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A connection between stress and development in the multicellular prokaryote *Streptomyces coelicolor* A3(2)

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Summary

Morphological changes leading to aerial mycelium formation and sporulation in the mycelial bacterium *Streptomyces coelicolor* rely on establishing distinct patterns of gene expression in separate regions of the colony. σ^H was identified previously as one of three paralogous sigma factors associated with stress responses in *S. coelicolor*. Here, we show that *sigH* and the upstream gene *prsH* (encoding a putative antisigma factor of σ^H) form an operon transcribed from two developmentally regulated promoters, *sigHp1* and *sigHp2*. While *sigHp1* activity is confined to the early phase of growth, transcription of *sigHp2* is dramatically induced at the time of aerial hyphae formation. Localization of *sigHp2* activity using a transcriptional fusion to the green fluorescent protein reporter gene (*sigHp2–egfp*) showed that *sigHp2* transcription is spatially restricted to sporulating aerial hyphae in wild-type *S. coelicolor*. However, analysis of mutants unable to form aerial hyphae (*bld* mutants) showed that *sigHp2* transcription and σ^H protein levels are dramatically upregulated in a *bldD* mutant, and that the *sigHp2–egfp* fusion was expressed ectopically in the substrate mycelium in the *bldD* background. Finally, a protein possessing *sigHp2* promoter-binding activity was purified to homogeneity from crude mycelial extracts of *S. coelicolor* and shown to be BldD. The BldD binding site in the *sigHp2* promoter was defined by DNase I footprinting. These data show that expression of σ^H is subject to temporal and spatial regulation during colony development, that this tissue-specific regulation is mediated directly by the developmental transcription factor BldD and suggest that stress and developmental programmes may be intimately connected in *Streptomyces* morphogenesis.

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Introduction

In unicellular prokaryotes, chromosome duplication is typically followed by formation of the division septum, cytokinesis and cell separation. However, in vegetatively growing *Streptomyces*, chromosome duplication is not followed by septation and cell separation. This generates a substrate mycelium of branching hyphal filaments that grows across and into the agar. Later, presumably in response to nutrient limitation, the substrate mycelium supports the development of specialized aerial hyphae that grow out of the aqueous environment of the substrate mycelium and into the air (Kelemen and Buttner, 1998), a phase of differentiation that requires the action of a number of 'bald' (*bld*) developmental regulatory genes, one of which, *bldD* (Merrick, 1976; Elliot *et al.*, 1998), is relevant to the work described here. When extension of the aerial hyphae stops, their multigenomic tips undergo synchronous, multiple septation to give rise to unigenomic prespore compartments (Schwedock *et al.*, 1997; Chater, 1998).

Localized morphological differences within the 'tissues' of the mycelial *Streptomyces* colony suggest that each has its own developmental programme. The spatial pattern of expression of individual genes in the different 'tissues' can now be defined by transcriptional fusions to an enhanced variant of the green fluorescent protein (EGFP). By this means, transcription of *sigF* (encoding the late spore-specific sigma factor σ^F; Potúčková *et al.*, 1995) and transcription from one of two *ftsZ* promoters were shown to be restricted to sporulating aerial hyphae (Sun *et al.*, 1999; Flärdh *et al.*, 2000). Conversely, transcription of *redD*, encoding a pathway-specific activator for the biosynthetic genes specifying the antibiotic undecylprodigiosin, was restricted to the substrate hyphae (Sun *et al.*, 1999), the site of undecylprodigiosin biosynthesis. The hyphae forming these distinctive 'tissues' may be subject to different environmental and metabolic stress conditions.

In the unicellular bacterium, *Bacillus subtilis*, diverse stresses, such as heat shock, salt stress and ethanol, induce a common set of proteins, called the general stress proteins (GSPs; Hecker *et al*., 1996; Hecker and Völker, 1998; Völker *et al*., 1999; Price, 2000). In this organism, the general stress response is controlled by σ^B (Price, 2000), and the vast majority of GSP genes ($>$ 100; Hecker *et al*., 1996; Völker *et al*., 1999) are induced from σ^B-dependent promoters. In addition to these non-specific GSPs, each stress induces a set of specific proteins that may confer specific protection. In contrast, two-dimensional gel analysis of changes in the *Streptomyces coelicolor* proteome after induction with various stresses shows that such treatments induce mutually exclusive stimulons (Vohradsky *et al*., 1997; 2000). Importantly, many osmotic shock and heat shock proteins are transiently induced during growth in liquid medium and differentiation on solid medium in *Streptomyces* (Bucca *et al*., 1995; Puglia *et al*., 1995; Vohradsky *et al*., 1997; 2000).

Recently, we identified three paralogous sigma factors (σ^H, σ^I and σ^J), closely related to *Bacillus subtilis* σ^B, that are associated with stress responses in *S. coelicolor*. σ^H was identified biochemically through its ability to direct *in vitro* transcription of a heterologous promoter, *ctc*, which is part of the σ^B general stress response regulon in *B. subtilis* (Viollier *et al*., submitted). When introduced into *S. coelicolor*, the *ctc* promoter was induced by salt shock, and the level of salt induction was significantly reduced in a *sigH* null mutant. The *ctc* promoter was also induced during the transition between exponential growth and stationary phase in *S. coelicolor* grown in liquid culture in the absence of exogenous stress. Unexpectedly, *sigH* was found to encode two N-terminally distinct isoforms, σ^{52} and σ^{37}, which arise from translation initiation at two distinct in frame start codons (Viollier *et al*., submitted). Based on their apparent molecular weights and their ability to direct transcription of the *ctc* promoter, these two isoforms of σ^H almost certainly correspond to two sigma factors identified by Westpheling *et al*. (1985) in the original description of RNA polymerase heterogeneity in *Streptomyces*. The open reading frame (*prsH*) lying immediately upstream of *sigH* encodes a putative antisigma factor (PrsH stands for putative regulator of SigH), suggesting that σ^H may be regulated post-translationally (Viollier *et al*., submitted). Here, we show that transcription of the *sigH* operon is subject to temporal and spatial regulation during *S. coelicolor* colony development, and that this tissue-specific regulation is mediated directly by the developmental transcription factor BldD.

Results

Transcription of sigH *is developmentally regulated*

Initial S1 nuclease mapping experiments showed that *prsH* and *sigH* form an operon transcribed from two promoters, initiating transcription 72 bp (*sigHp1*) *and* 133 bp (*sigHp2*) upstream of the *prsH* GTG start codon (Fig. 1). In *B. subtilis*, one of the two promoters of the *sigB* operon depends on σ^B itself (Yang *et al*., 1996). In the case of *sigH*, however, neither promoter resembles the *ctc* promoter that is transcribed by σ^H, and S1 nuclease protection analysis showed that both *sigHp1* and *sigHp2* were active in a constructed *sigH* null mutant (data not shown).

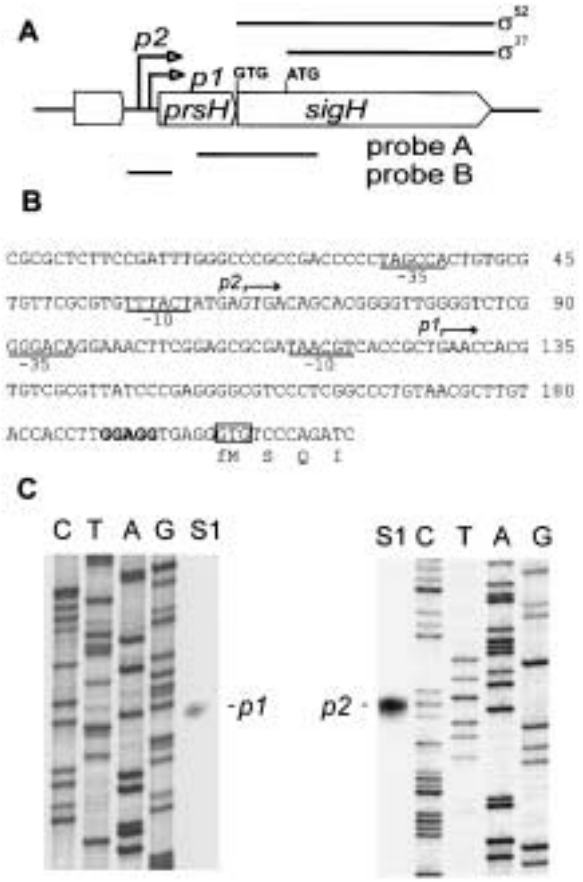

Fig. 1. Transcriptional organization of the *prsH–sigH* operon.
A. Genetic organization of the *sigH* locus showing the two N-terminally distinct isoforms of σ^H, σ^{52} and σ^{37}, which arise from translation initiation at two distinct in frame start codons (Viollier *et al*., submitted). Initial S1 nuclease mapping experiments using probe A gave full-length protection, indicating that all *sigH* transcription initiated upstream of the *Pst*I site internal to *prsH*.
B. Nucleotide sequence of the promoter region of the *prsH–sigH* operon showing the *sigHp1* and *sigHp2* transcription start points, their putative −10 and −35 sequences (underlined), the putative ribosome binding site (in bold) and the start of the *prsH* coding sequence.
C. High-resolution S1 nuclease mapping of the 5′ ends of the *sigH* transcripts using probe B. RNA was isolated from wild-type *S. coelicolor* A3(2) grown for 72 h on agar. Lanes labelled C, T, A and G represent a dideoxy sequencing ladder generated using the same radiolabelled oligonucleotide that was used to make S1 mapping probe B.

Transcription of the *sigH* operon was monitored by S1 mapping during differentiation of wild-type *S. coelicolor* on solid medium and during growth in liquid culture (Fig. 2). Transcription from *sigHp1* was readily detectable during all stages of liquid growth (Fig. 2A), but was seen only at the early time points on solid medium (Fig. 2A), suggesting that *sigHp1* activity was associated with vegetative growth. In contrast, the *sigHp2* transcript was undetectable during growth in liquid (apart from a faint signal in late stationary phase; Fig. 2A) and during vegetative growth in surface-grown cultures (Fig. 2B). However, on solid media, *sigHp2* expression was strongly induced at the time of aerial hyphae formation and continued throughout sporulation (Fig. 2B). The time course of RNA samples used in this study was the same as that used by Kelemen *et al*. (1996) to analyse the transcription of *sigF*, which encodes a sigma factor required for spore maturation. *sigF* transcription is confined to spore compartments and is blocked by mutations that prevent sporulation septum formation (Kelemen *et al*., 1996; Sun *et al*., 1999). Comparison of the two sets of data showed that the appearance of the *sigHp2* transcript precedes that of the *sigF* transcripts.

sigHp2 *is active only in the aerial hyphae*

The absence of *sigHp2* transcripts in liquid culture (Fig. 2A), conditions that do not support differentiation of *S. coelicolor*, and the temporal correlation of *sigHp2* promoter activity with the formation of aerial hyphae in surface-grown cultures (Fig. 2B) prompted us to examine the spatial location of *sigHp2* activity within differentiating colonies. A *sigHp2*–EGFP transcriptional fusion was generated using the reporter plasmid pIJ8660 (Sun *et al*., 1999), which integrates site specifically into the *S. coelicolor* chromosome at the phage ΦC31 *attB* site. The resulting *sigHp2*–EGFP reporter plasmid, pIJ6910, was introduced into the morphologically wild-type *S. coelicolor* strain 916, and *sigHp2* activity was monitored at different developmental stages using confocal fluorescence microscopy (Fig. 3).

sigHp2-mediated green fluorescence was detected transiently between 44 h and 60 h and only in the aerial parts of the colonies (Fig. 3). Thus, *sigHp2* activity not only correlated temporally with aerial growth, but was also spatially restricted to the aerial mycelium. Most of the fluorescence was localized to spore chains (Fig. 3), together with a few examples of apparently undifferentiated aerial hyphae. Most of the undifferentiated aerial hyphae and all the vegetative hyphae were non-fluorescent. The simplest interpretation of these results is that, in colonies of wild-type *S. coelicolor*, the *sigHp2* promoter is primarily active in the spores. However, because of the long half-life of the EGFP protein (Corish and Tyler-Smith, 1999) and the results described below, it is possible that the *sigHp2* promoter is activated just before sporulation septation.

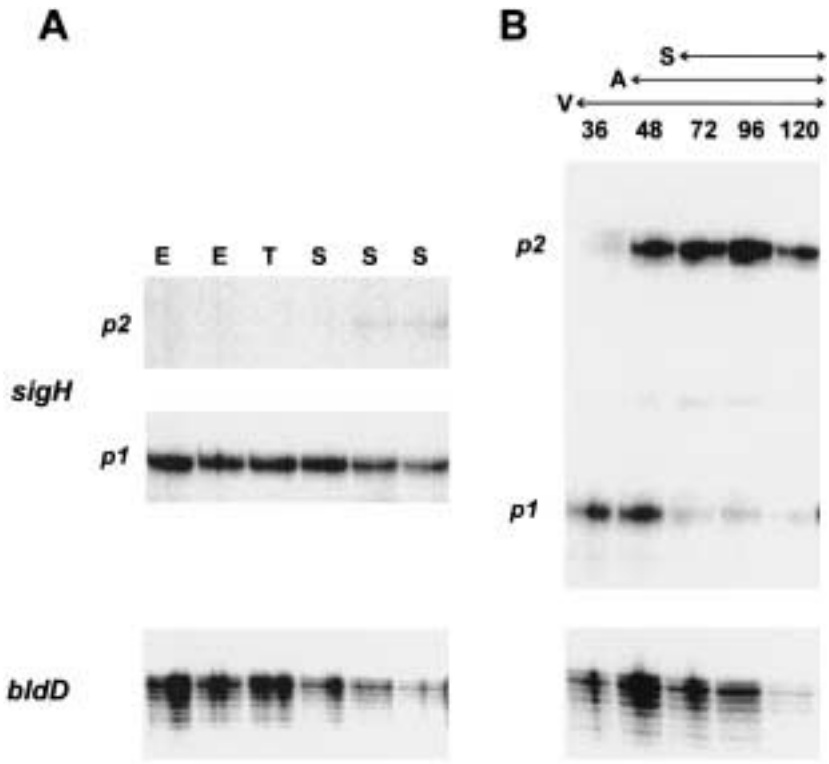

Fig. 2. A. S1 nuclease protection analysis of *sigH* and *bldD* transcription during growth of wild-type *S. coelicolor* in liquid medium [TSB containing 5% (w/v) PEG]. Mycelium was harvested at different time points during exponential growth (E), transition phase (T) and stationary phase (S).
B. S1 nuclease protection analysis of *sigH* and *bldD* transcription during development of wild-type *S. coelicolor* on solid medium (MM + mannitol). The time points (h) at which mycelium was harvested for RNA isolation and the presence of vegetative mycelium (V), aerial mycelium (A) and spores (S), as judged by microscopic examination, are shown.

Sporulation septum formation is not required for sigHp2 *activity*

The localization of *sigHp2*–EGFP fluorescence to spores raised the possibility that *sigHp2* expression might depend on genes required for sporulation. To address this question, *sigH* transcription was monitored in time courses of RNA samples isolated from six sporulation mutants, *whiA*, *B*, *G*, *H*, *I* and *J*, grown on solid medium. These six 'early' *whi* mutants are specifically affected in the initiation of sporulation, and three (*whiA*, *B and G*) are completely devoid of sporulation septa. Strong *sigHp2* activity was detected in all six *whi* mutants, showing that *P2* promoter activity does not depend on the formation of sporulation septa (data not shown).

sigH *is expressed ectopically in a* bldD *mutant*

To see whether transcription of the *sigH* operon was affected by any of the *bld* mutations that prevent the formation of aerial hyphae, RNA was isolated from *bldA*, *B*, *C*, *D*, *G and H* mutants grown on R5 solid medium. As these *bld* mutations exist in a complicated variety of genetic backgrounds (Table 1), only major variations in *sigH* mRNA levels with respect to wild-type *S. coelicolor* were considered potentially significant. The level of the transcript for *hrdB*, which encodes the principal, essential sigma factor of *S. coelicolor*, was monitored as an internal normalization control.

Transcription from *sigHp2* was detectable, but reduced, in five of the *bld* mutants, whereas transcription from *sigHp1* seemed unaffected (Fig. 4A; data not shown). However, strikingly, *sigHp2* transcription was dramatically upregulated in the *bldD* mutant J774 (Fig. 4A). To eliminate the possibility that differences in genetic background could account for this effect, *sigH* transcription was examined in RNA isolated from the original *bldD* isolate, 1169 (Merrick, 1976), and its congenic parent, 916; again, *sigHp2* transcription was dramatically upregulated (data not shown). Moreover, in 1169 (*bldD*), *sigHp2* transcripts were readily detectable in RNA isolated from liquid cultures under conditions in which no *sigHp2* transcripts were detected in 916 (*bldD*$^+$), indicating that repression of *sigHp2* in liquid medium depends, directly or indirectly, on *bldD*. Although transcription from *sigHp1* also appeared to be upregulated in J774 in Fig. 4A, in further independent experiments, *sigHp1* upregulation was not observed consistently in the *bldD* background.

Deregulation of *sigHp2* in a *bldD* background was also shown through analysis of *sigHp2*–EGFP reporter plasmid, pIJ6910, in 1169 (*bldD*). Like many *bld* mutants, 1169 has a bald phenotype on R5 medium, but forms aerial hyphae and sporulates on minimal medium containing mannitol as carbon source (Merrick, 1976). Strong *p2*-mediated green fluorescence was detected in all tissues of a sporulating colony of 1169/pIJ6910 grown on minimal medium containing mannitol (Fig. 3). Ectopic expression of the *sigHp2* promoter in the substrate mycelium was also detected when 1169/pIJ6910 was grown on R5 medium, when no aerial mycelium was formed (data not shown).

To see whether deregulation of the *sigHp2* promoter

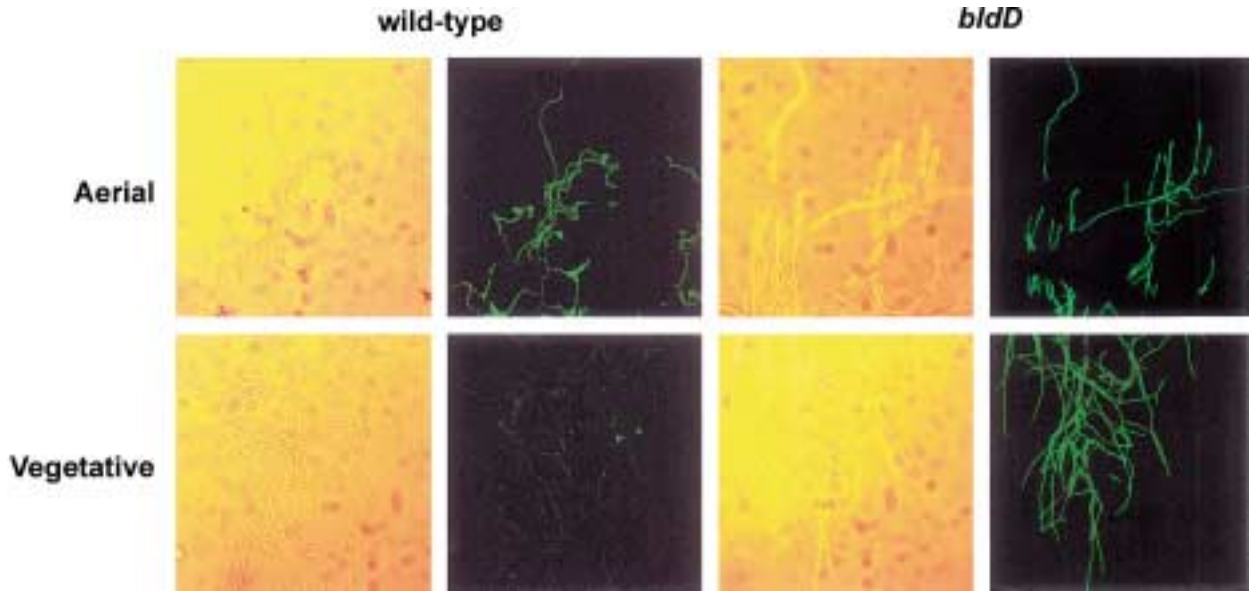

Fig. 3. Localization of *sigHp2* activity using the *egfp* reporter gene. Confocal microscope images of 916 (*bldD*$^+$; morphologically wild type) and 1169 (*bldD*), both carrying *sigHp2–egfp,* are shown. Fluorescence images are on the right. Strains were inoculated adjacent to a coverslip inserted at an angle of ≈ 60° into an MM agar plate containing 0.5% (w/v) mannitol (a carbon source that permits sporulation of the *bldD* mutant). Cultures were incubated for 72 h, and images were taken of the aerial and vegetative mycelium attached to the coverslip.

Table 1. Derivatives of *S. coelicolor* A3(2) used in this work.

Strain	Genotype	Reference
Wild type	Pgl$^+$ SCP1$^+$ SCP2$^+$	Kieser *et al.* (2000)
916	*hisA1 mthB2 pheA1 strA1* SCP1^{NF} SCP2*	Merrick (1976)
J1700	*bldA39 hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$	Kieser *et al.* (2000)
J669	*bldB43 mthB2 cysD18 agaA7* SCP1^{NF} SCP2*	Merrick (1976)
J660	*bldC18 mthB2 cysD18 agaA7* SCP1^{NF} SCP2*	Merrick (1976)
1169	*bldD53 hisA1 mthB2 pheA1 strA1* SCP1^{NF} SCP2*	Merrick (1976)
J774	*bldD53 cysA15 pheA1 mthB2 strA1* SCP1^{NF} SCP2*	Merrick (1976)
WC103	*bldG103 hisA1 uraA1 strA1* Pgl$^-$ SCP1$^-$ SCP2$^-$	Champness (1988)
WC109	*bldH109 hisA1 uraA1 strA1* Pgl$^-$ SCP1$^-$ SCP2$^-$	Champness (1988)

correlated with overexpression of σ^H, mycelial extracts were prepared from liquid-grown cultures of 1169 (*bldD*) and 916 (*bldD*$^+$). Immunoblot analysis of these extracts using σ^H polyclonal antiserum showed that both *sigH* primary translation products, σ^{52} and σ^{37}, were present at higher abundance in the *bldD* mutant (Fig. 4B).

The sigHp2 *promoter is a direct BldD regulatory target*

Biochemical experiments were initiated to identify the regulatory protein(s) mediating the temporal and spatial control of *sigHp2* activity. Gel retardation assays using crude mycelial extracts prepared from 916 (*bldD*$^+$) detected an activity able to retard the migration of a 127 bp fragment carrying both *sigH* promoters (*sigHp*). Importantly, this binding activity was absent from the *bldD* mutant 1169 grown under the same conditions (data not shown). The *sigHp*-binding activity was subsequently purified from *S. coelicolor* 916 crude extracts using a procedure that relies primarily on a site-specific DNA affinity column as the final chromatographic step (Folcher *et al.*, 2001). The *sigHp*-binding activity (as monitored by gel retardation) was partially purified by ammonium sulphate precipitation, DEAE–Sepharose chromatography and heparin–Sepharose chromatography, and then applied to a *sigHp* affinity matrix. After washing the column extensively, retained proteins were eluted stepwise with increasing concentrations of NaCl (0.2–1 M). These fractions were separated by SDS–PAGE and stained with Coomassie brilliant blue (Fig. 5A). A major band of 18 kDa correlated strongly with the gel-retarding activity; this protein, presumed to represent the *sigHp*-binding activity, was transferred to a polyvinylidene difluoride (PVDF)–nylon membrane, and its N-terminal sequence was determined by Edman degradation. The sequence obtained [S(S)EYAKQLGAKLRAIRTQQ] was identical to the predicted N-terminal sequence of BldD (Elliot *et al.*, 1998), but lacking the N-terminal *N*-formylmethionine.

To confirm that BldD bound *sigHp*, the *bldD* gene was cloned in an *Escherichia coli* expression plasmid (pET11c) to generate pVHP364. *sigHp*-binding activity, not present in the vector-containing host [BL21(DE3)/

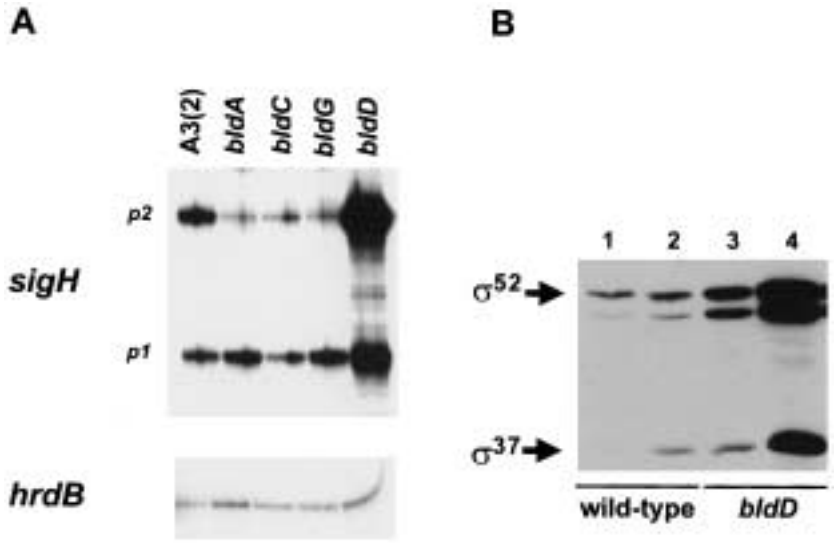

Fig. 4. A. S1 nuclease protection analysis of transcription of *sigH and hrdB* in representative mutants of four *bld* genes and in wild-type *S. coelicolor*. Mycelium was harvested for RNA isolation after 60 h growth on R5, a solid medium on which all the *bld* mutants fail to produce aerial mycelium, but on which the wild type sporulates normally. B. Immunodetection of σ^H in crude extracts of 916 (*bldD*$^+$) and 1169 (*bldD*). Crude extracts (10 μg) were prepared from 916 (lanes 1 and 2) and the *bldD* mutant (lanes 3 and 4) grown in J liquid medium (Puglia *et al.*, 1995) for 12 h (lanes 1 and 3) and 36 h (lanes 2 and 4) and subjected to immunoanalysis using the σ^H antibody. The two primary translation products of σ^H (σ^{37} and σ^{52}) are indicated.

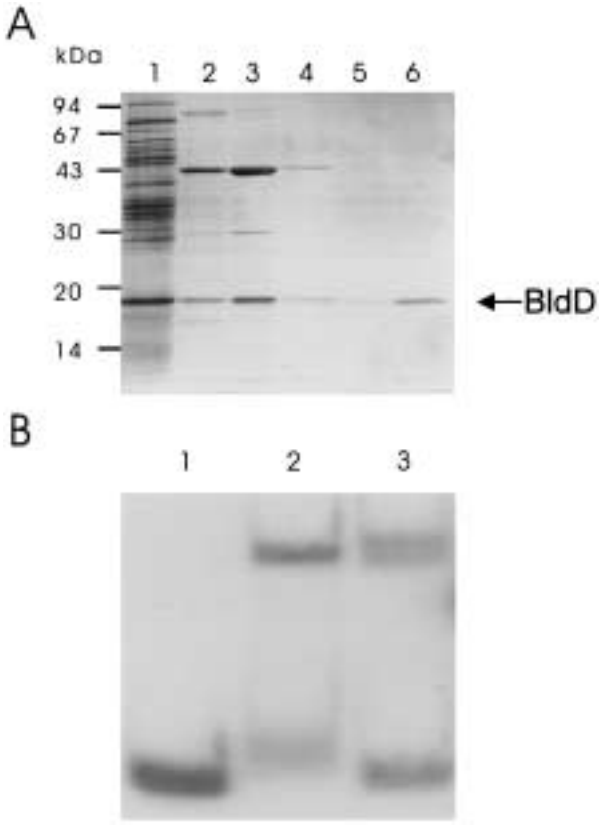

Fig. 5. A. The *sigHp*-binding activity in *S. coelicolor* 916 crude extracts was partially purified by sequential ammonium sulphate precipitation, DEAE–Sepharose chromatography and heparin–Sepharose chromatography. The active heparin–Sepharose fractions were applied to a *sigHp* affinity matrix, and proteins that interacted non-specifically were competed off the matrix using poly-(dI–dC)·poly-(dI–dC) (lane 1). Retained proteins were then eluted using increasing concentrations of NaCl in loading buffer (200 mM, lane 2; 400 mM, lane 3; 600 mM, lane 4; 800 mM, lane 5; and 1 M, lane 6). Samples (80 μl) of each eluted fraction (1ml) were resolved on a 15% (w/v) SDS–polyacrylamide gel and stained with Coomassie brilliant blue. The arrow on the right indicates BldD. The mobilities and molecular weights of the protein standards are shown on the left.
B. A 127 bp fragment carrying both *sigH* promoters (lane 1) was ^{32}P labelled and incubated with BldD purified from either *S. coelicolor* (lane 2) or *E. coli* (lane 3), and DNA–protein complexes were separated from unbound DNA on a TBE–polyacrylamide gel.

pET11c; data not shown], was detected in crude extracts of BL21(DE3)/pVHP364 after induction with IPTG. When the recombinant BldD was purified from *E. coli* (as described for *S. coelicolor*), it retarded the 127 bp fragment in a manner indistinguishable from native BldD (Fig. 5B).

To localize the BldD binding site, the *sigH* promoter region was subcloned to provide fragments corresponding to *p1* (Fig. 1, co-ordinates 64–138) or *p2* (Fig. 1, co-ordinates 9–78 or 1–106). BldD only retarded the *sigHp2* fragments, placing its binding site between nucleotide co-ordinates 9 and 78. The site was mapped more precisely by DNase I footprinting of the template strand (Fig. 6A); BldD protected a 44 bp sequence extending from −34 to +10 with respect to the *sigHp2* transcriptional start site (Fig. 6B). The BldD footprints on the *bldDp* (Elliot and Leskiw, 1999) and *sigHPp2* promoters are compared in Fig. 6B. Both footprints are of a similar size (42–44 bp), extending approximately from the −35 hexamer to about 10 bp downstream of the transcription start site. However, comparison of the sequences within these two footprints does not suggest a clear-cut consensus sequence for BldD binding (Fig. 6B).

The sigHp2 *and* bldD *promoters are differentially regulated*

Like *sigHp2*, the *bldD* promoter is repressed directly by the BldD protein (Elliot and Leskiw, 1999). However, the expression patterns of these two promoters are different. Although *sigHp2* is active only in the aerial mycelium of wild-type *S. coelicolor* (Figs 2 and 3A), the *bldD* promoter is active during vegetative growth (Elliot *et al*., 1998). To confirm that these apparent differences are real and did not arise from differences in growth conditions or genetic background, *bldD* transcription was monitored in the same RNA samples that were used to monitor *sigH* expression during development in both solid and liquid grown cultures (Fig. 2). Although *sigHp2* transcripts were detected only during aerial mycelium formation in surface-grown cultures of wild-type *S. coelicolor* and were hardly detectable in liquid culture, *bldD* transcription was readily detectable in all stages of liquid and surface-grown cultures.

Heat shock and ethanol also induce sigHp2 *activity*

The implication that σ^H is involved in the osmotic shock response (Viollier *et al*., submitted) prompted us to analyse the response of the *sigH* promoters to other stresses [Fig. 7; while this work was in progress, similar analyses were reported by Kormanec *et al*. (2000)]. Although *sigHp1* was not affected by any of the stresses applied, *sigHp2* was strongly induced by heat shock and, to a lesser extent, by ethanol, but not by osmotic or oxidative stresses. To see whether these effects on the *sigHp2* promoter might be mediated through the expression of *bldD*, *bldD* transcription was monitored in the same RNA samples. *bldD* transcription was not affected by any of the applied stresses (Fig. 7). Given that BldD represses its own promoter (Elliot *et al*., 1998; Elliot and Leskiw, 1999) as well as *sigHp2*, if BldD were inactivated by heat or ethanol, *bldD* transcription might also be expected to be affected by these stresses. It is more likely, therefore, that the stress induction of *sigHp2* is mediated by other, as yet unidentified, regulatory protein(s).

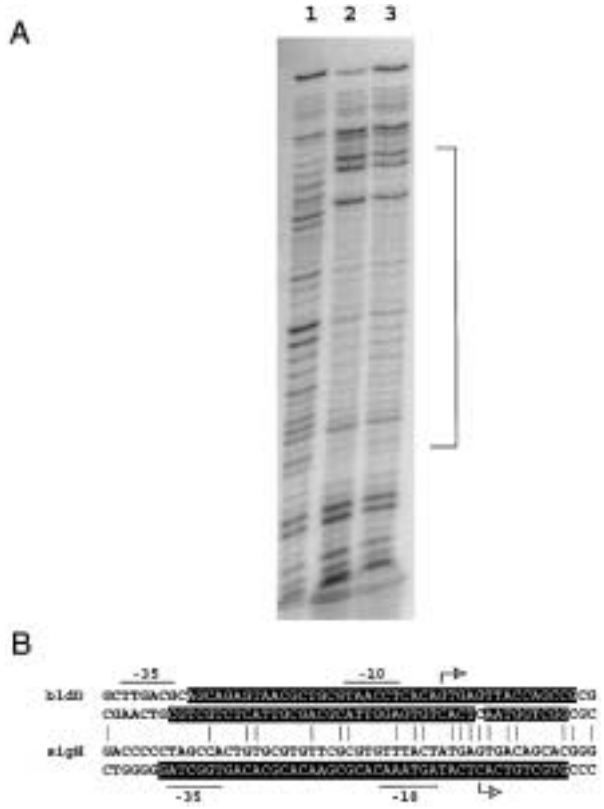

Fig. 6. Localization of the BldD binding site by DNase I footprint analysis.
A. A fragment carrying the *sigHp2* promoter (co-ordinates 1–106; Fig. 1B) was end labelled on the template strand, incubated alone (lane 1) or with BldD (50 pmol, lanes 2 and 3) purified from *S. coelicolor* (Fig. 5A) and then treated with DNase I for 5 (lanes 1 and 3) or 10 (lane 2) min (see *Experimental procedures*).
B. Comparison of the BldD footprints on the *bldD* (Elliot and Leskiw, 1999) and *sigHp2* promoters. The extent of the DNase I-protected regions are highlighted, transcription start points are marked by arrows, and putative −10 and −35 promoter hexamers are underlined or overlined.

Discussion

sigH *transcription is developmentally regulated*

The activities of the two *sigH* promoters are strictly correlated with morphological development; the formation of aerial hyphae is accompanied by the strong induction of *sigHp2* and the disappearance of *sigHp1* transcripts. In addition, *sigHp2* transcripts are virtually undetectable in liquid culture, conditions in which *S. coelicolor* does not differentiate. Using a *sigHp2*–EGFP transcriptional fusion, *sigHp2*-mediated fluorescence was found to be absent from the substrate mycelium, but was detected in spore chains and in a few aerial hyphae that were not overtly septating (without specific staining, septation can only be inferred indirectly, as the spore compartments begin to round off). However, monitoring transcription from *sigHp2* in early *whi* mutants showed that the activation of *sigHp2* does not depend on the formation

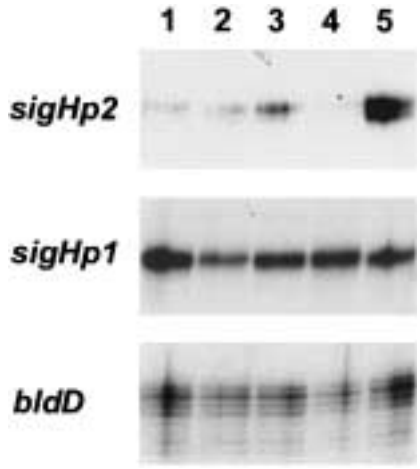

Fig. 7. S1 nuclease protection analysis of the effects of different stress conditions on transcription of *sigH* and *bldD*. Wild-type *S. coelicolor* was grown in liquid MM containing 5% PEG to late exponential phase and exposed to 0.5 M NaCl (lane 2), 5% (v/v) ethanol (lane 3), 1 mM H_2O_2 (lane 4) or temperature upshift to 41°C (lane 5) for 20 min. As a control, a sample was left untreated for 20 min (lane 1).

of sporulation septa. These observations suggest that the developmental activation of *sigHp2* might be associated with an earlier event, such as the cessation of growth of the aerial hyphae, rather than the induction of sporulation *per se*.

BldD is a regulator of the spatial organization of gene expression

bldD is one of 13 known loci required for aerial hyphae formation in *S. coelicolor*. On rich media, there is evidence for the involvement of a complex signalling cascade in the initiation of aerial mycelium formation, involving at least five different extracellular signals (Willey *et al*., 1993; Kelemen and Buttner, 1998). The steps in the signalling pathway have been defined genetically, and *bldD* is required to sense or respond to the last extracellular signal in the cascade (Willey *et al*., 1993). BldD acts as a repressor of its own promoter, *bldDp* (Elliot *et al*., 1998; Elliot and Leskiw, 1999), as well as *sigHp2*. Both promoters are strongly upregulated in a *bldD* mutant and, in both cases, BldD protects regions of similar sizes (42–44 bp) extending approximately from the −35 hexamer to about 10 bp downstream of the transcription start site (Fig. 6; Elliot *et al*., 1998; Elliot and Leskiw, 1999).

Recently, more targets for direct BldD repression have been identified (Elliot *et al*., 2001; J. Tenor, unpublished), including *bldN*, which encodes a sigma factor required for the initiation of aerial mycelium formation (Bibb *et al*., 2000), and *whiG*, which encodes a sigma factor that plays a critical role in triggering sporulation in aerial hyphae (Chater *et al*., 1989). Here, we show that BldD regulates

tissue-specific expression of *sigH*; the *sigHp2* promoter is repressed by BldD in vegetative hyphae, but this repression is released in sporulating aerial hyphae. Given the involvement of BldD in regulating these three key sigma factor genes, it seems clear that *bldD* is a critical regulator of biological processes associated with development, acting as a repressor of developmental genes during vegetative growth.

How is BldD activity regulated?

Our data show that BldD repression of *sigHp2* is relieved at a certain point in the development of aerial hyphae. How is this relief of repression achieved? Many explanations are possible, including modification or proteolysis of BldD. In this regard, it is interesting to note that the Clp ATP-dependent protease plays an essential role in aerial mycelium formation in *Streptomyces* (de Crécy-Lagard *et al*., 1999). Analysis of the BldD amino acid sequence may yield important clues for other models. Although simple BLAST searches did not identify significant BldD homologues (Elliot *et al*., 1998), PSI-BLAST and PFAM searches indicate that the N-terminal 69 amino acid residues of BldD show statistically significant similarity to the HTH-3 family of DNA-binding domains (PFAM 01381; *E*-value 1.6×10^{-11}). Members of the HTH-3 family include SinR, which acts as a tetrameric repressor that inhibits the expression of genes essential for entry into sporulation in *B. subtilis*, including *spo0A* and *spoIIA* (Mandic-Mulec *et al*., 1995). Thus, both BldD and SinR seem to act during vegetative growth to repress genes involved in development. The repressor activity of SinR is abolished through interaction with a partner protein, SinI, which disrupts the SinR tetramer through the formation of a SinI–SinR heterodimer (Lewis *et al*., 1998). BldD might be inactivated similarly through an interaction with a cognate partner protein. Finally, the only known *bldD* mutant allele (*bldD53*) carries a Tyr to Cys mutation at residue 62 (close to the C-terminal end of the proposed HTH-3-related DNA-binding domain), which led Elliot *et al*. (1998) to speculate that BldD might be regulated by phosphorylation.

The regulatory pathways controlling morphogenesis and stress responses in S. coelicolor *are connected*

The results reported here suggest that stress and development may be intimately connected in *S. coelicolor*. *sigH* was initially studied because of its involvement in stress responses. The smaller form of σ^H, σ^{37}, was purified by virtue of its ability to direct *in vitro* transcription of a heterologous promoter, *ctc* (Westpheling *et al*., 1985; Viollier *et al*., submitted), which is part of the σ^B general stress response regulon in *B. subtilis* (Price, 2000). When introduced into *S. coelicolor*, the *ctc* promoter is induced by salt shock, and the level of induction is significantly reduced in a *sigH* null mutant (Viollier *et al*., submitted). In addition, *sigHp2* is substantially induced by both heat shock and ethanol. However, it is now clear that *sigH* expression is also subject to developmental regulation, mediated by *bldD*, a gene forming part of the developmental cascade leading to the morphogenesis of a new cell type, the aerial hyphae.

The discovery that BldD spatially and temporally controls the *p2* promoter of this stress-inducible gene (*sigH*) has two important implications. First, it identifies BldD as a regulator of tissue-specific gene expression during *Streptomyces* development and suggests that the restriction of *sigHp2* activity to a specific stage and region during morphogenesis is achieved by modulating BldD activity in a tissue-specific manner. Secondly, it serves as a paradigm for linkage between stress response and developmental programmes. It seems unlikely that the connection between *sigH* expression and colony morphogenesis will prove to be unique. Instead, it suggests a more general regulatory organization, in which tissues with different developmental fates may have specialized stress response systems, reminiscent of the distinct heat shock stimulons induced during different physiological phases in liquid culture (Puglia *et al*., 1995).

Although it is clear that morphological development influences the expression of *sigH*, these results also raise the question as to whether stress response systems might influence developmental functions. Our recent findings suggest that the *prsH sigH* operon can influence development, perhaps through cross-talk with other sigma factors; although a *sigH* mutant sporulates normally, *a prsH sigH* double mutant is conditionally bald (P. H. Viollier, unpublished data), implying that PrsH must have a role beyond the regulation of σ^H. PrsH is homologous to SpoIIAB and RsbW, antisigma factors that regulate σ^F and σ^B, respectively, in *B. subtilis* (Alper *et al*., 1996; Yang *et al*., 1996), and PrsH probably plays a similar role as it co-purifies with σ^H (P. H. Viollier and A. Weihofen, unpublished data). However, unlike *B. subtilis*, the *S. coelicolor* genome encodes at least eight σ^B-type proteins. Two of the corresponding genes (*sigI* and *sigJ*) are adjacent to putative antisigma factor and anti-antisigma factor genes, three (*sigH*, *sigL* and *sigM*) are adjacent only to an antisigma factor gene, and three (*sigG*, *sigK* and *sigN*) are adjacent to neither. This genetic organization raises the possibility of cross-talk between the products of these loci, giving the potential for a complex stress response system that could be integrated into the network of developmental genes. Together with previous results (Vohradsky *et al*., 2000), the data presented here support the concept that evolutionary pressures have

caused stress-regulatory systems to become adopted as part of a developmental programme in *S. coelicolor*.

Experimental procedures

Bacterial strains, plasmids, growth conditions and conjugal plasmid transfer from E. coli *to* Streptomyces

The *S. coelicolor* strains used are shown in Table 1. For RNA isolation from liquid cultures, tryptic soy broth (TSB) containing 5% PEG8000 (Sigma) was inoculated with spores of wild-type *S. coelicolor* A3(2), and mycelium were collected at different stages of growth. For RNA isolation from solid cultures, spores of *S. coelicolor* A3(2) or mycelial fragments of the *whi* and *bld* mutants were inoculated onto sterile cellophane disks on the agar surface of R5 (Kieser *et al*., 2000) or minimal medium (MM; Kieser *et al*., 2000) containing 0.5% (w/v) mannitol as carbon source and appropriate auxotrophic supplements. Unmethylated plasmids were conjugated from the *dam dcm hsdS E. coli* strain ET12567 (MacNeil *et al*., 1992) into *S. coelicolor* as described by Ryding *et al*. (1999).

RNA isolation and S1 nuclease mapping

RNA was isolated and S1 nuclease mapping was performed using 30 μg of RNA as described by Kelemen *et al*. (1996). All the probes were uniquely labelled on single 5′ ends. *sigH* probe A (Fig. 1A) was a *Bss*HII–*Hin*dIII fragment (Fig. 1A) uniquely labelled at the *Bss*HII site; the *Hin*dIII site was derived from the polylinker of pUC18. The remaining probes were generated by polymerase chain reaction (PCR) using pairs of cold and radiolabelled oligonucleotides. Oligonucleotides were radiolabelled on their 5′ ends using [γ-^{32}P]-ATP (3000 Ci mmol^{-1}) and T4 polynucleotide kinase. The oligonucleotides used were: for *sigH* probe B (Fig. 1A), 5′-CTAG CCACTGTGCGTGTTCGCGTG-3′ and 5′-GCAGCACCGAC AGGTAGGCACC-3′ (labelled); for *bldD*, 5′-ACCTTAAAGGA CGTCGGATATGCG-3′ and 5′-CGAGCTGTTTGGCGTATT CGCTGG-3′ (labelled); and for *hrdB*, 5′-CGGCCGCAAGGT ACGAGTTGATGA-3′ and 5′-GCCATGACAGAGACGGACT CGGCG-3′ (labelled). The sequencing ladders were generated by dideoxy chain termination using the same radiolabelled oligonucleotide primer that was used to generate *sigH* probe B.

GFP fusions and monitoring GFP expression

A 314 bp DNA fragment carrying the *sigHp2* promoter was amplified by PCR using the oligonucleotide primers 5′-ACCC CGGTACCGTGCTGTCACTCATAGTAAACACG-3′ and 5′-GGCACGGATCCGGTGCAGCTGAGGTTGTGTCTCG-3′ and chromosomal DNA as the template. The PCR product was cleaved with *Bam*HI and *Kpn*I and cloned into pIJ8660 (Sun *et al*., 1999) that had been cut with the same enzymes to generate pIJ6910; the insert was confirmed by sequencing. pIJ6910 and the parent vector, pIJ8660, were introduced into *S. coelicolor* 916 (*bldD*$^{+}$) and 1169 (*bldD*) by conjugation from *E. coli*. Confocal microscopy was carried out using a Leica TCS NT confocal microscope (488 nm argon laser, × 63 objective, 0.5 numerical aperture dry PL Fluotar).

Purification of BldD from S. coelicolor

BldD was purified from *S. coelicolor* crude extracts using gel retardation as an assay. Unless otherwise stated, all purification steps were performed at 4°C. *S. coelicolor* 916 mycelium (200 g wet weight) grown to early exponential phase in a fermentor containing 50 l of J medium (Puglia *et al*., 1995) was lysed by sonication in 600 ml of TA buffer containing 50 mM NaCl and Complete^{TM} protease inhibitors (Roche Biochemicals). The cellular debris was removed by centrifugation for 30 min at 15 000 r.p.m. in a GSA rotor (Sorvall). The supernatant was clarified further by ultracentrifugation for 45 min at 45 000 *g*. Proteins that precipitated in 30% (w/v) ammonium sulphate were removed by centrifugation, and the ammonium sulphate concentration of the supernatant was brought to 60% (w/v). Precipitated proteins were collected by centrifugation and resuspended in 400 ml of TA buffer [10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, 10% glycerol] supplemented with 50 mM NaCl. The sample was dialysed in TA buffer to give a conductivity equal to 50 mM NaCl. Two fractionation steps were performed with fast protein liquid chromatography (FPLC). First, the sample was loaded onto a 100 ml DEAE Fast Flow column (XK26/50; Amersham Pharmacia) that had been equilibrated in buffer TA containing 50 mM NaCl. Retained proteins were eluted in 540 ml of TA buffer by applying a linear NaCl gradient from 50 mM to 600 mM. Fractions containing *sigHp*-binding activity were pooled and diluted in TA buffer to give a conductivity equal to 100 mM. This sample was then applied to a 25 ml heparin Cl-6B Sepharose column (XK16/20; Amersham Pharmacia) that had been equilibrated in buffer TA containing 50 mM NaCl. After extensive washing, bound proteins were eluted with a 500 ml linear gradient ranging from 50 mM to 600 mM NaCl in buffer TA. Fractions that contained activity were dialysed and concentrated by ultrafiltration (YM10; Amicon) to 15 ml in buffer TA supplemented with 50 mM NaCl. Subsequently, affinity chromatography was performed using the DNA affinity column containing the *sigH* upstream region (described below). Immediately after incubation of the partially purified sample with the DNA affinity column, proteins that bound unspecifically were removed by performing a wash in the loading buffer (TA buffer containing 50 mM NaCl) that contained 0.2 mg ml^{-1} competitor DNA [poly-(dI–dC)·poly-(dI–dC)]. Thereafter, bound proteins were eluted by washing the magnetic beads stepwise in TA buffer with increasing NaCl concentrations. After each washing step, the NaCl concentration was raised by 200 mM until a final NaCl concentration of 1 M was reached. The 1 M NaCl wash was performed twice to maximize recovery of specifically bound protein. Three such elution cycles were performed until no gel-retarding activity remained in the flowthrough.

Preparation of a sigHp *DNA affinity column*

pVHP378 is a derivative of the vector pJS14 carrying a 127 bp fragment of DNA (5′-TTCCG ATTTGGGCCC……. TCACCGCTGAACCAC-3′) that contains both the *sigH* promoters (*sigHp*). Approximately 2 mg of a 300 bp biotinylated DNA fragment including this 127 bp sequence was

prepared by setting up 200 PCR reactions using universal primer, biotinylated reverse primer and pVHP378 as the template. Unincorporated biotinylated primer was separated from the biotinylated PCR product by perfusion chromatography on a Poros HQ/F (Roche Biochemicals). The biotinylated *sigHp* fragment was coupled to streptavidin-coated magnetic particles (Roche Biochemicals) as described by Folcher *et al*. (2001). Briefly, 1 ml of streptavidin-coated magnetic particles was incubated with 4 ml of the *sigHp* fragment (0.5 mg ml^{-1}) in TA buffer for 1 h with gentle agitation. The magnetic particles were washed three times in TA buffer containing 1 M NaCl and then resuspended in TA buffer containing 50 mM NaCl.

Expression of bldD *in* E. coli

The *bldD* gene was PCR amplified, cloned as an *Nde*I–*Eco*RI fragment into pET11c (Novagen) and its correct sequence confirmed. This plasmid, pVHP364, was used to overexpress *bldD* in *E. coli* BL21(DE3) (Novagen). An overnight culture of BL21(DE3)/pVHP364 grown at 37°C in LB was diluted 1:100 in LB, grown to an OD_{600} of 0.4 at room temperature and then induced with 1 mM IPTG. Cells were harvested after 4 h at room temperature. BldD was purified from *E. coli* as described for *S. coelicolor*.

DNase I footprinting

A fragment containing *sigHp2* (co-ordinates 1–106; Fig. 1B) was PCR amplified using primers that introduced terminal *Hin*dIII and *Eco*RV sites and cloned into pGEMT easy (Promega) to create pJLT12. The fragment was released from pJLT12 by digestion with *Hin*dIII and *Eco*RV and end-labelled on the 3′ end of the template strand by filling in the *Hin*dIII site using the Klenow fragment of DNA polymerase (New England BioLabs) and [α-^{32}P]-dATP. The radiolabelled probe (5 pmol) was incubated for 30 min at room temperature with ≈ 50 pmol of BldD (purified from *S. coelicolor*) in 20 μl of TA buffer containing 5 μg of poly-(dI–dC)·poly-(dI–dC) (Pharmacia). The DNA was partially digested with DNase I (Roche Biochemicals) at room temperature, precipitated and washed in ethanol, resuspended in 10 μl of stop solution (98% deionized formamide, 10 mM EDTA) and heat denatured (90°C for 5 min, then immediately placed on ice). Digestion products were resolved on an 8% (w/v) polyacrylamide gel and visualized by autoradiography.

Gel mobility shift assays

The probe used for the gel retardation assays was a ^{32}P-labelled 127 bp fragment carrying both *sigH* promoters, isolated from pVHP385 (pVHP385 is a derivative of the vector pOK12 carrying a 127 bp *Bam*HI–*Pml*I fragment). Protein samples were mixed with the labelled probe (2–5 ng) in a total volume of 20 μl of TA buffer containing 50 mM NaCl and 2 μg of competitor DNA [poly-(dI–dC)·poly-(dI–dC)] for 5 min at room temperature. The reaction mixtures were then resolved on a non-denaturing 5% (w/v) polyacrylamide gel in TBE buffer (90 mM Tris, 90 mM borate and 2 mM EDTA, pH 8) run at room temperature and constant voltage (7 V cm^{-1}) for 2–4 h. After migration, gels were dried and the bands visualized by autoradiography.

Immunoblotting

Crude mycelial lysates containing 20 μg of protein were mixed with SDS–PAGE sample buffer, boiled, run on a 10% (w/v) SDS–polyacrylamide gel and transferred to PVDF membrane (Millipore) for immunodetection using the ECL Plus system (Amersham). Anti-σ^H antiserum (Viollier *et al*., submitted) was used as the primary antibody (1:10 000 dilution) and peroxidase-conjugated pig anti-rabbit IgG (Dako; 1:10000 dilution) as the secondary antibody.

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