 2004).

Once aerial hyphae have emerged, the commitment to sporulation requires two identified RNA polymerase sigma factors: WhiG controls the initiation of sporulation (Mendez and Chater, 1987; Chater *et al*., 1989; Kelemen *et al*., 1996) and SigF (Potuckova *et al*., 1995; Kelemen *et al*., 1996) governs late sporulation events, including control of the *whiEp2* promoter for production of the grey spore pigment (Kelemen *et al*., 1998). SigF belongs to a family of nine sigma factors [SigF, G, H, I, J=B, K, L, M, N (Kelemen *et al*., 2001; Lee *et al*., 2004a)], all highly homologous to SigB of *Bacillus subtilis*. SigB is the central regulator of stress response in *B. subtilis*, controlling more than 100 genes, and is itself regulated by a complex mechanism including an antagonist, anti-sigma factor, RsbW, and an anti-anti-sigma factor, RsbV (reviewed in Price, 2000).

Although SigF of *S. coelicolor* has not been implicated in any form of stress response, several other members of the SigB-like sigma family have been shown to be involved both in morphogenesis and in responses to specific stresses, suggesting a connection between stress and development in *S. coelicolor*. Transcription of *sigH* was shown, on the one hand, to be induced by heat, ethanol and osmotic stresses, and on the other hand to be directly targeted by BldD, a developmental repressor (Kelemen *et al*., 2001; Sevcikova *et al*., 2001; Viollier *et al*., 2003). Production of another member of the SigB-like sigma family, SigB [= SigJ (Kelemen *et al*., 2001; Viollier *et al*., 2003)], was induced during osmotic stress (Cho *et al*., 2001; Viollier *et al*., 2003) and a constructed *sigB* mutant was also impaired in formation of aerial hyphae when grown on solid medium with a high osmolite concentration (Cho *et al*., 2001).

Interestingly, one of the nine SigB-like sigma factor genes, *sigN*, is located immediately downstream of *sigF* on the chromosome of *S. coelicolor*, raising the possibility that the biological role of *sigN* is connected to that of *sigF*. Two sporulation specific sigma factors, SigE and SigG, are encoded by adjacent genes, *spoIIGB* and *spoIIIG*, on the *B. subtilis* chromosome (Masuda *et al*., 1988; Karmazyn-Campelli *et al*., 1989). While SigE controls early sporulation in the mother cell, SigG, expression of which depends on SigE, directs transcription in the spore compartment at late stages of sporulation (reviewed in Errington, 1993).

Here we address the biological role of *sigN* and its potential link to *sigF* in *S. coelicolor.* In doing so we discovered that SigN was involved in controlling aerial development and we have defined, for the first time, a novel basal compartment, the 'subapical stem', located at the interface between the aerial hyphae and the vegetative mycelium.

Results

A sigN *null mutant is conditionally defective in development*

In order to identify the role of *sigN* we generated a *sigN* knockout mutant using PCR targeting (Gust *et al*., 2003).

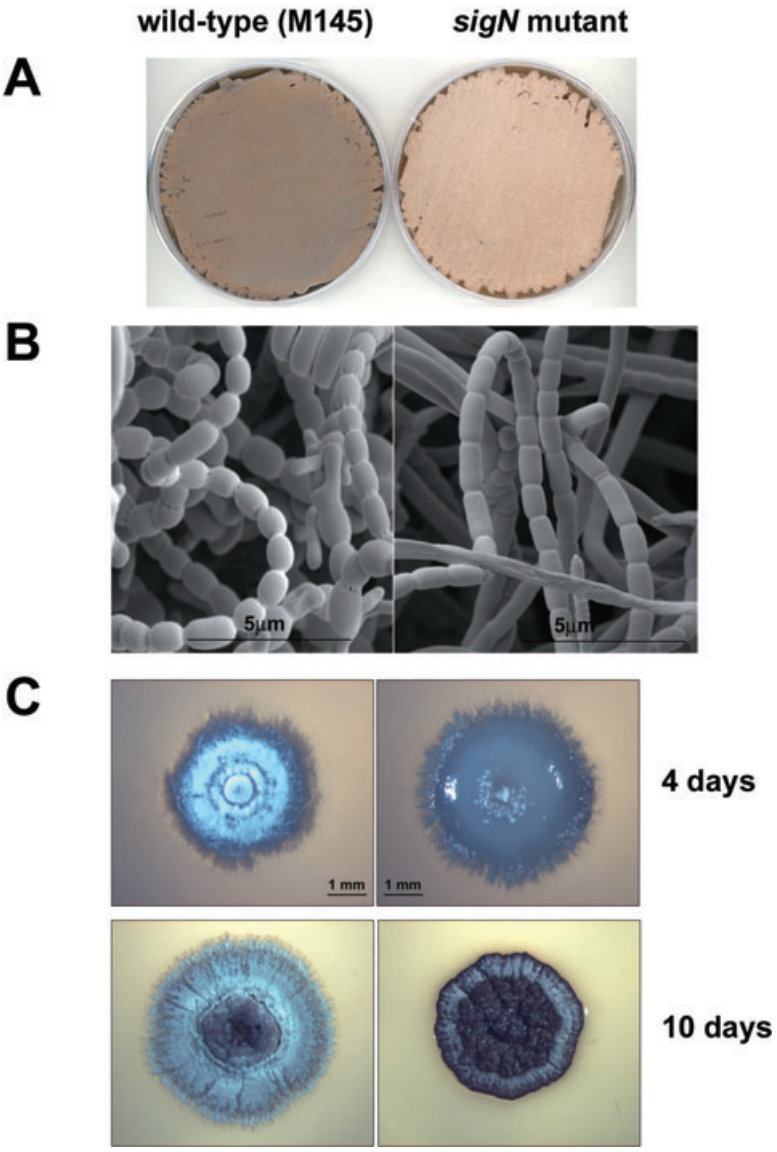

Fig. 1. Phenotypic analysis of the *sigN* mutant.
A. The *sigN* mutant (K100) and the wild-type M145 grown on SF-glucose medium for 5 days. Production of aerial hyphae is greatly delayed in K100 compared with M145.
B. Scanning electron micrographs showing the thin, elongated spores of the *sigN* mutant. K100 and M145 were grown for 3 days on SF-glucose medium.
C. Single colonies of K100 and M145 grown on minimal medium containing 1% glucose. Stereomicroscope images were taken after 4 and 10 days growth at 30°C.

The oligonucleotide primers were designed in such way that, apart from the first 13 amino acids (aa) and the last 9 aa, the complete coding sequences for *sigN* were removed in the mutant allele. A representative *sigN* null mutant was designated K100.

The phenotype of the *sigN* mutant was indistinguishable from the wild type when grown on a wide range of media including minimal medium containing 0.5% mannitol (MM-mannitol), soya flour medium containing 1% mannitol (SFM) and during osmotic, heat, cold or oxidative stress. However, K100 exhibited a delay in development when grown on glucose as carbon source. K100 had a 'pale' grey appearance on soya flour containing glucose (SF-glucose) instead of mannitol (Fig. 1A), and scanning electron microscopy (SEM) confirmed that the development of K100 was delayed compared with the wild type (Fig. 1B). The *sigN* mutant spores produced on SF-glucose were thinner and longer than wild-type spores, although after 5 to 6 days growth the spores became rounded, similar to the wild type. Moreover, the *sigN* mutant colonies were bald, even when grown for long periods, on MM medium containing 1% glucose (MM-glucose), a medium on which the classical *bld* mutants were identified (Fig. 1C). However, unlike many classical *bld* mutants, the *sigN* mutant did not fail to produce the pigmented antibiotics, actinorhodin and undecylprodigiosin, and K100 was indistinguishable from the wild-type *S. coelicolor* M145 on the rich medium, R2YE, a medium on which classical *bld* mutants produce no aerial mycelium. The *sigN* phenotype was complemented when a single copy of the *sigN* gene was introduced *in trans* into K100 using pAT20.

The fact that *sigN* lies downstream of *sigF* on the chromosome raised the question of a potential functional link between these two sigma factors. To assess such a link, we created a *sigF* mutant (K101) and a *sigF-sigN* double mutant (K102) using PCR targeting with appropriate primers. As expected, the phenotype of K101 (pale greenish-grey aerial mycelium) was indistinguishable from that of a previously described *sigF* mutant, J1978 (Kelemen *et al*., 1998). Both K101 and J1978 were generated in the M145 background and, while in K101 the complete *sigF* sequence was replaced by the apramycin resistance cassette (apramycin resistance gene and *oriT*), in J1978 part of *sigF* was replaced by the thiostrepton resistance gene. The pale greenish-grey phenotype of K101 (*sigF*) was observed on a wide variety of media (SFM, MM-mannitol, R2YE, SF-glucose or MM-glucose) and was preserved even after prolonged growth (6–7 days). In contrast, the delayed development of K100 (*sigN*) was exclusive to media containing glucose and the most severe impairment, lack of emergence of aerial hyphae, was observed only on MM-glucose medium. The *sigF-sigN* double mutant, K102, exhibited the characteristic *sigF* phenotype when grown on MM-mannitol or SFM medium. On SF-glucose medium K102 developed more slowly than K101 or the wild-type M145 strain, but after 5 days had a grey-green appearance and further analysis by light microscopy confirmed that it showed the characteristic features of *sigF*, such as small, round spores often with irregular septation (Potuckova *et al*., 1995). On MM-glucose medium, however, K102 (*sigF-sigN*) was indistinguishable from K100 (*sigN*) and had a bald phenotype.

Transcription of sigN *is initiated from two promoters*

For the initial transcription analysis S1 nuclease protection assays were performed using RNA from *S. coelicolor* A3(2) grown on solid MM-mannitol medium together with a radioactively labelled *sigN*-specific probe. The uniquely labelled 5′ end of the probe was internal to the *sigN* coding sequences. The same RNA samples were previously used to monitor expression of several developmental genes, including *sigF*, *whiG* (Kelemen *et al*., 1996), *sigH* (Kelemen *et al*., 2001), *whiH* (Ryding *et al*., 1998) and *hrdB* (Kelemen *et al*., 1996). Monitoring transcription of *hrdB*, encoding the principal sigma factor in *S. coelicolor*, was used as a control.

sigN transcription was found to be initiated from two distinct promoters, *sigNP1* and *sigNP2*, and both were upregulated during aerial hyphae formation (Fig. 2A). Because *sigN* lies immediately downstream of *sigF* and is oriented in the same direction, we had to exclude the possibility of *sigN* being co-transcribed from a promoter upstream or within *sigF*. S1 nuclease analysis using a probe with non-specific DNA at its 3′ end confirmed that no *sigN* transcription originated upstream of *sigNP2* under the conditions tested (growing on SFM medium at 30°C, for 1–4 days; data not shown). High-resolution S1 mapping was performed to identify the two transcriptional start points (Fig. 2B). The *sigN* promoter sequences of *S. coelicolor* are shown on Fig. 2C.

Because both *sigN* promoters were upregulated during aerial development (Fig. 2A), we wanted to test whether transcription from either of the *sigN* promoters was restricted to the aerial hyphae. We therefore monitored *sigN* transcription during growth in liquid medium, where no morphological differentiation of wild type occurs. While *sigNP2* activity was readily detectable, no transcription from *sigNP1* was identified throughout liquid growth (Fig. 3A), suggesting that *sigNP1* activity might be specifically confined to the aerial hyphae. We also monitored the two *sigN* promoters in several *whi* and *bld* mutants. Neither promoter depended on any of the early *whi* genes tested: *whiG*, *whiH*, *whiA* and *whiB* (data not shown). While transcription from *sigNP2* was apparent in all the samples, *sigNP1* activity was absent in all the *bld* mutants tested (Fig. 3A), including some of the original point mutants (*bldD*-J774, *bldC*-J660 and *bldG*-WC103) together with constructed *bldC* and *bldD* null mutants (Elliot *et al*., 2003b; Hunt *et al*., 2005). The dependence of *sigNP1* on all *bld* mutants tested, the coincidence of *sigNP1* transcription with aerial hyphae formation on solid medium and the absence of *sigNP1* activity during growth in liquid medium where *S. coelicolor* fails to differentiate, all suggested that *sigNP1* transcription was specific to the aerial hyphae.

Transcription of sigN *is autoregulated but does not depend on* sigF

Transcription of *sigB* of *B. subtilis* is initiated from a promoter controlled by SigB itself (Kalman *et al*., 1990). In *S. coelicolor*, autoregulation controls transcription of at least two other members of the SigB-like sigma factor subfamily, *sigBP1* (Cho *et al*., 2001) and the P3 promoter of *sigH* (Sevcikova *et al*., 2001). In addition, two SigB-like sigma factors from *Streptomyces ambofaciens*, HasL and HasR, control their own promoters (Roth *et al*., 2004). The promoter of *sigNP2* resembles *hrdB*-dependent promoters, albeit with a slightly longer spacer of 20 bp, whereas the *sigNP1* sequences show high similarity to the consensus promoter sequence recognized by SigB of *B. subtilis* (Boylan *et al*., 1991; Haldenwang, 1995; Price, 2000; Figs 2C and 5C). In order to test whether *sigN* was autoregulated we performed S1 nuclease protection assays using RNA from the wild-type strain M145 and the isogenic *sigN* mutant, K100 (Fig. 3B). Samples were taken at regular intervals rep-

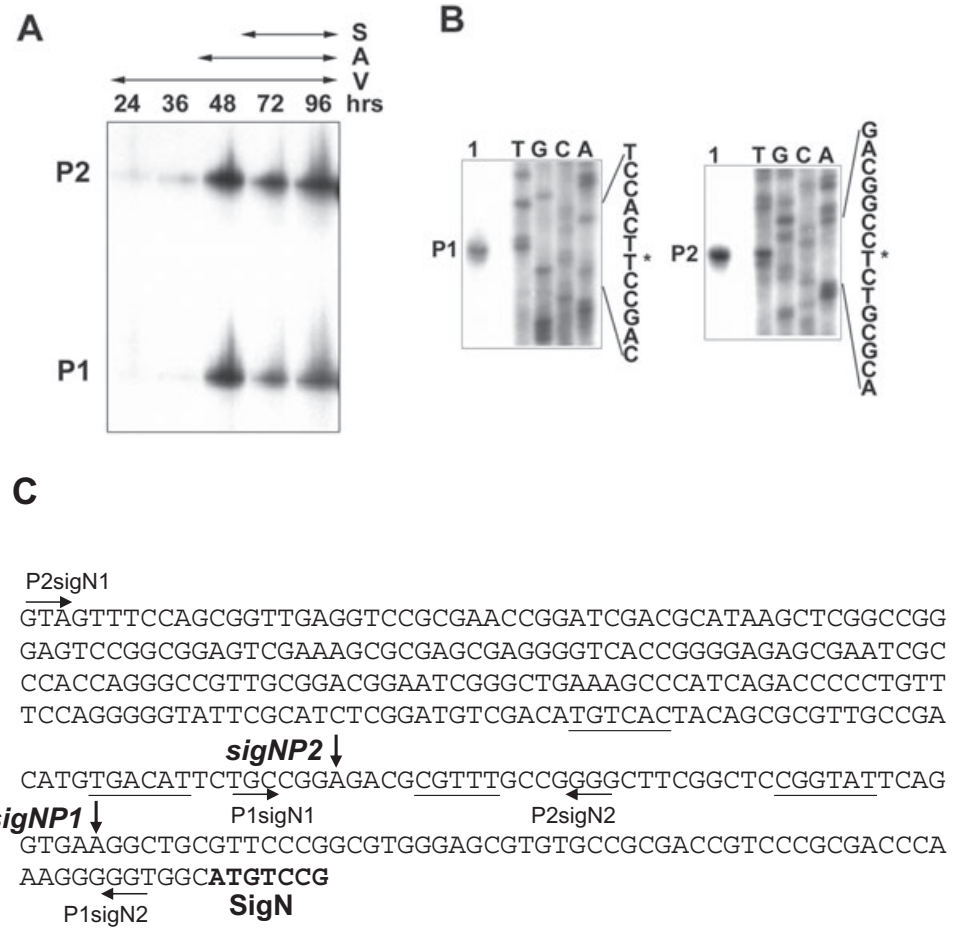

Fig. 2. Transcription analysis of *sigN*.
A. S1 nuclease protection assays were performed using 40 μg of RNA from *S. coelicolor* A3(2) grown on MM-mannitol. Samples were taken at different stages during development at 24, 36, 48, 72 and 96 h after inoculation. The presence of vegetative mycelium (V), aerial mycelium (A) and spores (S) is indicated. The *sigN* probe was generated by PCR using SIGN4 and ^{32}P-labelled SIGN5 primers. The same RNA was used by Kelemen *et al*. (1996) where the control using *hrdB*-specific probe was shown.
B. High-resolution S1 mapping of the two *sigN* promoters. Lane 1: protected DNA; lanes T, G, C and A were generated using dideoxy chain terminator sequencing with SIGN5 primer. * indicate transcription start points.
C. Sequences around the *sigN* promoters. Vertical arrows mark the transcriptional start points. Putative −10 and −35 promoter sites are underlined. The SigN coding sequences are shown in bold. Horizontal arrows indicate the 5′ ends of the *S. coelicolor* sequences within the oligonucleotides used to generate the different *sigN–egfp* transcriptional fusions.

resenting distinct phases during morphological differentiation from cultures grown on solid SFM medium. Because the *sigN* mutant lacks sequences downstream of the first 13 aa, the probe was generated using a labelled oligonucleotide (SigN7) internal to sequences present in the *sigN* mutant. Transcription from both *sigNP1* and *sigNP2* was readily detectable in the samples from M145 but no *sigN* transcription was detected in the *sigN* mutant. The absence of *sigNP1* activity in K100, together with the *sigNP1* promoter sequences (Figs 2C and 5C), suggests that SigN directs transcription from the *sigNP1* promoter and *sigN* is therefore autoregulated. It is less straightforward to explain why *sigNP2* was affected in the *sigN* mutant. Previous analysis of the promoters *sigHp1* and *sigHp2* showed similar apparent 'co-upregulation' in the *bldD* mutant (Kelemen *et al*., 2001), where only one of the promoters, *sigHp1*, was a direct target for repression by BldD. Transcription of *hrdB* was readily detectable in all samples tested, confirming that the absence of the *sigN*-specific signal was not due to poor RNA preparations.

In order to test whether expression of the two adjacent sigma factor genes, *sigN* and *sigF*, were interdependent, we monitored transcription of *sigN* and *sigF* in the *sigF* and *sigN* mutants respectively (Fig. 4). Transcription of *sigN* in the wild-type strain was indistinguishable from that in the *sigF* mutant. Similarly, transcription of *sigF* was unaffected in the *sigN* mutant, implying that despite their proximity on the chromosome, there is no interdependence between *sigN* and *sigF* expression.

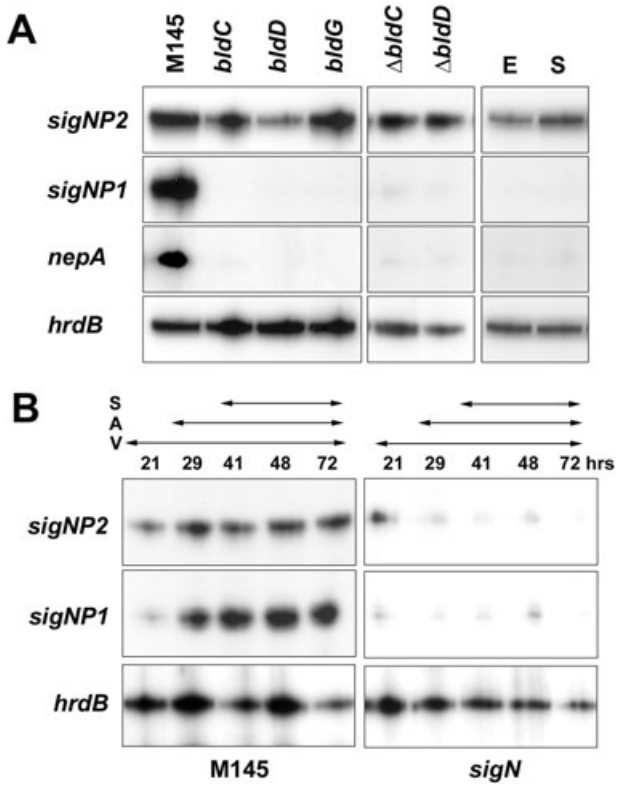

Fig. 3. Transcriptional analysis of *sigN*.
A. S1 nuclease assays were performed using 40 μg of RNA from wild-type *S. coelicolor* together with several *bld* point mutants, *bldC* (J660), *bldD* (J774) and *bldG* (WC103) together with constructed Δ*bldC* (J2168) and Δ*bldD* null mutants, grown on R2YE medium for 60 h (left and middle) or from wild-type *S. coelicolor* grown to exponential (E) and stationary (S) phase in liquid TSB+PEG medium at 30°C (right). The *sigN* probe was generated by PCR using SIGN4 and ^{32}P-labelled SIGN7 primers. The *nepA* probe was generated using 4002.2 and ^{32}P-labelled 4002.1 oligonucleotides. To assess the RNA preparations, *hrdB* probe was used as a control on all panels.
B. S1 nuclease protection assays were performed using 40 μg of RNA from *S. coelicolor* M145 (wild type) and *sigN* mutant (K100) grown on solid SFM medium. Samples were taken at different stages during development at 21, 29, 41, 48 and 72 h after inoculation. The *sigN* probe was the same as in (A). The presence of vegetative mycelium (V), aerial mycelium (A) and spores (S) is indicated.

nepA*, encoding a small extracellular protein, depends on* sigN

Preliminary microarray analysis (K.A. Dalton and G.H. Kelemen, unpubl. data) using the PCR-based *S. coelicolor* microarrays (Bucca *et al.*, 2003; http://www.surrey.ac.uk/SBMS/Fgenomics/) and the same RNA samples used to monitor the *sigN* promoters (Fig. 3B) identified *SCO4002* as a potential SigN target. In order to confirm these preliminary results we performed S1 nuclease protection assays to monitor *SCO4002* transcription in the wild-type M145 and *sigN* mutant grown on solid SFM medium (Fig. 5A). Transcription of *SCO4002* was initiated from a single promoter and coincided with the formation of aerial mycelium during colony development. No *SCO4002* transcription was observed during vegetative growth or at late stages of sporulation. This suggested that the transient expression of *SCO4002*, like that of *sigNP1*, was restricted to the aerial hyphae. Importantly, transcription of *SCO4002* depended on *sigN* (Fig. 5A) because no *SCO4002* transcription was detectable in RNA samples from the *sigN* mutant. In order to confirm that the lack of *SCO4002* transcription was due to the absence of *sigN* in K100, we showed that *SCO4002* promoter activity was restored in K100 containing pAT20 carrying the wild-type *sigN* allele *in trans* (Fig. 5B). The promoter sequences of *nepA* and *sigNP1* are compared in Fig. 5C. Most of the RNA used in the S1 nuclease protection assay was taken from mycelium grown on mannitol-containing medium routinely used in the laboratory, allowing comparisons with previous experiments monitoring *sigF* or

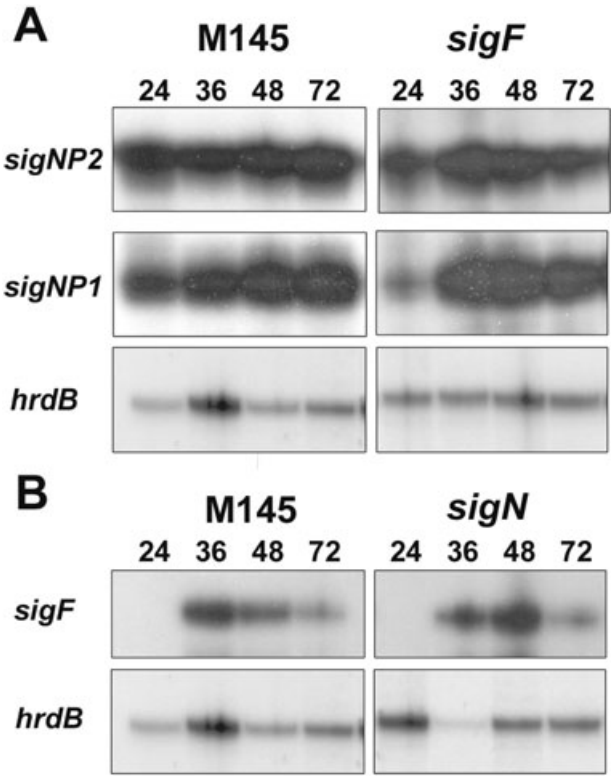

Fig. 4. Monitoring transcription of *sigN* and *sigF*. S1 nuclease protection assays were performed using 40 μg of RNA from *S. coelicolor* M145 (wild type), the *sigF* mutant, K102 (A) and the *sigN* mutant, K100 (B) grown on solid SFM medium. Strains from which the RNA samples were analysed are shown above the panels. Samples were taken at different stages during development at 24, 36, 48 and 72 h after inoculation. All the probes (shown on the left of the panels) were generated using PCR according to *Experimental procedures*; the *sigN* probe was created using SIGN4 and ^{32}P-labelled SIGN7 primers.

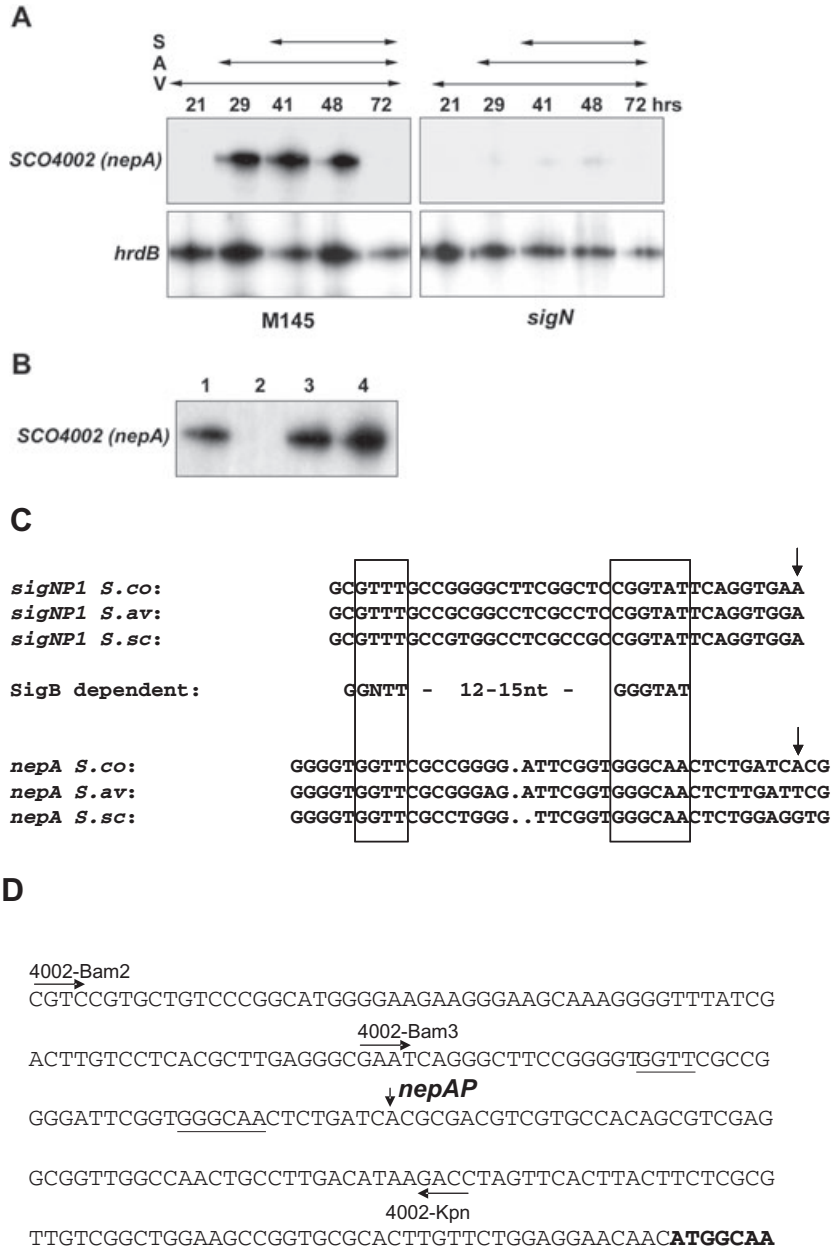

Fig. 5. Transcription of *nepA* (*SCO4002)* depends on *sigN*.
A. S1 nuclease protection assays were carried out on the same set of RNA samples as shown in Fig. 3B. We used 40 μg of RNA from *S. coelicolor* M145 (wild type) and *sigN* mutant (K100) grown on solid SFM medium. Samples were taken at different stages during development at 21, 29, 41, 48 and 72 h after inoculation. The presence of vegetative mycelium (V), aerial mycelium (A) and spores (S) is indicated.
B. RNA (40 μg) was taken from: lane 1, *S. coelicolor* M145 (wild type) at 41 h after inoculation; lane 2, the *sigN* mutant (K100) at 41 h after inoculation; lanes 3 and 4, the *sigN* mutant complemented with pAT20 at 36 and 48 h after inoculation. All strains were grown on SFM solid medium. Probes on both (A) and (B) were generated using PCR and are shown on the left of the panels. The *nepA* probe was generated using 4002.2 and ^{32}P-labelled 4002.1 primers.
C. Promoter sequence alignments. Promoter sequences of *sigNP1* and *SCO4002* are shown from *S. coelicolor* (*S. co*), *S. avermitilis* (*S. av*) and *S. scabies* (*S. sc*). Only the *S. coelicolor* promoter sequences are based on experimental analysis; sequences from *S. avermitilis* and *S. scabies* were taken from upstream regions of the appropriate *sigN* and *nepA* homologues (http://avermitilis.ls.kitasato-u.ac.jp/http://www.sanger.ac.uk/Projects/S_scabies/). Transcriptional start points are marked with an arrow. The consensus SigB-dependent promoter sequences of *B. subtilis* are shown for comparison (Haldenwang, 1995) and the putative −10 and −35 sequences are boxed.
D. Sequences at the 5′ end of *nepA*. The transcriptional start point is marked by a vertical arrow. Horizontal arrows indicate the 5′ ends of the *S. coelicolor* sequences within the oligonucleotides used to generate the different *nepA–egfp* fusions. Primers 4002-Bam2 or 4002-Bam3 were used together with 4002-Kpn to create pK11 or pK12 respectively. The *nepA* coding sequences are shown in bold.

hrdB transcription. We tested transcription of *sigN* and *nepA* using two independent RNA samples from wild-type M145, and *sigN* and *bldC* mutants grown on solid medium containing glucose. The transcription patterns of *sigN* and *nepA* were independent of the carbon source (data not shown), so we did not conduct an extensive analysis of RNA extracted from mycelium grown on glucose containing medium.

SCO4002 encodes a putative secreted protein of 108 aa. Searches for homologous proteins using PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) revealed proteins only among streptomycetes: SAV4213 in *Streptomyces avermitilis* (http://avermitilis.ls.kitasato-u.ac.jp/) and a yet unannotated protein derived from the translation of sequences from *Streptomyces scabies* (http://www.sanger.ac.uk/Projects/S_scabies/). SAV4213 contains only 78 aa and the *S. scabies* protein 101 aa. Alignment of the three protein sequences (Fig. 6A) revealed that the first 31 aa and the last 47 aa are highly conserved among all three proteins. Between these highly conserved regions lies an extension of 29 aa and 25 aa exclusive to *S. coelicolor* and to *S. scabies* respectively. SIGNALP (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al.*, 2004) predicted the highly conserved N-terminal 31 aa as a signal peptide with probability 1.000, and a cleavage site between positions 29 (A) and 30 (D) with probability

A

```
S.coelicolor          MASIRTARVVAAVAALPLAAALLGGVATADNGAFADDGSNATAASASAGIIGSGVGDDNN 60
S.avermitilis         MASIRTARVLAAVAALPLAAALFTGVAAADN---------------------------- 31
S.scabies             MASIRTARVLAAVSAIPLAAALFAGVAAADNGSFADNGSNAGVAN----LVGSGVGRDNS 56
                      ********* *** * ******  *** ***

S.coelicolor          GNSSTTQQQAVGSGASNQSNTAQVDGSAFTSINQGNDNVAVTFSPLWW 108
S.avermitilis         GNSAITYQQAVGFGASNQSNTAQVNGSPFTTINQKNENVAVNFGNLW  78
S.scabies             GNSSTSQQQAIGSGASNQNNTAQVNGFGLTAIDQK--NAVVNFADLW  101
                      ***    *** * ***** ***** *   * * *   *  * *  **
```

B

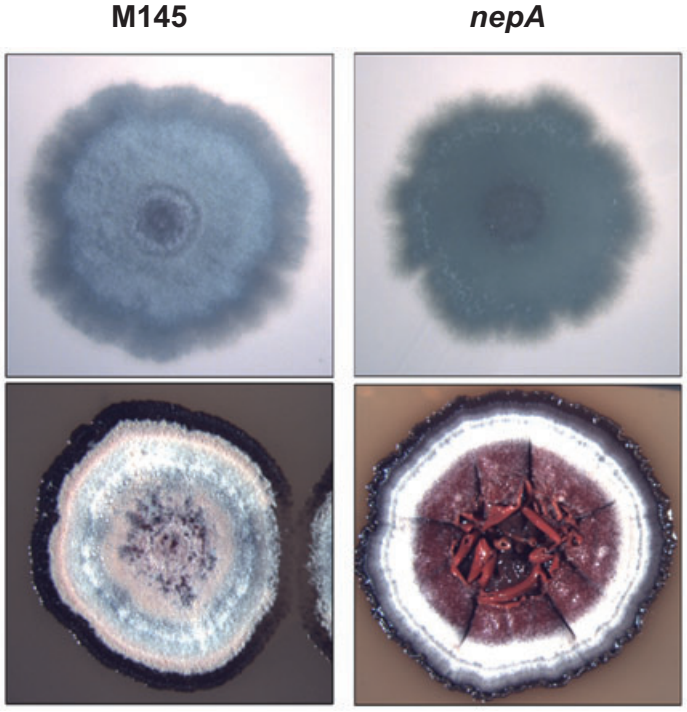

Fig. 6. The *nepA* mutant phenotype.
A. Sequences of NepA from *S. coelicolor*, *S. avermitilis* and *S. scabies* are compared using CLUSTALW (http://www.ebi.ac.uk/clustalw/). The position of the predicted signal peptide cleavage site is indicated with an arrow. Identical residues in all three sequences are marked with asterisks.
B. Single colonies of *S. coelicolor* M145 (wild type), and the *nepA* mutant were grown on minimal medium containing 1% glucose and stereomicroscope images were taken after 5 and 12 days growth at 30°C.

0.97. This suggests secreted proteins of 49 aa (*S. avermitilis*), 72 aa (*S. scabies*) and 79 aa (*S. coelicolor*). However, the highly conserved residues within these sequences comprise 5–26 aa, which might suggest further processing, generating an even shorter, secreted oligopeptide. Based on the bioinformatic analysis and the fact that *SCO4002* depended on *sigN*, we designated *SCO4002* as *nepA* (sigma N-dependent extracellular oligopeptide).

Using PCR targeting (Gust *et al.*, 2003) we replaced *nepA* with the apramycin resistance cassette in *S. coelicolor* M145. A representative *nepA* mutant was designated as K106 and was further characterized. The morphology of the K106 mutant was monitored on several solid media (including SFM, MM-mannitol, MM-glucose and R5 media) and its phenotype resembled that of the *sigN* mutant. The progression of aerial development of K106, as compared with the wild-type strain, was delayed when grown on MM-glucose, although this delay was less severe than that of the *sigN* mutant, allowing aerial hyphae formation at the edges of the colonies. Similarly to the *sigN* phenotype, this developmental delay was most prominent when K106 was grown as single colonies (Fig. 6B).

SigN directs transcription from the ctc *promoter of* B. subtilis *in vitro, but not from* sigNP1 *or* nepAP

In order to demonstrate the sigma factor activity of SigN *in vitro*, we overexpressed SigN with an N-terminal His extension in *Escherichia coli* using the vector pET28a (Novagen). During the purification trials His-SigN was found in the insoluble fraction and was purified using denaturing conditions of the Ni-NTA kit (Qiagen) as

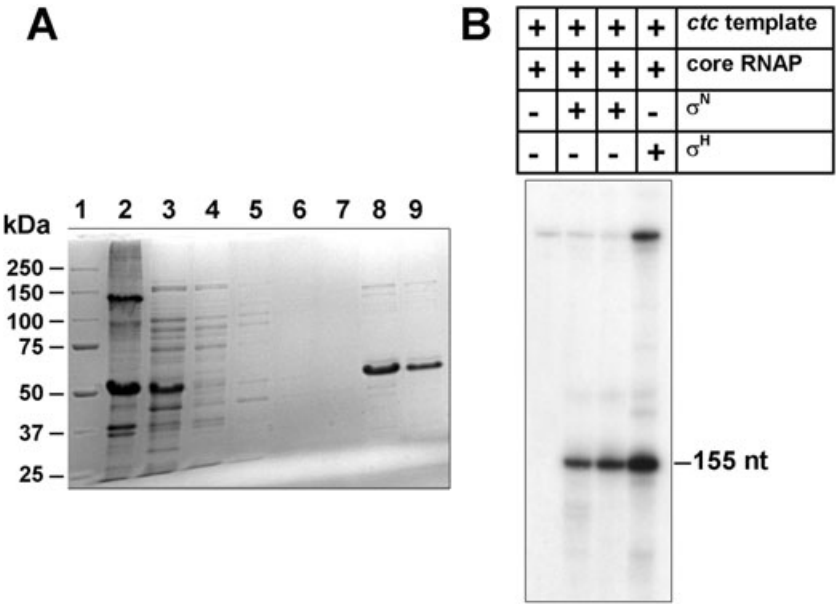

Fig. 7. *In vitro* run-off transcription using RNA polymerase holoenzyme containing SigN.
A. pK20 carrying *sigN* in pET28a was introduced into *E. coli* BL21 (DE3)pLysS (Novagen). Cultures of BL21(DE3)pLysS/pK20 were grown at 37°C to an OD_{600} = 0.4 and after induction with 1 mM IPTG for a further 4 h. After harvesting the cells, His-SigN was purified under denaturing conditions (8 M urea) using the Ni-NTA Spin Kit (Qiagen) according to the manufacturer's instruction. Lane 1, protein size markers, sizes shown on the left of the panel; lane 2, total extract after harvesting the mycelium; lane 3, sample loaded onto the Ni-NTA column; lanes 4–7, washes with buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris-Cl, pH = 6.3; lanes 8 and 9, samples eluted with buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris-Cl, pH = 4.5. Samples were run on an 8% SDS-PAGE gel and stained with Coomassie brilliant blue.
B. Transcription from the *ctc* promoter of *B. subtilis* was tested using a 340 bp EcoRI–BamHI fragment from pMI349 (Igo and Losick, 1986) together with *E. coli* core RNA polymerase (RNAP) in the presence or absence of SigN or SigH (Viollier *et al*., 2003). The 155 nt transcript produced was analysed on 6% polyacrylamide-7M urea sequencing gel.

shown on Fig. 7A. The apparent molecular weight estimated on SDS-PAGE was higher than that predicted from the His-SigN sequence (36.6 kDa). This aberrant migration is characteristic of many sigma factors and is believed to result from their acidic pI (Haldenwang, 1995). 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*. Core RNA polymerase from *E. coli* together with His-SigN was sufficient to produce a specific transcript from *Pctc* (promoter of *ctc*) in an *in vitro* run-off transcription assay (Fig. 7B), demonstrating the sigma factor activity of His-SigN. However, when we used template DNA carrying either the *sigN* or the *nepA* promoters, His-SigN failed to initiate transcription *in vitro* (data not shown). Moreover, no transcription was detected from either the *sigN* or the *nepA* promoters when *in vitro* run-off transcription was attempted using unfractionated RNA polymerase holoenzyme (kindly provided by Luis Servin-Gonzalez and Mark Buttner) purified from liquid cultures of *S. coelicolor* (data not shown). This suggested either that the holoenzyme that initiates transcription from the *sigN* and *nepA* promoters was absent from the preparation, or that an additional missing transcription factor was required for successful initiation at the *sigN* and *nepA* promoters.

The location of nepA *transcription defines a novel compartment, the 'subapical stem', within the aerial hyphae*

Transcription of *nepA* was transiently induced during aerial hyphae formation (Fig. 5A), so we tested whether it depended on some of the *bld* genes, *bldC*, *bldD* and *bldG.* Transcription of *nepA* was absent in all the *bld* mutants tested (Fig. 3A), suggesting that, like *sigNP1*, *nepAP* expression was likely to be restricted to the aerial hyphae. To confirm this, we created a *nepA–egfp* transcriptional fusion plasmid, pK10, by introducing a PCR-generated *nepA* promoter fragment into pIJ8660 (Sun *et al*., 1999). pK10 was introduced into *S. coelicolor* M145 by conjugation and one of the representative exconjugants, K103, was monitored by fluorescence microscopy for green fluorescence during different

stages of development on solid SFM or MM-mannitol medium containing apramycin.

Transcription of *nepA* was completely absent during vegetative growth (Fig. 8) and was exclusive to the aerial compartment. High-resolution images taken from samples grown alongside a coverslip slide indicated that *nepA* transcription was not present even in the early aerial hyphae prior to sporulation. Moreover, fluorescence from the *nepA–egfp* fusion was detectable only in the part of the aerial hyphae immediately adjacent to the spore chains and enclosed by the nearest septa at the interface between the vegetative and aerial parts of the colony (Fig. 8B and C). Previously, aerial hyphae-specific transcription has been shown for *sigHp2* (Kelemen *et al*., 2001) and some of the chaplin genes, such as *chpH* (Claessen *et al*., 2003; Elliot *et al*., 2003a). However, both *sigHp2*- and *chpH*-specific green fluorescence was localized to the apical part of the aerial hyphae, including, at later stages, the spore chains. The location of *nepA* transcription specifies a novel compartment we designated as the 'subapical stem', which for the first time enables us to define the interface between aerial and vegetative hyphae. To test whether enclosure of the stem depends on sporulation, we monitored *nepAP*-specific fluorescence in one of the *whi* mutants, *whiG* (J2400). Even in a non-sporulating strain, such as the *whiG* mutant, *nepAP* activity was not detected in the entire length of the aerial hyphae but was restricted to a compartment enclosed by septation (Fig. 8D). This suggests that neither *nepA* transcription nor formation of the stem requires sporulation, *per se*.

The fact that, although *nepA* depended on *sigN*, SigN was not sufficient for transcription *in vitro* from the *nepA* promoter raised the possibility that initiation of *nepA* transcription required some activator besides the RNA polymerase holoenzyme. Generating further *egfp* constructs, pK11 and pK12, enabled us to test the promoter activities of DNA fragments of different length upstream of the transcriptional start point (tsp). While pK10 carried 360 bp upstream of *nepA* tsp, pK11 and pK12 contained 126 bp and 55 bp upstream sequences respectively. Transcription of *nepA* judged by the green fluorescence of K104, carrying pK11, was indistinguishable in strength and location from that of K103, carrying pK10. However, despite the fact that in pK12 the 55 bp upstream sequences contained both the putative −10 and −35 promoter sites, no *nepA*-specific fluorescent signal was detectable in *S. coelicolor* M145 carrying pK12 (data not shown). Probably the extra 71 bp in pK11 permits the binding of one or more additional proteins that are needed for promoter activation. This would be consistent with the negative results of the *in vitro* run-off transcription experiments, in which such additional factors were not expected to be present.

We attempted to dissect the *sigN* promoters by generating *egfp* fusions carrying only *sigNP1*, or *sigNP2* or both promoters (details in *Experimental procedures* and Fig. 2C). Because *sigNP1* is 40 nt downstream of *sigNP2*, the construct carrying *sigNP1* on its own contained 46 bp upstream of the P1 tsp, including the putative promoter site. After introducing the generated constructs into *S. coelicolor* M145 no fluorescence signal was detected when *sigNP1–egfp* was monitored. This suggested that one or more additional transcription factor(s) is required for transcription from the *sigNP1* promoter. Fluorescence signals specific to *sigNP2* and *sigNP1+2* were detectable, albeit extremely weak (Fig. S1). The weak fluorescence signal of *sigNP2–egfp* was present in both the substrate and aerial compartments, while the *sigNP1+2–egfp* fusion clearly indicated an increased signal in the subapical stem compartment of the aerial hyphae. This suggested that transcription of *nepA* and *sigNP1* coincided not only temporally, as shown by the S1 nuclease analysis (Figs 3B and 5A), but also spatially to the subapical stem of the aerial hyphae.

Discussion

sigN, *a new member of the* bld *gene family*

Similarly to the classical *bld* mutants, mutation in *sigN* resulted in a bald phenotype when colonies were grown on MM-glucose as carbon source, and the *bld* phenotype was 'rescued' when *sigN* colonies were grown on medium containing the less well-utilized carbon source, mannitol. However, unlike the classical *bld* mutants, which are also blocked in production of aerial hyphae on a rich medium, R2YE, the *sigN* colonies were indistinguishable from wild type on R2YE medium. Mutations in the *bld* genes often result in the failure to produce secondary metabolites, some of which are coloured blue (actinorhodin) or red (undecylprodigiosin). Although we observed some slight differences in pigmentation of the *sigN* mutant when grown on media containing glucose (SF-glucose or MM-glucose; Fig. 1), colonies of the *sigN* mutant never lacked pigmentation.

One of the classical *bld* genes, *bldG*, encodes a putative anti-anti-sigma factor homologous to RsbV or SpoIIAA of *B. subtilis* that could, in principle, control the activity of an RNA polymerase sigma factor (Bignell *et al*., 2000). Activation of SigB and SigF in *B. subtilis* are the most studied examples of post-translational sigma factor activation via protein–protein interactions. The sigma factor (SigB or SigF) is held inactive by binding to an antagonist protein or anti-sigma factor (RsbW or SpoIIAB). In turn, the anti-anti-sigma factor (RsbV or SpoIIAA) can abolish this interaction by making a complex with the anti-sigma factor and releasing the sigma factor

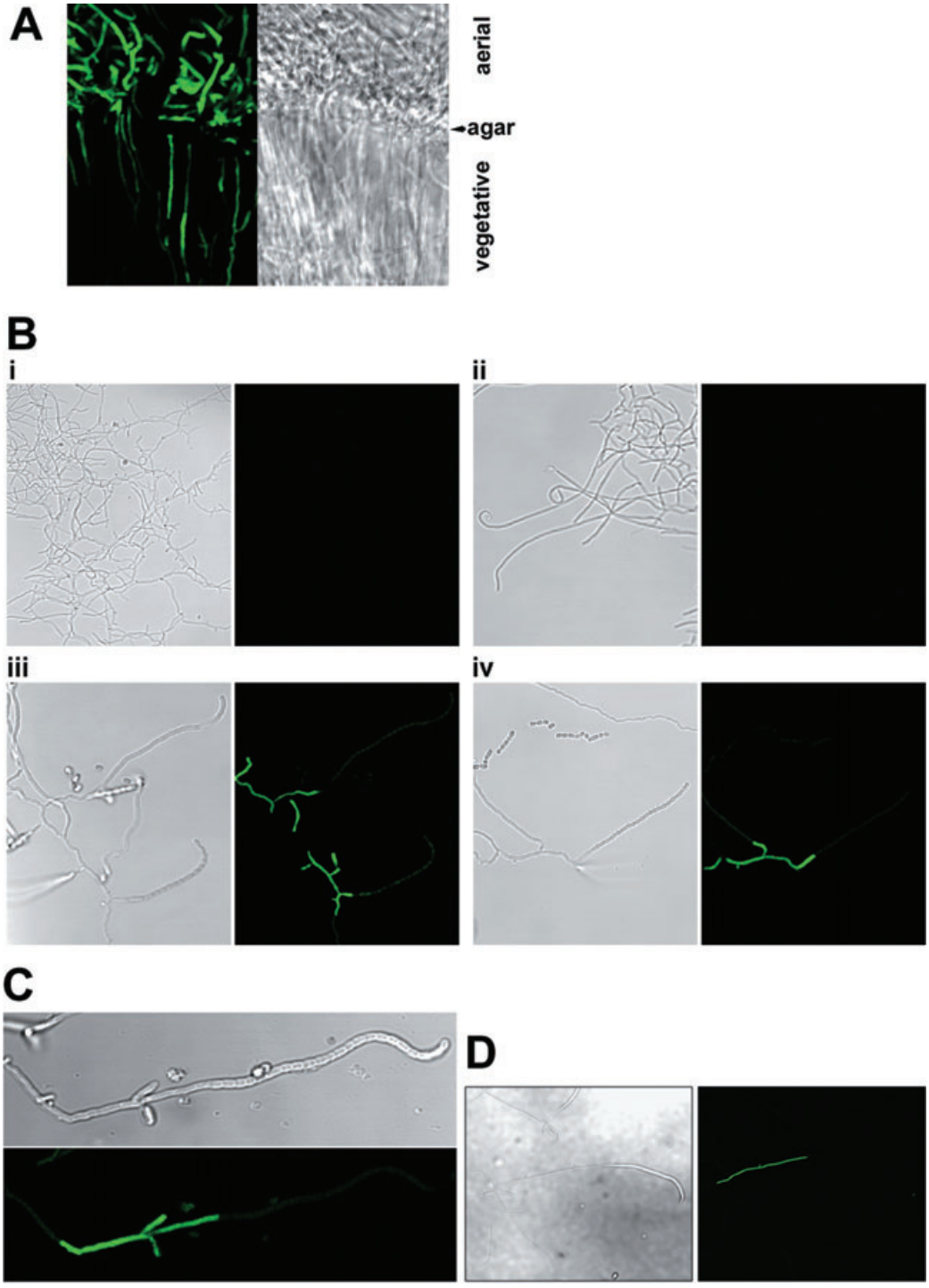

Fig. 8. Monitoring *nepA*-specific green fluorescence.
A–C. *S. coelicolor* M145 carrying pK10, a derivative of pIJ8660 with a *nepA–egfp* transcriptional fusion, was grown on minimal medium containing mannitol in the presence of apramycin to keep the selection for pK10.
A. A cross-section of a colony is shown with the fluorescence image on the left and the light microscopy control on the right.
B. Colonies were grown for 24–48 h alongside a microscope coverslip slide that was removed and viewed without fixation. Samples of (i) vegetative hyphae, (ii) early aerial development, (iii) and (iv) aerial filaments during sporulation are shown with fluorescence images on the right and light microscopy control on the left.
C. Close-up image of a sporulating aerial filament.
D. *whiG* carrying pK10 was grown on MM-mannitol in the presence of apramycin alongside a microscope coverslip. After 48 h the coverslip was removed and viewed without fixation.
On (C) and (D) the fluorescence image is shown on the bottom or right and light microscopy control on the top or left, respectively.

to initiate transcription. Complex formation between the anti-anti-sigma factor and the anti-sigma factor depends on phosphorylation of RsbV or SpoIIAA by RsbW or SpoIIAB respectively (Benson and Haldenwang, 1993; Duncan and Losick, 1993; Min *et al*., 1993; Alper *et al*., 1994; Duncan *et al*., 1996). Next to *bldG* there is an open reading frame that could function as a sigma factor antagonist, and phosphorylation of BldG has been shown both *in vivo* and *in vitro* (Hesketh *et al*., 2002; Bignell *et al*., 2003). However, while in *B. subtilis* the three genes encoding SpoIIAA, SpoIIAB and SigF or RsbV, RsbW and SigB are adjacent on the chromosome, there is no sigma factor gene in the vicinity of *S. coelicolor bldG* (Bignell *et al*., 2000).

Could SigN be controlled by an anti-sigma factor, which in turn is controlled by BldG? Analysis of the gene organization around the nine SigB-like sigma factor genes identified *sigF*, *sigN*, *sigK* and *sigG* with no adjacent putative anti-sigma, or anti-anti-sigma genes. The presence of more than 40 genes encoding putative anti-sigma factors and at least 17 genes encoding putative anti-anti-sigmas (C. Reynold, pers. comm.) in the *S. coelicolor* genome suggests that the physical proximity of genes encoding partner proteins might not be critical. Furthermore, some degree of promiscuity is inevitable: some sigmas might be controlled by multiple anti-sigmas, or conversely some anti-sigmas might control the activity of multiple sigmas. Similarly, multipartnerships can also be expected between the anti-sigmas and anti-anti-sigmas. The choice of a partnership might be highly dependent on the availability and activity of the partners, which, in turn, could be controlled by physiological state, nutrient availability or environmental stimuli. While the polycistronic gene organization of the *spoIIA* operon of *B. subtilis* allows equimolar production of the interacting proteins, the potential multipartnership in *S. coelicolor* might not require a 1:1 ratio for any single partner and a different, more complex mechanism is expected to control the availability of any given sigma factor.

The bald phenotype of the *sigN* mutant is compatible with the hypothesis that BldG might be an antagonist of an anti-SigN protein under the physiological condition of growth in the presence of glucose. If so, SigN might not be the only sigma factor regulated by BldG, because, besides being bald, the *bldG* mutant failed to produce the pigmented antibiotics, a characteristic not shared with the *sigN* mutant. Further experiments are needed to establish the link, if any, between SigN and BldG.

SigN is not sufficient to initiate transcription at sigNP1 *and* nepA *promoters*

Comparison of the promoter sequences of *sigNP1* and *nepA* with the consensus SigB-dependent promoters (Fig. 5C; Haldenwang, 1995) displayed strong similarity in the −35 sequences and a somewhat lower but significant similarity in the −10 sequences, implying that these promoters could be targets for a SigB-like sigma factor. Transcription from both *sigNP1* and *nepA* depended on *sigN in vivo*, suggesting that SigN might control these promoters directly. However, run-off transcription using RNA polymerase holoenzyme containing SigN failed to initiate transcription at these promoters *in vitro*, although successfully initiated transcription from the *ctc* promoter of *B. subtilis* under the same conditions. In addition, no transcripts were generated from the *sigN* and *nepA* templates when we used unfractionated RNA polymerase holoenzyme purified from liquid cultures of *S. coelicolor*, a condition in which SigN is expected to be produced via the *sigNP2* promoter. All the above leads to two possible conclusions: (i) that SigN does not directly interact with these promoters but indirectly controls their expression, or (ii) that SigN is necessary but not sufficient for transcription initiation from the *sigNP1* and *nepA* promoters. Indeed, monitoring fluorescence using transcriptional *egfp* fusions of *nepA* and *sigN* demonstrated no *in vivo* promoter activity from DNA fragments carrying little more than the −10 and −35 sequences of *sigNP1* or *nepA*, suggesting that transcription from these promoters requires some additional upstream DNA, perhaps to interact with transcription factor(s). Only when these transcription factors are identified can we begin to address which conclusion above is correct.

The striking similarities within the promoter recognition domains of the nine SigB-like sigma factors (Fig. S2) raise some fundamental questions. First, do these sigma factors recognize distinct sequences at their specific target sites? Although computer searches (Lee *et al*., 2004b) identified several putative *S. coelicolor* promoters predicted to be recognized by SigB [= SigJ (Viollier *et al*., 2003)], only two promoters, *sigBP1* and *catBP*, have been shown both *in vitro* and *in vivo* to be targets for SigB (Lee *et al*., 2004b). Also, the *B. subtilis ctc* promoter was transcribed *in vitro* by SigF (Kelemen *et al*., 1998), SigH (Viollier *et al*., 2003), SigN (shown in this article), SigM, SigJ and SigI (unpublished results from G.H. Kelemen's laboratory) – in fact by all the SigB-like sigma factors of *S. coelicolor* that have been tested. This leads to the second fundamental question: how can these sigma factors with potentially overlapping promoter specificities exhibit distinct biological functions? Besides regulating the activity of the sigma factors both temporally and spatially, one possible answer could entail 'weak' target promoters that require sigma factor-specific transcriptional activators for successful transcription initiation. Besides SigN, this could also explain the way in which SigF controls its targets, as SigF controls *whiEP2 in vivo*, but failed to transcribe from *whiEP2*

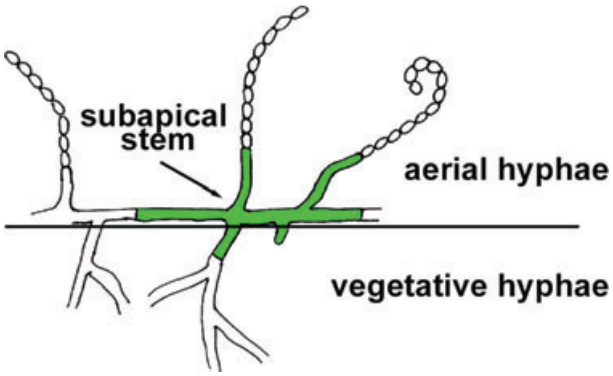

Fig. 9. The novel compartment, the subapical stem (green), separates the vegetative mycelium from the aerial tip compartment.

in vitro (Kelemen *et al*., 1998; Homerova *et al*., 2000; Novakova *et al*., 2004).

What is the role of the subapical stem compartment during colony development?

Correct localization of proteins controlling bacterial development is essential to their biological function (Shapiro and Losick, 1997; 2000). Previous transcriptional fusions to *egfp* localized the spatial patterns of expression of several *Streptomyces* genes largely to two distinct 'tissues', the aerial and the vegetative hyphae. Transcription specific to aerial hyphae has been shown for *sigHp2* (Kelemen *et al*., 2001) and for some of the chaplin genes, such as *chpH* (Claessen *et al*., 2003; Elliot *et al*., 2003a). In contrast, transcription of *redD*, encoding a pathway-specific activator for production of the red-pigmented antibiotic, undecylprodigiosin, was confined to vegetative hyphae (Sun *et al*., 1999). Interestingly, both *sigHp2*- and *chpH*-specific green fluorescence was localized to the apical part of the aerial hyphae, including, at later stages, the spore chains. Sporulating aerial hyphae were also the location for transcription of *sigF* (Sun *et al*., 1999) and one of the two *ftsZ* promoters (Flardh *et al*., 2000). In this article we have shown that transcription of *nepA* was exclusive to a novel, developmentally distinct location, the subapical stem of the aerial hyphae. The subapical stem compartment is defined by our analysis as a hyphal segment that is enclosed by septa, at least one of which is the septum separating the spore chains from the non-sporulating hyphal filament in wild-type colonies (Fig. 9). Although we have never detected *nepA*-specific fluorescence prior to sporulation in the wild-type strain, the presence of *nepA* transcription in a non-sporulating mutant, *whiG*, confirmed that *nepA* transcription was not dependent on sporulation, *per se*. While the three *bld* genes, *bldC*, *bldD* and *bldG*, were required for *nepA* or *sigNP1* transcription and, presumably, for the emergence of the stem, the earliest acting of known essential sporulation genes, *whiG*, was not needed for the formation of the stem compartment.

It is not known exactly how transformation of the aerial hyphae into chains of spores begins, although it was speculated that the formation of a special septum could enclose the part of the aerial hyphae that is converted into spores (Flardh *et al*., 1999; Kwak *et al*., 2001). This special septum would divide the aerial hyphae into two subcompartments, the stem and the tip, with distinct fates. The fate of the tip compartment is transformation into spore chains during normal development, while the fate of the subapical stem is still unknown. Early characterization of the organization of the aerial mycelium has described the spore-bearing cells as 'fertile' and the non-sporulating hyphal fragments as 'sterile' (Wildermuth, 1970); the latter, we believe, might correspond to the subapical stem. Here, for the first time, we have assigned a specific function to this compartment, the upregulation of *sigN* expression together with the production of NepA. Because *nepA* encodes a putative secreted protein or peptide it is conceivable that the subapical stem, at least in part, is involved in signalling between the aerial tip compartment and the vegetative mycelium. In *Streptomyces*, signalling has been shown to control both development (the *bld* signalling cascade) and antibiotic production (γ-butyrolactones) and signalling in bacteria is implicated in a wide range of biological phenomena such as competence, quorum sensing or biofilm formation (see review by Bassler and Losick 2006). C-factor, a 17 kDa protein, is involved in cell-to-cell communication essential for fruiting body formation and sporulation of *Myxococcus xanthus* (Kim and Kaiser, 1990a,b; Lobedanz and Sogaard-Andersen, 2003). Meanwhile, the complex programming of sporulation in *B. subtilis* relies on cross-talk between the mother cell and fore-spore compartments, where communication is achieved via secreted proteins, SpoIIR and SpoIVD (reviewed in Losick and Stragier, 1992; Rudner and Losick, 2001).

Could NepA be part of the *bld* signalling cascade? The first signal in the hierarchy of extracellular communication between a set of *bld* mutants was predicted to be a peptide (Nodwell and Losick, 1998). However, the *nepA* mutant restored aerial hyphae formation to the *bldJ* mutant (data not shown) that is predicted to be blocked in production of signal1. The *nepA* mutant was indistinguishable from the wild-type strain and exhibited extracellular complementation when grown next to *bldJ*, *bldA*, *bldC*, *bldD* or *bldH* on R2YE medium (data not shown). This suggests that either *nepA* is located to the 'right' of *bldD* or it is not part of the originally described *bld* cascade.

According to the early paper by Wildermuth (1970) the 'sterile' aerial fragments, likely to be identical to the sub-

Table 1. Bacterial strains used in this work.

Strain	Genotype or sequence	Reference or source
S. coelicolor		
A3(2)	SCP1$^+$ SCP2$^+$	Kieser *et al.* (2000)
M145	SCP1$^-$ SCP2$^-$	Kieser *et al.* (2000)
J660	*bldC18 mthB2 cysD18 agaA7* SCP1^{NF} SCP^{2*}	Merrick (1976)
J774	*bldD53 cysA15 pheA1 mthB2 strA1* SCP1^{NF} SCP^{2*}	Merrick (1976)
WC103	*bldG103 hisA1 uraA1 strA1* Pgl$^-$ SCP1$^-$ SCP2$^-$	Champness (1988)
J2168	Δ*bldC* derivative of M145	Hunt *et al.* (2005)
Δ*bldD*	Δ*bldD* derivative of M600	Elliot *et al.* (2003b)
K100	Δ*sigN*::*apr* derivative of M145	This work
K101	Δ*sigF*::*apr* derivative of M145	This work
K102	Δ*sigFN*::*apr* derivative of M145	This work
K103	M145/pK10	This work
K104	M145/pK11	This work
K105	M145/pK12	This work
K106	Δ*nepA*::*apr* derivative of M145	This work
J1978	Δ*sigF*::*thio* derivative of M145	Kelemen *et al.* (1998)
J2400	Δ*whiG::hyg* derivative of M145	Flardh *et al.* (1999)

apical stem compartment described in this article, frequently showed signs of lysis. Recently, two rounds of orderly process of cell disintegration have been demonstrated in *Streptomyces antibioticus*: the first round taking place in the young substrate mycelium while the second round affecting the aerial mycelium (Manteca *et al.*, 2006). It is conceivable to speculate that SigN might be involved in the ordered programme of cell death in *S. coelicolor* : the *sigNP2*-specific expression controlling events in the substrate mycelium, whereas the stem-specific, *sigNP1*-driven expression affecting the breakdown of the subapical stem compartment. Further microarray analysis of the *nepA* and *sigN* mutants grown on MM-glucose will be required to further elucidate the role of *nepA* and *sigN* during development together with the distinct developmental programme of the subapical stem compartment.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli BW25113 (Datsenko and Wanner, 2000) carrying pIJ790 was the host for recombination between the tailored apramycin resistance cassette and the target gene to generate knockout mutants. ET12567 (MacNeil *et al.*, 1992) containing pUZ8002 (Kieser *et al.*, 2000) was used to transfer plasmids or cosmids from *E. coli* into *S. coelicolor* by conjugation. *E. coli* DH5α (Hanahan, 1983) was used for routine cloning. The strains of *S. coelicolor* used in this work are listed in Table 1. *Streptomyces* strains were grown at 30°C on SFM, MM-glucose or MM-mannitol solid media or in tryptic soya broth liquid medium (Kieser *et al.*, 2000). In order to test the *sigN* mutant phenotype we also used a SF-glucose solid medium prepared like SFM, except that mannitol was replaced by 1% glucose. pIJ82 is a derivative of pSET152 (Bierman *et al.*, 1992) in which the apramycin resistance gene is replaced with the hygromycin resistance gene (H.M. Kieser, pers. comm.). pIJ82 was used to generate the construct for complementing K100 (*sigN::apr*).

Generation of knockout mutants

Mutant alleles of *sigN* and *sigF* were generated in cosmid D57 (a gift from H.M. Kieser) because in this cosmid, both *sigN* and *sigF* lie more than 7 kb from the cosmid ends. D57 was part of the original cosmid library, but did not become a member of the ordered set, where *sigN* is represented by two overlapping cosmids, 2St10A7 and 2StD60 (Redenbach *et al.*, 1996). To create the *nepA (SCO4002)* null allele we used cosmid 2St10A7. Knockout mutants were generated using PCR targeting (Gust *et al.*, 2003). The apramycin resistance cassette (apramycin resistance gene and *oriT*) was PCR-amplified using appropriate primers: for the *sigN* mutant, D13sigN1 and D13sigN2; for the *sigF* mutant, SigFknock.1 and SigFknock.2; for the *sigF-sigN* double mutant, SigFknock.1 and D13sigN2; for the *nepA* mutant, 4002-knock1 and 4002-knock2 (see Table 2). The PCR cassettes were then introduced into cosmid D57 or 2St10A7 in BW25113 carrying pIJ790 (Datsenko and Wanner, 2000) to generate D57/*sigN::apr*; D57/*sigF::apr*; D57/*sigFN::apr* and 2St10A7/*nepA::apr* mutants in *E. coli*. After the mutant alleles were passaged through the *dcm-dam*- ET12567 strain (MacNeil *et al.*, 1992) containing pUZ8002 they were introduced into *S. coelicolor* M145 by conjugation. The double-cross-over exconjugants were tested for resistance to apramycin and sensitivity to kanamycin. To confirm the mutants, chromosomal DNA was isolated and analysed by Southern blot hybridization.

Complementation of the sigN *mutation*

A ~1.4 kb BamHI–KpnI DNA fragment containing the entire *sigN* gene including its promoter sequences but no adjacent genes was first introduced into pIJ2925 (Janssen and Bibb, 1993) to generate pAT19. The *sigN* fragment was liberated from pAT19 using BglII, and introduced into the BamHI site

Table 2. List of oligonucleotides used in this work.

	Name	Oligonucleotide sequence
A	D13sigN1	CATGTCCGCAGAACAGGGCAGCTCGAAGGTGCTCGCGCTC**ATTCCGGGGATCCGTCGACC**
	D13sigN2	CGGCGCGGGCTCAGTCGGAGATGAGACCCTCGCGCAGCTG**TGTAGGCTGGAGCTGCTTC**
	SigFknock.1	ACGGAAGAGTTGACAGATCACGAGGTGGAGTTGACCGTG**ATTCCGGGGATCCGTCGACC**
	SigFknock.2	GCGCTTTCGACTCCGCCGGACTCCCGGCCGAGCTTATGC**TGTAGGCTGGAGCTGCTTC**
	4002-knock1	CTGGAAGCCGGTGCGCACTTGTTCTGGAGGAACAACATGCTAGC**ATTCCGGGGATCCGTCGACC**
	4002-knock2	CGGCGCGGCAGCGCACCCGCGCCGGGCCCCCTCAGCTCAGCTAGC**TGTAGGCTGGAGCTGCTTC**
B	4002-Kpn	TAAGT**GGTACC**GGTCTTATGTCAAGGCAGTTGG
	4002-Bam	GGTGC**GGATCC**CGCAGCGCCGGGAGCTTTGTCC
	4002-Bam2	GGTGC**GGATCC**CGTCCGTGCTGTCCCGGCATGG
	4002-Bam3	GGTGC**GGATCC**CGAATCAGGGCTTCCGGGGTGG
	P1sigN1	ATGTG**GGATCC**TGCCGGAGACGCGTTTGCC
	P1sigN2	GCGGA**GGTACC**ACCCCCTTTGGGTCGCGGG
	P2sigN1	GCTAC**GGATCC**GTAGTTTCCAGCGGTTGAG
	P2sigN2	GAGCC**GGTACC**CCGGCAAACGCGTCTCCGG
C	SIGN4	CTGGTGCGCCACCTGCTCGTCC
	SIGN5*	TCTCGCTCTCCGCGAGCGCGAGC
	SIGN7*	GGACATGCCACCCCCTTTGG
	SIGF15	GAACATCCGGCGCACCAGGCG
	SIGF16*	CGGCGCGGGCGGTGCTTGAGGCGC
	HRDB1*	GCCATGACAGAGACGGACTCGGCG
	HRDB2	CGGCCGCAAGGTACGAGTTGATGA
	4002.1*	GAGCAGCGGCGAGCGGAAGG
	4002.2	CAGCGCCGGGAGCTTTGTCC
D	SigNover1	GATAC**CATATG**TCCGCAGAACAGGGCAGCTCG
	SigNover2	GATAC**GAATTC**GGTCGCCGTGCTGTCGTACGGG

(A) Primers used to generate knockout mutations. Nucleotides corresponding to the apramycin resistance template are shown in bold. (B) Primers for generating *egfp* transcriptional fusions. Restriction endonuclease sites are shown in bold (B and D). (C) Primers used to produce probes during S1 nuclease protection assays. (D) Primers used to overexpress SigN.

of pIJ82, to create pAT20. pAT20 was introduced into *S. coelicolor* M145 and K100 by conjugation and hygromycin-resistant exconjugants, carrying pAT20 integrated to the ΦC31 attachment site on the chromosome, were examined for their phenotype.

S1 nuclease mapping

Mycelium grown on solid medium covered with cellophane discs or in liquid medium 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 of RNA in each assay. Probes labelled with ^{32}P at their single 5′ end were generated using PCR with two oligonucleotides, one labelled at its 5′ end with [γ-^{32}P]-ATP using T4 polynucleotide kinase. Probes were generated using the following oligonucleotide pairs: *sigN* probes using SIGN4 and either SIGN5* or SIGN7*; *sigF* probes using SIGF15 and SIGF16*; *hrdB* probes using HRDB1* and HRDB2; *nepA* probes using 4002.1* and 4002.2 (asterisks mark the labelled oligonucleotides). The probe for *hrdB*, encoding the principal sigma factor in *S. coelicolor*, was used as a control to assess the RNA preparations. DNA fragments protected by RNA were separated on 6% sequencing gels. Transcriptional start points of *sigN* and *SCO4002* were 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 the nepA–egfp *and* sigN–egfp *transcriptional fusions*

Two appropriate primers, 4002-Kpn and 4002-Bam, were used to generate a PCR product containing KpnI and BamHI sites, together with sequences 55 bp downstream and 360 bp upstream of the transcriptional start point of *nepA*. This PCR product was introduced into pIJ8660 (Sun *et al*., 1999) as a BamHI–KpnI fragment generating pK10, which was introduced into *S. coelicolor* M145 by conjugation. Apramycin-resistant exconjugants were selected and one of the representative strains, K103, was used to monitor *nepA* transcription. In a similar way, we generated pK11 and pK12, carrying truncated *nepA* fragments containing 126 bp and 55 bp sequences upstream of the transcriptional start respectively. The truncated sequences were generated by PCR using 4002-Bam2 with 4002-Kpn and 4002-Bam3 with 4002-Kpn oligonucleotides. The positions of the oligonucleotides compared with that of the *nepA* promoter are shown in Fig. 5D. pK11 and pK12 were introduced into *S. coelicolor* M145, generating K104 and K105 respectively. As pIJ8660 integrates into the ΦC31 *attB* site in the *S. coelicolor* chromosome, to maintain selection for the presence of the plasmids, we propagated K103, K104 and K105 on medium containing apramycin.

To generate *sigN–egfp* fusions we used DNA fragments created by PCR using the following oligonucleotides: P1sigN1 and P1sigN2 to carry only *sigNP1*; P2sigN1 and P2sigN2 to carry *sigNP2* exclusively; or P1sigN1 and P2sigN2 to carry both *sigNP1* and *sigNP2* promoters. These

PCR products were introduced into pIJ8660 as BamHI–KpnI fragments and the constructs generated were moved to *S. coelicolor* M145 as described above. Fluorescence was monitored using a Leica TCS SP2 laser-scanning confocal microscope with a 63×, 1.4NA oil-immersion objective.

Overexpression and purification of His-SigN

The *sigN* gene was introduced into pET28a (Novagen) as an NdeI–EcoRI fragment generated by PCR using the primers SigNover1 and SigNover2 followed by the appropriate restriction digests. One of the clones confirmed by sequencing was designated pK20 and moved into *E. coli* BL21 (DE3)pLysS (Novagen). Cultures of BL21(DE3)pLysS/pK20 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-SigN 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_2PO_4 and 50 mM NaCl, pH = 8.0 buffer at 4°C. The His-SigN 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 of His-SigN, core RNA polymerase from *E. coli* (Cambio), [α-^{32}P]-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. Templates carrying the *sigN* and *nepA* promoters were generated by PCR using the SIGN4, SIGN7 and 4002.1, 4002.2 oligonucleotide pairs respectively.

Scanning electron microscopy

Single colonies grown on solid medium were immobilized to the aluminium SEM stub using O.C.T. compound (BDH Laboratory Supplies, Poole, UK). The stub was then immediately plunged into liquid nitrogen slush at approximately –210°C to cryo-preserve the material. The sample was transferred onto the cryo-stage of a CT1500HF cryo-transfer system (Gatan, Oxford, UK) attached to a Philips XL30 FEG scanning electron microscope (Philips Electron Optics, FEI UK, Cambridge, UK). Sublimation of surface frost was performed at –95°C for 3 min before sputter-coating the sample with platinum for 2 min at 10 mA, below –110°C. After sputter-coating, the sample was moved onto the cryo-stage in the main chamber of the microscope, held at approximately –140°C. The sample was viewed at 3 kV and digital TIFF files were stored.

Acknowledgements

We thank Kim C. Findlay for assistance with the scanning electron microscopy, Dylan Sweetman for the stereomicroscopy images of *Streptomyces* colonies, Colin Smith and his laboratory for training and help in transcriptomics, Luis Servin-Gonzalez and Mark Buttner for RNA polymerase holoenzyme preparations from *S. coelicolor*, and Kate Bowdrey for her contribution to the overexpression of SigN. We are very grateful to David Hopwood, Keith Chater, Matthew Hutchings and Andrew Johnston for their valuable comments on the manuscript. This work was supported by BBSRC Grant G15940 to G.H.K.

References

Alper, S., Duncan, L., and Losick, R. (1994) An adenosine nucleotide switch controlling the activity of a cell type-specific transcription factor in *B. subtilis*. *Cell* **77:** 195–205.

Bassler, B.L., and Losick, R. (2006) Bacterially speaking. *Cell* **125:** 237–246.

Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) Improved prediction of signal peptides: SIGNALP 3.0. *J Mol Biol* **340:** 783–795.

Benson, A.K., and Haldenwang, W.G. (1993) *Bacillus subtilis* sigma B is regulated by a binding protein (RsbW) that blocks its association with core RNA polymerase. *Proc Natl Acad Sci USA* **90:** 2330–2334.

Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schoner, B.E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116:** 43–49.

Bignell, D.R., Warawa, J.L., Strap, J.L., Chater, K.F., and Leskiw, B.K. (2000) Study of the *bldG* locus suggests that an anti-anti-sigma factor and an anti-sigma factor may be involved in *Streptomyces coelicolor* antibiotic production and sporulation. *Microbiology* **146:** 2161–2173.

Bignell, D.R., Lau, L.H., Colvin, K.R., and Leskiw, B.K. (2003) The putative anti-anti-sigma factor BldG is post-translationally modified by phosphorylation in *Streptomyces coelicolor*. *FEMS Microbiol Lett* **225:** 93–99.

Boylan, S.A., Thomas, M.D., and Price, C.W. (1991) Genetic method to identify regulons controlled by nonessential elements: isolation of a gene dependent on alternate transcription factor sigma B of *Bacillus subtilis*. *J Bacteriol* **173:** 7856–7866.

Bucca, G., Brassington, A.M., Hotchkiss, G., Mersinias, V., and Smith, C.P. (2003) Negative feedback regulation of *dnaK*, *clpB* and *lon* expression by the DnaK chaperone machine in *Streptomyces coelicolor*, identified by transcriptome and *in vivo* DnaK-depletion analysis. *Mol Microbiol* **50:** 153–166.

Buttner, M.J., Fearnley, I.M., and Bibb, M.J. (1987) The agarase gene (Daga) of *Streptomyces-Coelicolor* a3(2) – nucleotide-sequence and transcriptional analysis. *Mol Gen Genet* **209:** 101–109.

Champness, W.C. (1988) New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *J Bacteriol* **170:** 1168–1174.

Chater, K.F. (2001) Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? *Curr Opin Microbiol* **4:** 667–673.

Chater, K.F., and Horinouchi, S. (2003) Signalling early developmental events in two highly diverged *Streptomyces* species. *Mol Microbiol* **48:** 9–15.

Chater, K.F., Bruton, C.J., Plaskitt, K.A., Buttner, M.J., Mendez, C., and Helmann, J.D. (1989) The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with the motility sigma factor of *B. subtilis*. *Cell* **59:** 133–143.

Cho, Y.H., Lee, E.J., Ahn, B.E., and Roe, J.H. (2001) SigB, an RNA polymerase sigma factor required for osmoprotection and proper differentiation of *Streptomyces coelicolor*. *Mol Microbiol* **42:** 205–214.

Claessen, D., Wosten, H.A., van Keulen, G., Faber, O.G., Alves, A.M., Meijer, W.G., and Dijkhuizen, L. (2002) Two novel homologous proteins of *Streptomyces coelicolor* and *Streptomyces lividans* are involved in the formation of the rodlet layer and mediate attachment to a hydrophobic surface. *Mol Microbiol* **44:** 1483–1492.

Claessen, D., Rink, R., de Jong, W., Siebring, J., de Vreugd, P., Boersma, F.G., *et al.* (2003) A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. *Genes Dev* **17:** 1714–1726.

Claessen, D., Stokroos, I., Deelstra, H.J., Penninga, N.A., Bormann, C., Salas, J.A., *et al.* (2004) The formation of the rodlet layer of streptomycetes is the result of the interplay between rodlins and chaplins. *Mol Microbiol* **53:** 433–443.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Delic, V., Pigac, J., and Sermonti, G. (1969) Detection and study of cosynthesis of tetracycline antibiotics by an agar method. *J Gen Microbiol* **55:** 103–108.

Duncan, L., and Losick, R. (1993) SpoIIAB is an anti-sigma factor that binds to and inhibits transcription by regulatory protein sigma F from *Bacillus subtilis*. *Proc Natl Acad Sci USA* **90:** 2325–2329.

Duncan, L., Alper, S., and Losick, R. (1996) SpoIIAA governs the release of the cell-type specific transcription factor sigma F from its anti-sigma factor SpoIIAB. *J Mol Biol* **260:** 147–164.

Elliot, M.A., and Talbot, N.J. (2004) Building filaments in the air: aerial morphogenesis in bacteria and fungi. *Curr Opin Microbiol* **7:** 594–601.

Elliot, M.A., Karoonuthaisiri, N., Huang, J., Bibb, M.J., Cohen, S.N., Kao, C.M., and Buttner, M.J. (2003a) The chaplins: a family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *Streptomyces coelicolor*. *Genes Dev* **17:** 1727–1740.

Elliot, M.A., Locke, T.R., Galibois, C.M., and Leskiw, B.K. (2003b) BldD from *Streptomyces coelicolor* is a non-essential global regulator that binds its own promoter as a dimer. *FEMS Microbiol Lett* **225:** 35–40.

Errington, J. (1993) *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol Rev* **57:** 1–33.

Flardh, K., Findlay, K.C., and Chater, K.F. (1999) Association of early sporulation genes with suggested developmental decision points in *Streptomyces coelicolor* A3(2). *Microbiology* **145:** 2229–2243.

Flardh, K., Leibovitz, E., Buttner, M.J., and Chater, K.F. (2000) Generation of a non-sporulating strain of *Streptomyces coelicolor* A3(2) by the manipulation of a developmentally controlled *ftsZ* promoter. *Mol Microbiol* **38:** 737–749.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci USA* **100:** 1541–1546.

Haldenwang, W.G. (1995) The sigma factors of *Bacillus subtilis*. *Microbiol Rev* **59:** 1–30.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166:** 557–580.

Hesketh, A.R., Chandra, G., Shaw, A.D., Rowland, J.J., Kell, D.B., Bibb, M.J., and Chater, K.F. (2002) Primary and secondary metabolism, and post-translational protein modifications, as portrayed by proteomic analysis of *Streptomyces coelicolor*. *Mol Microbiol* **46:** 917–932.

Homerova, D., Sevcikova, B., Sprusansky, O., and Kormanec, J. (2000) Identification of DNA-binding proteins involved in regulation of expression of the *Streptomyces aureofaciens* sigF gene, which encodes sporulation sigma factor sigma(F). *Microbiology* **146:** 2919–2928.

Hopwood, D.A. (1967) Genetic analysis and genome structure in *Streptomyces coelicolor*. *Bacteriol Rev* **31:** 373–403.

Hunt, A.C., Servin-Gonzalez, L., Kelemen, G.H., and Buttner, M.J. (2005) The *bldC* developmental locus of *Streptomyces coelicolor* encodes a member of a family of small DNA-binding proteins related to the DNA-binding domains of the MerR family. *J Bacteriol* **187:** 716–728.

Igo, M.M., and Losick, R. (1986) Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. *J Mol Biol* **191:** 615–624.

Janssen, G.R., and Bibb, M.J. (1993) Derivatives of pUC18 that have BglII sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. *Gene* **124:** 133–134.

Kalman, S., Duncan, M.L., Thomas, S.M., and Price, C.W. (1990) Similar organization of the *sigB* and *spoIIA* operons encoding alternate sigma factors of *Bacillus subtilis* RNA polymerase. *J Bacteriol* **172:** 5575–5585.

Karmazyn-Campelli, C., Bonamy, C., Savelli, B., and Stragier, P. (1989) Tandem genes encoding sigma-factors for consecutive steps of development in *Bacillus subtilis*. *Genes Dev* **3:** 150–157.

Kelemen, G.H., and Buttner, M.J. (1998) Initiation of aerial mycelium formation in *Streptomyces*. *Curr Opin Microbiol* **1:** 656–662.

Kelemen, G.H., Brown, G.L., Kormanec, J., Potuckova, L., Chater, K.F., and Buttner, M.J. (1996) The positions of the sigma-factor genes, *whiG* and *sigF*, in the hierarchy controlling the development of spore chains in the aerial hyphae of *Streptomyces coelicolor* A3(2). *Mol Microbiol* **21:** 593–603.

Kelemen, G.H., Brian, P., Flardh, K., Chamberlin, L., Chater, K.F., and Buttner, M.J. (1998) Developmental regulation of transcription of *whiE*, a locus specifying the polyketide spore pigment in *Streptomyces coelicolor* A3(2). *J Bacteriol* **180:** 2515–2521.

Kelemen, G.H., Viollier, P.H., Tenor, J., Marri, L., Buttner, M.J., and Thompson, C.J. (2001) A connection between stress and development in the multicellular prokaryote *Streptomyces coelicolor* A3(2). *Mol Microbiol* **40:** 804–814.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000) *Practical Streptomyces genetics.* Norwich, UK: John Innes Foundation.

Kim, S.K., and Kaiser, D. (1990a) C-factor: a cell–cell signaling protein required for fruiting body morphogenesis of *M. xanthus*. *Cell* **61:** 19–26.

Kim, S.K., and Kaiser, D. (1990b) Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein. *Proc Natl Acad Sci USA* **87:** 3635–3639.

Kodani, S., Hudson, M.E., Durrant, M.C., Buttner, M.J., Nodwell, J.R., and Willey, J.M. (2004) The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene *ramS* in *Streptomyces coelicolor*. *Proc Natl Acad Sci USA* **101:** 11448–11453.

Kwak, J., Dharmatilake, A.J., Jiang, H., and Kendrick, K.E. (2001) Differential regulation of ftsZ transcription during septation of *Streptomyces griseus*. *J Bacteriol* **183:** 5092–5101.

Lee, E.J., Cho, Y.H., Kim, H.S., Ahn, B.E., and Roe, J.H. (2004a) Regulation of sigmaB by an anti- and an anti-anti-sigma factor in *Streptomyces coelicolor* in response to osmotic stress. *J Bacteriol* **186:** 8490–8498.

Lee, E.J., Cho, Y.H., Kim, H.S., and Roe, J.H. (2004b) Identification of sigmaB-dependent promoters using consensus-directed search of *Streptomyces coelicolor* genome. *J Microbiol* **42:** 147–151.

Lobedanz, S., and Sogaard-Andersen, L. (2003) Identification of the C-signal, a contact-dependent morphogen coordinating multiple developmental responses in *Myxococcus xanthus*. *Genes Dev* **17:** 2151–2161.

Losick, R., and Stragier, P. (1992) Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. *Nature* **355:** 601–604.

MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H., and MacNeil, T. (1992) Analysis of streptomyces-avermitilis genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111:** 61–68.

Manteca, A., Fernandez, M., and Sanchez, J. (2006a) Cytological and biochemical evidence for an early cell dismantling event in surface cultures of *Streptomyces antibioticus*. *Res Microbiol* **157:** 143–152.

Masuda, E.S., Anaguchi, H., Yamada, K., and Kobayashi, Y. (1988) Two developmental genes encoding sigma factor homologs are arranged in tandem in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **85:** 7637–7641.

Mendez, C., and Chater, K.F. (1987) Cloning of whiG, a gene critical for sporulation of *Streptomyces coelicolor* A3(2). *J Bacteriol* **169:** 5715–5720.

Merrick, M.J. (1976) A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. *J Gen Microbiol* **96:** 299–315.

Min, K.T., Hilditch, C.M., Diederich, B., Errington, J., and Yudkin, M.D. (1993) Sigma F, the first compartment-specific transcription factor of *B. subtilis*, is regulated by an anti-sigma factor that is also a protein kinase. *Cell* **74:** 735–742.

Nodwell, J.R., and Losick, R. (1998) Purification of an extracellular signaling molecule involved in production of aerial mycelium by *Streptomyces coelicolor*. *J Bacteriol* **180:** 1334–1337.

Novakova, R., Bistakova, J., and Kormanec, J. (2004) Characterization of the polyketide spore pigment cluster whiESa in *Streptomyces aureofaciens* CCM3239. *Arch Microbiol* **182:** 388–395.

Pope, M.K., Green, B.D., and Westpheling, J. (1996) The *bld* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilization, morphogenesis and cell–cell signalling. *Mol Microbiol* **19:** 747–756.

Potuckova, L., Kelemen, G.H., Findlay, K.C., Lonetto, M.A., Buttner, M.J., and Kormanec, J. (1995) A new RNA polymerase sigma factor, sigma F, is required for the late stages of morphological differentiation in *Streptomyces* spp. *Mol Microbiol* **17:** 37–48.

Price, C.W. (2000) Protective function and regulation of the general stress response in *Bacillus subtilis* and related gram-positive bacteria. In *Bacterial Stress Responses*. Storz, G., and Hengge-Aronis, R. (eds). Washington, DC: American Society for Microbiology, pp. 179–197.

Redenbach, M., Kieser, H.M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H., and Hopwood, D.A. (1996) A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol Microbiol* **21:** 77–96.

Rigali, S., Nothaft, H., Noens, E.E., Schlicht, M., Colson, S., Muller, M., *et al.* (2006) The sugar phosphotransferase system of *Streptomyces coelicolor* is regulated by the GntR-family regulator DasR and links *N*-acetylglucosamine metabolism to the control of development. *Mol Microbiol* **61:** 1237–1251.

Roth, V., Aigle, B., Bunet, R., Wenner, T., Fourrier, C., Decaris, B., and Leblond, P. (2004) Differential and cross-transcriptional control of duplicated genes encoding alternative sigma factors in *Streptomyces ambofaciens*. *J Bacteriol* **186:** 5355–5365.

Rudner, D.Z., and Losick, R. (2001) Morphological coupling in development: lessons from prokaryotes. *Dev Cell* **1:** 733–742.

Ryding, N.J., Kelemen, G.H., Whatling, C.A., Flardh, K., Buttner, M.J., and Chater, K.F. (1998) A developmentally regulated gene encoding a repressor-like protein is essential for sporulation in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **29:** 343–357.

Sevcikova, B., Benada, O., Kofronova, O., and Kormanec, J. (2001) Stress-response sigma factor sigma(H) is essential for morphological differentiation of *Streptomyces coelicolor* A3(2). *Arch Microbiol* **177:** 98–106.

Shapiro, L., and Losick, R. (1997) Protein localization and cell fate in bacteria. *Science* **276:** 712–718.

Shapiro, L., and Losick, R. (2000) Dynamic spatial regulation in the bacterial cell. *Cell* **100:** 89–98.

Sun, J., Kelemen, G.H., Fernandez-Abalos, J.M., and Bibb, M.J. (1999) Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* **145:** 2221–2227.

Susstrunk, U., Pidoux, J., Taubert, S., Ullmann, A., and Thompson, C.J. (1998) Pleiotropic effects of cAMP on germination, antibiotic biosynthesis and morphological development in *Streptomyces coelicolor*. *Mol Microbiol* **30:** 33–46.

Takano, E., Tao, M., Long, F., Bibb, M.J., Wang, L., Li, W., *et al.* (2003) A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *Streptomyces coelicolor*. *Mol Microbiol* **50:** 475–486.

Viollier, P.H., Kelemen, G.H., Dale, G.E., Nguyen, K.T., Buttner, M.J., and Thompson, C.J. (2003) Specialized osmotic stress response systems involve multiple SigB-like

sigma factors in *Streptomyces coelicolor*. *Mol Microbiol* **47:** 699–714.
Wildermuth, H. (1970) Development and organization of the aerial mycelium in *Streptomyces coelicolor*. *J Gen Microbiol* **60:** 43–50.
Willey, J., Santamaria, R., Guijarro, J., Geistlich, M., and Losick, R. (1991) Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor*. *Cell* **65:** 641–650.
Willey, J., Schwedock, J., and Losick, R. (1993) Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*. *Genes Dev* **7:** 895–903.
Willey, J.M., Willems, A., Kodani, S., and Nodwell, J.R. (2006) Morphogenetic surfactants and their role in the formation of aerial hyphae in *Streptomyces coelicolor*. *Mol Microbiol* **59:** 731–742.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Monitoring *sigN*-specific green fluorescence. *S. coelicolor* M145 carrying *sigNP2–egfp* (A) and *sigNP1+2–egfp* (B) fusions were grown for 48 h on minimal medium containing mannitol alongside a microscope coverslip slide that was removed and viewed without fixation. Samples of (i) vegetative hyphae and (ii) aerial filaments during sporulation are shown with fluorescence images on the left or at the bottom and light microscopy control on the right or at the top. Arrows indicate the subapical stem compartments within the aerial hyphae.

Fig. S2. Multiple alignment of the nine SigB-like sigma factors of *S. coelicolor*. CLUSTALW (http://www.ebi.ac.uk/clustalw/) with default parameters was used to generate multiple alignment of the following: SigB_B.su,BG10735, P06574; SigF,SCO4035, P37971; SigG,SCO7341, O52313; SigH,SCO5243, Q7AKF9; SigI,SCO3068, Q7AKM6; SigJ,SCO0600, Q7AKS4; SigK,SCO6520, O86702; SigL,SCO7278, Q9X7S2; SigM,SCO7314, Q9K4K0; SigN,SCO4034, Q9ADM4 – shown in the order of name on figure; genomic identifier as in SubtiList (http://genolist.pasteur.fr/SubtiList/) or SCODB (http://streptomyces.org.uk/); UniProt accession number. The 2.4 and 4.2 domains involved in promoter recognition are boxed.

This material is available as part of the online article from http://www.blackwell-synergy.com

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

