Specialized osmotic stress response systems involve multiple SigB-like sigma factors in *Streptomyces coelicolor*

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Summary

Whereas in *Bacillus subtilis*, a general stress response stimulon under the control of a single sigma factor (SigB) is induced by different physiological and environmental stresses (heat, salt or ethanol shock), in *Streptomyces coelicolor*, these environmental stresses induce independent sets of proteins, and its genome encodes nine SigB paralogues. To investigate possible functions of multiple *sigB*-like genes in *S. coelicolor*, *Pctc*, a promoter routinely used to assay SigB activity *in vivo*, was analysed as a heterologous reporter. The fact that *Pctc* was activated by osmotic shock, but not by heat or ethanol, confirmed that stress response system(s) could operate independently in *S. coelicolor*. *Pctc* was also induced transiently during growth of liquid cultures, presumably by nutritional signals. We purified an RNA polymerase holoenzyme from crude extracts that catalysed specific transcription of *Pctc in vitro*. Its sigma subunit was identified as a product of the *sigH* gene, which is co-transcribed downstream of a putative antisigma factor gene (*prsH*). Although the *sigH* function was not needed for normal colony morphology, *prsH* was conditionally required for both aerial hyphae formation and regulation of antibiotic biosynthesis. Levels of two different *sigH*-encoded proteins were growth phase dependent but not significantly changed by osmotic stress, implying the important roles of post-translational regulatory elements such as PrsH. In addition, synthesis of three other SigH-like proteins was induced by osmotic stress, but not by ethanol or heat. Two of them were genetically assigned to *sigH* homologous loci *sigI* and *sigJ* and shown to be independently regulated. This family of SigH-like proteins displayed different osmotic response kinetics. Thus, in contrast to many other bacteria, *S. coelicolor* uses an osmotic sensory system that can co-ordinate the activity of multiple paralogues to control the relative activity of promoters within the same stress stimulon. Such specialized stress response systems may reflect adaptive functions needed for colonial differentiation.

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Introduction

In their soil habitat and during their developmental programme, *Streptomyces* are challenged with diverse nutritional and environmental stresses (see review by Chater, 2001). In response, they undergo a complex morphological differentiation programme coupled to changes in physiology and growth rates. After chromosome replication, daughter cells remain associated, generating an interconnected web of mould-like filaments (hyphae).

Developmental changes in *Streptomyces* are triggered by nutritional limitations and associated with polymer degradation. Growth of *Streptomyces* in liquid and solid medium is often discontinuous (Granozzi *et al.*, 1990; Süsstrunk *et al.*, 1998; Vohradsky *et al.*, 2000). An initial phase of rapid growth on solid media generates a dense filamentous network called the vegetative or substrate mycelium. The ensuing interruption of growth could result from the accumulation of toxic products, such as organic acids, or diauxic lag (Süsstrunk *et al.*, 1998; Viollier *et al.*, 2001). After a period of adaptation, the vegetative mycelium produces aerial hyphae and secondary metabolites (e.g. antibiotics) while undergoing a second round of rapid growth (Granozzi *et al.*, 1990; Süsstrunk *et al.*, 1998; J. Novotna *et al.*, unpublished). Depolymerization of storage compounds such as glycogen and trehalose may provide both carbon sources and turgor pressure supporting renewed growth and aerial mycelium formation (Bruton *et al.*, 1995; Martin *et al.*, 1997; Chater, 1998). These

osmotic and nutritional changes presumably require the activity of adaptive stress response systems.

In *Escherichia coli* and *Bacillus subtilis*, osmotic, heat, ethanol and acid shock induce a common group of general stress proteins. Although these treatments activate alternative sigma factors in *E. coli* (primarily σ^S, but also involving σ^H and σ^E; Gross, 1996; Muffler *et al*., 1997; Bianchi and Baneyx, 1999; Hengge-Aronis, 2002), *B. subtilis* uses only one (σ^B, SigB) (Hecker *et al*., 1996; Price, 2000).

Activation of the *sigB* pathway provides the most detailed and comparable paradigm for *Streptomyces* stress response networks. In *B. subtilis*, SigB directs the transcription of more than 200 genes (Völker *et al*., 1994; Hecker and Volker, 1998; Petersohn *et al*., 1999; Price, 2000), including a promoter upstream of its own operon (Benson and Haldenwang, 1993a). Exposure to salt stress induces an increase in SigB levels and activity (Boylan *et al*., 1993). SigB activity is regulated primarily by a post-translational control mechanism mediated through protein–protein interactions and phosphorylation states (Price, 2000). RsbW is an antisigma factor that inhibits SigB (Benson and Haldenwang, 1993b). The antiantisigma factor, RsbV, modulates the activity of RsbW. RsbW monitors a signal transduction network responding to environmental stresses such as osmotic, heat, ethanol or acid shock and an independent 'energy stress' system that is activated at the approach of stationary phase (Kang *et al*., 1998). The finding that two different RNA polymerase holoenzymes in *S. coelicolor* were able to transcribe *Pctc*, a *B. subtilis* SigB-specific promoter, suggested that alternative SigB-like proteins were present in *S. coelicolor* (Westpheling *et al*., 1985). Genome sequence analysis (Bentley *et al*., 2002) has shown recently that *S. coelicolor* encodes at least nine *sigB* homologues as well as numerous antisigma factors (RsbW orthologues) and antiantisigma factors (RsbV orthologues). To illustrate the complexity, five antisigma factors and two antiantisigma factor paralogues are localized in the vicinity of a single *sigB* homologue (G. H. Kelemen *et al*., manuscript in preparation).

These multiple sigma factor paralogues and their regulatory genes may underlie the complexity of environmental and energy stress responses in *S. coelicolor*. Unlike other bacteria in which various stresses induce the expression of similar sets of protein spots, in *S. coelicolor*, heat, salt and ethanol stimulons are composed of independent sets of proteins (Vohradsky *et al*., 2000). These global studies of liquid cultures also showed that synthesis of stress-induced proteins was dependent on nutritional conditions. For example, many cold, heat, ethanol and salt shock proteins were co-ordinately induced or repressed during T phase, a period of growth arrest shown recently to reflect a starvation response (J. Novotna *et al*., unpublished). This work suggested that independent environmental stress response systems were co-ordinated with physiological stress systems and led to the idea that stress regulatory elements controlling these individual stimulons may be connected to the *Streptomyces* morphological developmental programmes (Vohradsky *et al*., 2000; Kelemen *et al*., 2001; Nguyen *et al*., 2002).

Evidence in support of these concepts has been provided by recent analyses of *S. coelicolor* genes *sigB*, *sigF* and *sigH*, orthologues of *B. subtilis sigB*. The *S. coelicolor sigB* gene is induced by salt and plays a role in osmoprotection and erection of aerial mycelium (Cho *et al*., 2001). A strain with a mutated *sigH* allele is reported to have some abnormalities in spore formation and to be slightly osmosensitive (Sevcikova *et al*., 2001). The *sigH* operon is transcribed by promoters controlled by both environmental stress (heat, salt, ethanol) and developmental signals (Kormanec *et al*., 2000; Kelemen *et al*., 2001). The developmental transcription factor BldD mediates temporal and spatial regulation of *sigH* expression during colony differentiation (Kelemen *et al*., 2001). *sigH* is co-transcribed with a gene upstream encoding a putative regulator of SigH (*prsH*) that has significant similarity to *B. subtilis rsbW* (Benson and Haldenwang, 1993b) and binds SigH *in vitro* (P. Viollier *et al*., 2003; Sevcikova and Kormanec, 2002). Although its role as a possible stress response protein has not been explored, *sigF*, another *sigB* orthologue, is needed for spore maturation (Potuckova *et al*., 1995; Kelemen *et al*., 1996). Here, we show that multiple, previously uncharacterized SigB-like proteins are involved in osmotic and nutritional responses in *S. coelicolor*.

Results

Physiological and environmental stress activation of the B. subtilis ctc *promoter in* S. coelicolor

Transcription of all general stress response genes is dependent on SigB activity in *B. subtilis*. However, many stress-inducible promoters are also controlled by supplementary transcriptional regulatory proteins, not only in *Bacillus*, but also in *S. coelicolor* (Servant and Mazodier, 2001). Therefore, many studies of the general stress response in *Bacillus* have exploited a promoter regulated exclusively by SigB and not affected by other transcriptional regulators. This promoter, *Pctc*, often serves as a probe of the general response to environmental (heat, ethanol and salt) and energy stress (Tatti and Moran, 1984; Ray *et al*., 1985; Igo and Losick, 1986; Haldenwang, 1995). We chose to exploit this thoroughly characterized heterologous stress reporter system to monitor SigB-like activity in the absence of interfering transcriptional regulators.

To investigate whether *S. coelicolor* had a general stress response comparable to *B. subtilis* driven by at least one of its nine SigB-like proteins, we monitored the activity of *Pctc* fused to the *xylE* reporter gene (pXE-ctc). As in *B. subtilis*, *S. coelicolor Pctc* activity increased transiently during growth (Fig. 1A). The responses of *Pctc* to various stress-inducing chemicals and physical treatments was compared and analysed in more detail first using culture conditions used previously to define stress-specific stress stimulons in *S. coelicolor* (Vohradsky *et al.*, 2000). MT1110/pXE-ctc was grown to early exponential phase, and stress responses were induced by heat (37°C), NaCl (0.5 M), H_2O_2 (1 mM) or antibiotics (pristinamycin I or thiostrepton). Only treatment with NaCl induced XylE specific activity (a 20-fold increase; Fig. 1C). These changes were in response to osmotic shock rather than to salt-specific ionic stress, as a similar increase in XylE specific activity was observed after induction with 0.5 M sucrose (data not shown). To rule out effects unrelated to *Pctc* activity, including changes in DNA supercoiling, copy number of the reporter plasmid or XylE stability, a reporter construct comparable to pXE-ctc was made (pXEveg) in which expression of XylE was driven by the *B. subtilis veg* promoter (P_{veg}). *In vitro*, P_{veg} is transcribed by a *S. coelicolor* RNAP holoenzyme containing the housekeeping sigma factor HrdB (Brown *et al.*, 1992). XylE specific activities of MT1110/pXEveg did not increase in response to salt stress (data not shown). These experiments suggested that SigB-like activities were activated both during growth and as a specific osmotic shock stress response.

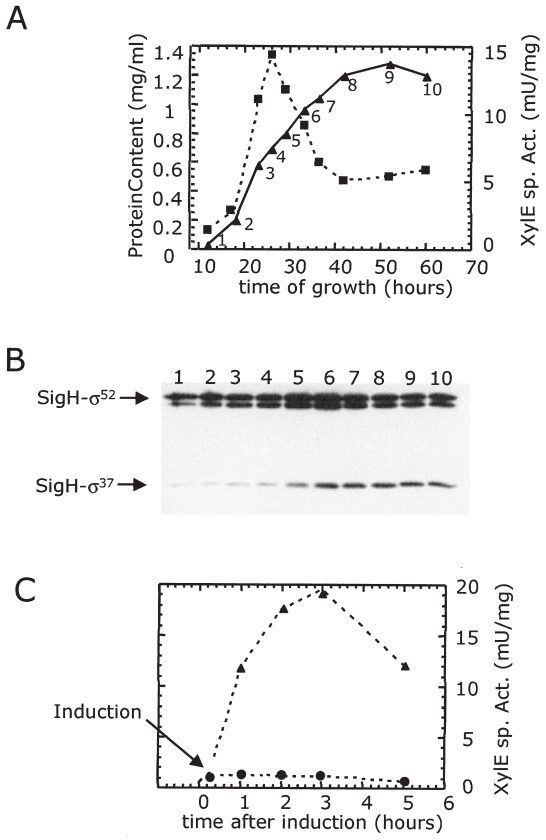

Fig. 1. Induction of a *Pctc-xylE* transcriptional fusion in *S. coelicolor* liquid cultures during growth and after osmotic shock.
A. YEME liquid medium was inoculated with a preculture of *S. coelicolor* M146/pXE-ctc and grown to stationary phase. At regular intervals, samples were withdrawn and analysed for XylE specific activity (squares) and protein content (triangles) to measure biomass accumulation.
B. The samples collected in (A) were analysed for relative changes in SigH levels during growth. Western immunoblots using the SigH antibody suggested that SigH was synthesized as two isoforms: either an ≈ 37 kDa product or N-terminally extended isoforms migrating at ≈ 52 kDa.
C. MG liquid medium was inoculated with a preculture of *S. coelicolor* MT1110/pXE-ctc grown in YEME and grown to early exponential phase (14 h) before treatment with NaCl (0.5 M), heat (37°C, circles) or H_2O_2 (1 mM). Samples were collected and assayed for XylE specific activity. Only treatment with 0.5 M NaCl (triangles) induced XylE specific activity to levels higher than those of the untreated control culture.

Purification of a Pctc*-specific sigma factor from* S. coelicolor *crude extracts*

RNA polymerase (RNAP) able to transcribe *Pctc in vitro* was purified from early stationary phase mycelia of *S. coelicolor* using sequential heparin affinity, Superose-6 gel filtration and MonoQ ion exchange chromatography steps. The final step used a DNA affinity matrix prepared by coupling *Pctc*, on a 340 bp biotin-labelled DNA fragment, to streptavidin-coated magnetic particles (see *Experimental procedures*; Folcher *et al.*, 2001). After washing the column with competitor DNA [poly-(dI–dC).poly-(dI–dC)], retained proteins were eluted stepwise with increasing NaCl concentrations (0.25 M, 0.5 M and 1 M). Fractions were analysed by SDS-PAGE (Fig. 2A) and by *in vitro* transcription using *Pctc* (Fig. 2B). *Pctc* transcribing activity and an essentially pure RNAP holoenzyme eluted at 0.25–1 M NaCl. These fractions contained proteins with mobilities of RNAP subunits β (≈ 150 kDa), β′ (≈ 150 kDa) and α (45 kDa). Another major band, presumed to be a sigma subunit, migrated at ≈37 kDa and was most abundant in the 0.5 M fraction. The N-terminal sequence of the 37 kDa protein determined by Edman degradation was: NH_2-(D/S)-E-H-E-R-H-A-D-G-H-A-P-X-(P/G)-R.

Its N-terminal sequence identified this RNAP subunit as SigH, a member of the SigF subfamily of *S. coelicolor* (G. H. Kelemen *et al.*, manuscript in preparation; Kormanec *et al.*, 2000), similar to SigB of *B. subtilis*. The corresponding open reading frame (ORF) initiated translation at an ATG and encoded a protein of 275 amino acids, designated SigH-σ^{37}. The apparent molecular mass of the protein estimated by SDS-PAGE (37 kDa) was significantly

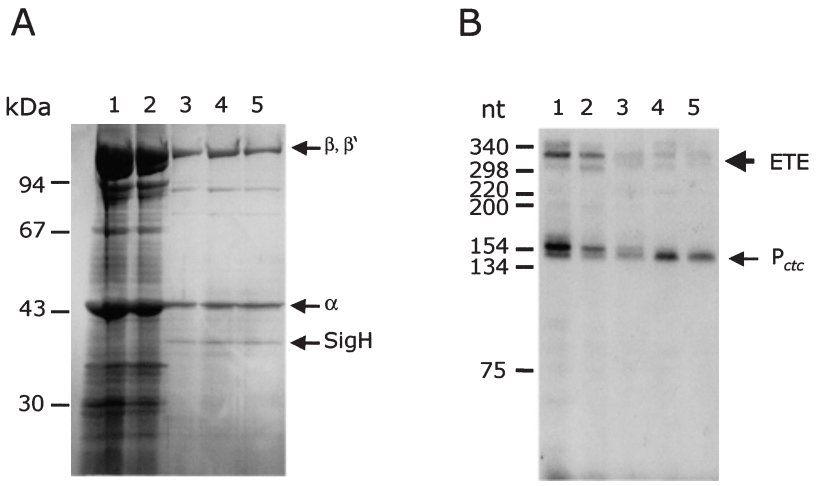

Fig. 2. Purification of SigH from *S. coelicolor* crude extracts.
A. *Pctc* affinity chromatography was used to purify SigH from *S. coelicolor* crude extracts. Representative fractions from each elution step were resolved on a 10% SDS-PAGE gel, which was subsequently stained with silver: flowthrough (lane 1), competitor DNA [poly-(dI–dC).poly-(dI–dC)] wash (lane 2), 0.25 M NaCl wash (lane 3), 0.5 M NaCl wash (lane 4) or 1 M NaCl wash (lane 5). The relative molecular masses (kDa) of protein standards are indicated on the left. The relative positions of the subunits of RNA polymerase (RNAP; α, β, β′), as well as the position of SigH, are shown on the right.
B. The *Pctc* transcriptional activity of fractions shown in (A). Sizes (nt, nucleotides) of a ^{32}P end-labelled DNA ladder (Roche Biochemicals) that served as standard are shown on the left. The arrows on the right indicate the size of the specific *ctc* transcripts (*Pctc*) as well as the unspecific end-to-end transcripts (ETE) of the template. A *Bam*HI–*Eco*RI fragment isolated from pUC-*ctc* was used as the template in these reactions. The direction of transcription was defined using alternative templates generated by PCR that extended through the universal or reverse primer sequences. The slightly larger *Pctc* transcripts were not observed when the gel was run at high temperature; we consider them as artifacts reflecting secondary structure rather than alternative start sites of different sigma factors.

higher than that predicted from the nucleotide sequence (31 kDa). Such aberrant migration of sigma factors has been described frequently and is presumed to be a result of their acidic pI (Haldenwang, 1995). The *sigH* ORF (accession no. AJ249450) is also translated from at least one upstream initiation site to generate 51 and 52 kDa isoforms (collectively referred to here as SigH-σ^{52}; P. H. Viollier *et al*., manuscript in preparation).

RNAP reconstituted with SigH initiated transcription of Pctc

To confirm that *S. coelicolor* SigH could direct RNAP core enzyme to initiate *Pctc*-specific transcription, SigH-σ^{37} and His6-SigH-σ^{37} (SigH with an N-terminal His tag) were overproduced in *E. coli* and purified to homogeneity (see *Experimental procedures*). Purified recombinant (native form without the His tag) and *S. coelicolor* SigH co-migrated on SDS-PAGE gels (not shown). In combination with either *S. coelicolor* or *E. coli* RNAP core enzyme (E), these recombinant SigH proteins catalysed specific transcription of *Pctc in vitro* (His6-SigH-σ^{37} results are shown in Fig. 3). SigH-reconstituted *S. coelicolor* RNAP holoenzyme was able specifically to retard the migration of a radiolabelled *Pctc*-DNA fragment in the presence of high amounts (10 μg) of competitor DNA [poly-(dI–dC).poly-(dI–dC); data not shown]. Neither SigH nor *E. coli* core

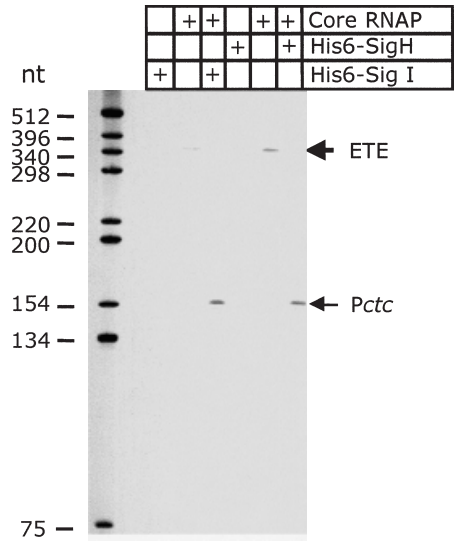

Fig. 3. Recombinant SigH and SigI catalyse *Pctc*-specific transcription *in vitro*. *In vitro* transcription assays were done using recombinant His6-SigH (His6-SigH-σ^{37}) and His6-SigI. Recombinant sigma factors (0.5 μg) were used to reconstitute active RNAP holoenzymes with 1 μg *S. coelicolor* RNAP core enzyme. His6-SigI and His6-SigH were incubated with *Pctc* in either the absence or the presence of core RNAP. The arrows on the right indicate the *Pctc* transcripts as well as the unspecific end-to-end transcripts (ETE) of the template by RNAP core enzyme.

RNAP alone was able to initiate transcription on *Pctc* (Fig. 3) or retard migration of *Pctc* (data not shown).

In vitro transcription experiments using the *dagA* (*S. coelicolor* agarase) promoters showed that the SigH-reconstituted RNAP holoenzyme was specific. It had been shown that at least three different *S. coelicolor* RNAP holoenzymes were responsible for initiating transcription at three of the four *dagA* promoters (Buttner *et al*., 1988). EsigH, reconstituted using *S. coelicolor* RNAP core, was not able to initiate transcription on any of these promoters (data not shown).

Changes in sigH *gene products during development in liquid cultures*

To determine whether the relative abundance of SigH changed during growth, samples collected at different times were analysed on immunoblots using the SigH antibody (specificity tests are described in *Experimental procedures*). SigH was expressed as two primary *sigH* translation products (P. H. Viollier *et al*., in preparation). Both SigH-σ^{37} and SigH-σ^{52} reached maximum levels just before entry into stationary phase (Fig. 1B). However, although SigH-σ^{52} was present throughout growth, only small amounts of SigH-σ^{37} accumulated in the early phases. The migration of SigH-σ^{52} and SigH-σ^{37} on SDS-PAGE corresponded roughly to the *Pctc* transcribing activities described previously (Westpheling *et al*., 1985).

The osmotic response depends on multiple SigH-like proteins

sigH null mutants (J2100 and BZ10, see *Experimental procedures*) were constructed to determine whether the gene plays a role in the osmotic shock response. As expected, the *sigH* mutant lacked both SigH-σ^{52} and SigH-σ^{37}. The salt tolerance of the wild type and the *sigH* mutants were indistinguishable on R2YE supplemented with 0.5 M NaCl or MS containing 1 M NaCl. *Pctc* was transcribed in the mutant strain carrying pXE-ctc and could be induced by development or salt shock. However, under the induced or uninduced conditions tested (the same as described in Fig. 1A and C), its activity was only reduced by 50% in the BZ10 mutant. This indicated the participation of other paralogous sigma factors.

NaCl-, sucrose-, ethanol-, heat- or cold-stressed cultures were screened on immunoblots for changes in SigH-related sigma factors (Fig. 4) that might correlate with stress-induced changes in *Pctc* expression. Although levels of SigH-σ^{52} and SigH-σ^{37} did not show significant changes during 60 min of osmotic shock (NaCl or sucrose), two SigH cross-reactive bands with apparent molecular masses of 44 kDa (P44) and 40 kDa (P40) accumulated transiently. Heat, ethanol or cold shock did not induce these proteins. The fact that the *sigH* mutant (J2100) accumulated wild-type levels of P40 and P44 in response to osmotic shock (Fig. 4) showed that these proteins were encoded by different loci and were not under *sigH* control.

SigH-like proteins are subunits of RNAP holoenzymes

Both P40 and P44 found in sucrose- or salt-induced cultures co-purified with RNAP. Purification was facilitated using *S. coelicolor* J1981, a strain engineered to encode a derivative of the β′ subunit of RNAP core enzyme (*rpoC*) containing a C-terminal hexahistidine tag (RpoC-His6) (Babcock *et al*., 1997). This allows isolation of the entire RNAP holoenzyme by Ni^{2+}-chelate affinity chromatography. Crude extracts prepared from sucrose- or salt-induced J1981 and its isogenic parent (M145) were incubated with a Ni^{2+}-NTA matrix and washed extensively. Retained proteins were eluted using increasing concentrations of imidazole, and fractions were assayed for SigH-like proteins on immunoblots (the results of the salt induction experiment are shown in Fig. 5). SigH (SigH-σ^{52} and

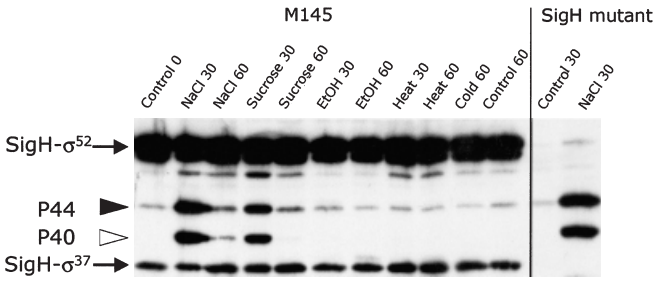

Fig. 4. Specific induction of SigH-like proteins by osmotic shock. Exponential phase cultures of M145 growing in J medium were left untreated (control) or subjected to various environmental stress treatments including: osmotic (0.5 M NaCl, 0.5 M sucrose), ethanol (4%), heat (37°C) or cold (14°C). Similarly prepared cultures of a *sigH* mutant derivative (J2100) were subjected to NaCl shock. After the indicated number of minutes, Western blots representing these cultures were probed with the SigH antibody. In addition to detecting SigH-σ^{52} and SigH-σ^{37}, two SigH-like cross-reacting proteins were detected (P40 and P44).

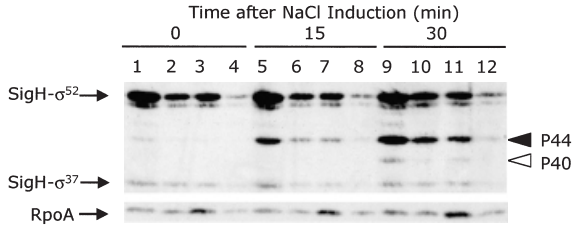

Fig. 5. SigH-like proteins induced by osmotic shock are RNA polymerase subunits. *S. coelicolor* J1981 (*rpoC-His6*) cultures grown to early exponential phase in J medium were induced with 0.5 M NaCl and sampled at the times indicated (min). Osmotically induced cultures (lanes 5 and 9) contained SigH cross-reacting bands not found in the same cultures before induction (lane 1). Aliquots of these samples were then adsorbed to a Ni^{2+}-chelate resin, and retained proteins were eluted with 20 mM (lanes 2, 6 and 10), 50 mM (lanes 3, 7 and 11) and 200 mM (lanes 4, 8 and 12) imidazole. All samples were then analysed on Western blots using the SigH antibody. SigH cross-reactive bands were not eluted by lower imidazole concentrations (not shown). As a control for the purification of RNAP, the same samples were probed with an antibody (kindly provided by J.-H. Roe) raised against the alpha subunit of the RNAP core enzyme (RpoA; shown at the bottom; Cho *et al.*, 1996). The positions of the two SigH isoforms are indicated on the left. The solid and open triangles mark the position of the two induced bands, P40 and P44 respectively.

SigH-σ^{37}) as well as P40 and P44 were detected in the same RNAP-containing fractions (20–200 mM imidazole).

Identification of the sigma factor genes corresponding to P40 and P44 was initiated by screening strains with mutations in two *sigB* homologues. *sigF* and *sigG* mutants accumulated P40 and P44 in response to salt shock (data not shown), suggesting that these proteins were new members of the SigB-like family. Phylogenetic analysis of amino acid sequences (G. H. Kelemen *et al.*, manuscript in preparation) implicated SigI (accession no, AJ249581) and SigJ (accession no. AJ249580) as likely candidates.

P44 is encoded by sigI

To determine whether P44 was encoded by *sigI*, the ORF was replaced by an allele encoding SigI fused to a C-terminal His-6 tag (generating a fusion protein of 46 kDa). This was done by homologous recombination between the mutated gene cloned in pVHP601 and *S. coelicolor* genomes (using both M145 and J1501). Immunoblot analysis of extracts prepared from M145::pVHP601 and J1501::pVHP601 (these strains produced similar results; only M145::pVHP601 is shown in Fig. 6) revealed that, although salt still induced accumulation of P40, P44 was replaced by a new cross-reacting protein that migrated with an apparent molecular mass of 46 kDa. The 46 kDa protein in crude extracts bound to a Ni^{2+}-chelate matrix under conditions in which neither P40 nor SigH were retained (Fig. 6). Together, these results demonstrated that P44 was encoded by *sigI*.

P40 represents at least two SigH-like proteins, one of which is encoded by sigJ

To explore the possibility that P40 was encoded by *sigJ*, the gene was replaced with an allele encoding a C-terminal His-6 fusion to SigJ (M145::pVHP602 and J1501::pVHP602) as described above for P44 (*sigI*). Salt induction of M145::pVHP602 (expressing SigJ-His6) led

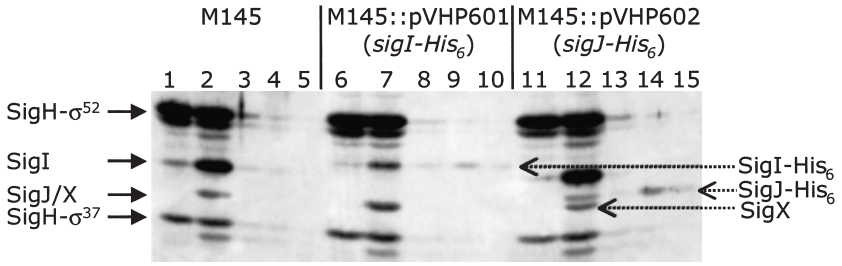

Fig. 6. Identification of the salt-induced genes encoding P40 and P44. M145 (wild type), M145::pVHP601 (*sigI-His6*) and M145::pVHP602 (*sigJ-His6*) were grown in J medium to early exponential phase and then induced by the addition of 0.5 M NaCl for 30 min. These and all subsequent samples were analysed by Western blots probed with the SigH antibody. Salt-induced cultures (lanes 2, 7 and 12) contained SigH cross-reacting bands not found in the same cultures before induction (lanes 1, 6 and 11). Crude extracts prepared from the induced culture were applied to a Ni^{2+}-chelate column and then eluted with 20 mM (lanes 3, 8 and 13), 50 mM (lanes 4, 9 and 14) and 200 mM (lanes 5, 10 and 15) imidazole. Specifically bound SigH cross-reactive bands were not eluted by lower imidazole concentrations (not shown). Note that the His6 tag increased the apparent molecular mass of P44 to 46 kDa (M145::pVHP601; SigI-His6). In contrast, the P40 band was made up of at least two co-migrating gene products. In M145::pVHP602 (SigJ-His6), only a portion of this band shifted to 42 kDa (SigJ-His6), revealing at least one additional 40 kDa cross-reacting band (SigX). The unidentified salt-induced band migrating just below SigH-σ^{37} was detected in M145-derived cultures, but not in the J1501 derivatives and therefore was not considered further. Note: the translational initiation site for the truncated *sigJ-His6* fragment used to replace the wild-type *sigJ* gene on pVHP602 was selected based on the database assignment of the N-terminal methionine. To exclude the possibility that *sigJ* initiated translation further downstream, resulting in a strain expressing both SigJ (P40) and SigJ-His6 (P42), a mutagenic plasmid was constructed (pVHP609) harbouring a *sigJ-His6* allele that contained a larger N-terminal truncation (42 versus 105 amino acids). Analysis of extracts from J1501::pVHP609 by immunoblotting or Ni^{2+}-chelate batch chromatography yielded results identical to J1501::pVHP602 or M145::pVHP602 (data not shown).

to the accumulation of P44, P40 and a new cross-reacting band with an apparent molecular mass of 42 kDa (presumably SigJ-His6; Fig. 6). Similar results were obtained with J1501::pVHP602. The fact that the 42 kDa protein, but not other cross-reacting proteins, bound to the Ni^{2+}-chelate resin identified it as SigJ-His6. Furthermore, these experiments suggested that at least two SigH-like proteins co-migrated on SDS-PAGE gels with an apparent molecular mass of ≈ 40 kDa. Finally, prolonged electrophoretic migration occasionally resolved the P40 band as two proteins, SigJ and another that we will provisionally refer to here as SigX (data not shown).

SigI-containing RNAP holoenzyme directed Pctc *transcription* in vitro

In order to test whether SigI contributed to the residual activity of *Pctc* in the absence of SigH, it was assayed for its ability to transcribe *Pctc in vitro*. When combined with core RNAP from *S. coelicolor*, both His-tagged SigH and SigI reconstituted similarly active RNAP holoenzymes that transcribed *Pctc in vitro* (Fig. 3). These results are consistent with the view that SigI (and possibly SigJ or SigX) was partially responsible for the residual activity of *Pctc* in the absence of SigH. If so, then osmotic induction of these alternative sigma factors should be independent of SigH. Indeed, osmotic induction of SigI and SigJ was not affected by the *sigH* mutation (Fig. 4).

Independent regulation of osmotically induced SigH-like proteins

Kinetic studies of SigH, SigI and SigJ/X induction showed that their patterns of accumulation were uncoupled (Fig. 7). SigI began to accumulate 10 min after induction, reaching highest levels between 15 and 30 min and then disappeared. The SigJ/X band reached highest levels later, between 30 and 60 min. In contrast, osmotic induction did not induce or change the relative intensities of SigH-σ^{52} or SigH-σ^{37} proteins in any of these media during the first 60 min; very small amounts of SigH-σ^{37} were only detected after 60 min. Similar asynchronous kinetic patterns were observed in all other media tested (Fig. 7), including J, TSB, TSBS (TSB supplemented with 10% sucrose), YEME or NMMP (minimal medium).

The RNAP inhibitor rifampicin was used to test whether these sigma factors were induced at the transcriptional level. J1981 or its rifampicin-resistant derivative, J1982, was treated with rifampicin and analysed for salt induction of P40 and P44 using the SigH antibody. Rifampicin prevented salt induction of these SigI, SigJ/SigX bands in strain J1981, but not in J1982 (data not shown).

Phenotypes of mutations in the sigH *operon*

In order to assess the potential role of *sigH* in the osmotic stress response and morphological development, we generated *sigH* null mutants in *S. coelicolor* A3(2) (six independent isolates) and J1508 (J2100) by replacing the wild-type gene with a mutant *sigH* allele containing the apramycin resistance cassette inserted into the sequence encoding the 3.1 domain of SigH (Fig. 8A; see *Experimental procedures*; all mutants were confirmed by Southern blot analysis). The *sigH* mutant allele in J1508 (J2100) was also moved by genetic crosses to M146 (BZ10; *Experimental procedures*). The colonial morphologies (growth, pigment formation and morphogenesis) of all *sigH* mutants were indistinguishable from those of their parents on a variety of solid media (R2YE, MR2YEM, SMMS, MS, MM or MM containing 0.5 M NaCl, 1.0 M NaCl or 1 M sucrose). Our results contrast with descriptions of the single *sigH* mutant that was constructed in *S. coelicolor* M145 and reported to have a severe defect in spore formation and slight osmotic sensitivity (Sevcikova *et al.*, 2001). These phenotypes might be dependent on a specific genetic background or disruption configuration.

Although our *sigH* mutants (BZ10 is shown in Fig. 8B, sector b) had no obvious abnormalities in colonial morphology compared with wild-type strains (J1916 is shown in Fig. 8B, sector a), deletion of both *prsH* and *sigH* generated a conditionally bald phenotype (Fig. 8A, sector c). Two different deletions were constructed in which regions

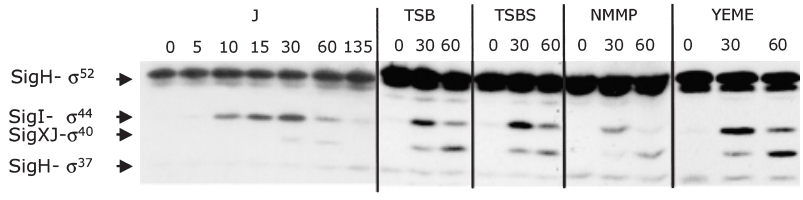

Fig. 7. Asynchronous osmotic induction of SigH, SigI and SigJ/X in different media. *S. coelicolor* A3(2) was grown to early exponential phase in various liquid media: J, tryptic soy broth (TSB), TSB containing 10% sucrose (TSBS), YEME or NMMP (minimal medium). Samples were collected before (time 0) or after various times (minutes) of NaCl (0.5 M) stress induction. Mycelia were lysed and analysed by immunoblotting using the SigH antibody to quantify SigH-σ^{37}, SigH-σ^{52}, SigI and SigJ/X.

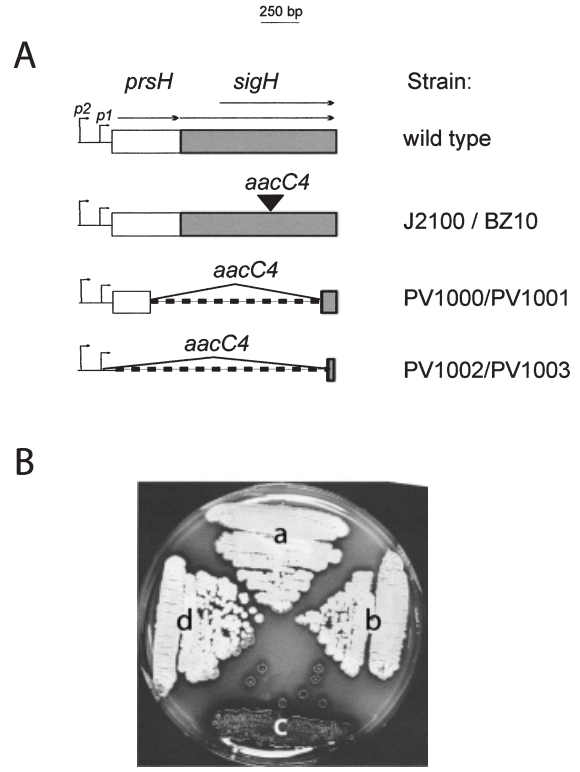

Fig. 8. *prsH*, but not *sigH*, is conditionally required for colonial development. Mutations in the *prsH/sigH* region of the *S. coelicolor* chromosome (MT1110 and J1916) were made using different strategies to inactivate either *sigH* alone or both *prsH* and *sigH*. Constructions (described in *Experimental procedures*) are illustrated in (A) and representative phenotypes in (B).
A. The *S. coelicolor prsH-sigH* loci of wild-type and mutant alleles and corresponding strain designations. At least two promoters (p1 and p2) upstream of *prsH* transcribe the *prsH-sigH* operon. Rectangles represent the *prsH* (white) and *sigH* open reading frames (grey). SigH is translated as SigH-σ^{37} (short arrow) and SigH-σ^{52} (long arrow) (P. H. Viollier *et al.*, unpublished). The apramycin resistance gene cassette (*aacC4*) was inserted into the *sigH* gene or used to replace deleted regions spanning *prsH* and *sigH* (dashed lines).
B. Wild-type strains (a, represented by J1916) sporulate rapidly and profusely on mannitol-based medium such as MR2YEM shown here. Strains containing the apramycin resistance gene inserted into the *sigH* gene (b; BZ10 shown here) differentiate like the wild type (albeit with a slight delay of <24 h). However, deletions that inactivated the adjacent *prsH* and *sigH* genes were unable to differentiate normally (PV1000 is shown in c). The sporulation defect of PV1000 was suppressed by unmapped spontaneous mutations (a representative is shown in d).

encoding *prsH* and *sigH* were replaced with the apramycin resistance gene (Fig. 8A; *Experimental procedures*). Both these alleles had the same phenotype in either MT1110 (PV1001, PV1003) or J1916 (PV1000 is shown in Fig. 8B, sector b, PV1002). Although morphological differentiation of the *prsH-sigH* deletion mutants was only delayed very slightly (by about 1 day) when cultured on standard R2YE medium, they were bald on mannitol-containing media. On MS (Fig. 8) or MR2YEM, *prsH-sigH* deletion mutants grew at the same rate but failed to produce aerial mycelium during the first week of growth. However, after further incubation of these colonies at room temperature for 2–3 weeks, a thin layer of aerial hyphae and spores appeared. Interestingly, when these spores were harvested and replated on the same medium, many colonies (≈ 50%) had reverted to the wild-type morphology, suggesting that they carried suppressor mutations (Fig. 8B, sector d). Polymerase chain reaction (PCR) analysis confirmed that these colonies still contained the *prsH-sigH* deletion, and Western blots using the SigH antibody did not detect SigH (not shown). PrsH was not required for synthesis of the pigmented antibiotics actinorhodin and undecylprodigiosin (on MS, MR2YEM and R2YE); instead, all *prsH-sigH* deletion mutants produced increased levels of blue pigment (actinorhodin; Fig. 8B, sector c).

As morphological development and normal regulation of antibiotic biosynthesis did not depend on *sigH*, the phenotype of this double mutant was presumed to result from the lack of *prsH* activity. This was verified by showing that supplying *prsH in trans* on a low-copy-number plasmid (pVHP391) restored morphological development and antibiotic biosynthesis to the mutants PV1000 and PV1001 (not shown). Thus, normal morphological and physiological development on mannitol-containing media required *prsH*, a *sigH* antisigma factor, but not *sigH* itself.

Discussion

The unusual specificity of stress response systems in S. coelicolor

Although most bacteria synthesize similar sets of proteins in response to various environmental stresses (osmotic, heat, ethanol), *S. coelicolor* activates stress-specific stimulons (Vohradsky *et al.*, 2000). Studies reported here demonstrated osmotic stress-specific control of transcription using *Pctc* and identified a family of osmotic stress-responsive sigma factors. Of the nine *S. coelicolor sigB*-like proteins, at least four were specifically induced by osmotic shock, and several were involved in the specific response of *Pctc* to osmotic shock.

In *B. subtilis*, *Pctc* transcription is determined exclusively by SigB, the activity of which is induced by various effectors of the environmental (heat, ethanol and salt) and energy stress responses. However, in *S. coelicolor*, this promoter was induced by osmotic stress, but not by other environmental stresses tested. Similarly, a *Pctc*-like promoter controlling the *catB* gene is induced by osmotic, but not ethanol or heat shock (Cho *et al.*, 2000). An alternative specificity is illustrated by a *sigH* promoter (*sigHp2*) that is not *Pctc* like, induced by heat and ethanol, but not by salt (Kormanec *et al.*, 2000; Kelemen *et al.*, 2001). An additional promoter upstream of *sigHp2* is induced only

by salt shock (Kormanec *et al*., 2000). The fact that the absolute levels of *sigH* products were not increased by any stress suggested that SigH activity relied primarily on osmosensitive post-translational control by PrsH.

Several osmotically induced sigma factors probably contribute to the osmotic induction of *Pctc.* Purification of SigH as the sigma subunit of the primary *Pctc*-binding RNAP, along with *in vitro* reconstitution studies (Fig. 3), suggested that SigH played a significant role in mediating *Pctc* induction. However, the levels of several additional mediators of the osmotic stress shock response, *sigI*, *sigJ* and *sigX*, were induced by osmotic stress, but not by heat or ethanol; this established the principle that specialized stress responses may rely on the activity of one or more specific sigma factors in *S. coelicolor*.

Independent control of four osmotically induced sigma factors

Western blot analyses of SigH, SigI and SigJ/SigX levels showed that each had its own unique osmotic induction profile, evidence of their distinct regulatory systems (Figs 5 and 6). Although SigH-σ^{52} and SigH-σ^{37} levels showed little or no response to osmotic shock, the patterns of SigI and SigJ/X induction and degradation were different. These dissimilar regulatory characteristics of *S. coelicolor sigH*-like genes may reflect partial specificity for certain concentrations or chemical classes of osmolytes or as yet undefined functional specificity. It is again important to note in this context that various osmotic signals are believed to be involved in the physiological changes that accompany the *Streptomyces* developmental programme (McBride and Ensign, 1987; Bruton *et al*., 1995; Martin *et al*., 1997; Chater, 1998). Similar kinetic analyses of stress-induced changes in the levels of SigB in *B. subtilis* have not been reported.

Although neither SigI nor SigJ was detected throughout growth of cultures under uninduced conditions, notable changes occurred in SigH proteins. SigH-σ^{52}, and especially SigH-σ^{37}, accumulated to high levels at the approach of stationary phase. *Pctc* was also induced during growth in complex liquid media, a phenomenon similar to that observed in *B. subtilis* (Igo and Losick, 1986; Benson and Haldenwang, 1993c), where it is presumed to be under the control of the RsbP-controlled 'energy depletion pathway' (Benson and Haldenwang, 1993c; Vijay *et al*., 2000). However, changes in SigH proteins did not correlate with *Pctc* activation (Fig. 1). Thus, the regulation of *Pctc* in response to osmotic shock and the approach of stationary phase in liquid cultures involve changes undetected on immunoblots, including control of SigH by antisigma factors (such as PrsH) and possibly other sigma factors that do not cross-react strongly with the SigH antibody.

Co-ordinated physiological and developmental control of stress response systems

A network linking stress stimulons to physiology had been revealed by global two-dimensional gel analyses of gene expression during different phases of growth in liquid media (Vohradsky *et al*., 2000). The heat shock stimulon, as well as the kinetics of response of its individual protein members, is defined by physiological conditions at the time of induction (Puglia *et al*., 1995). An underlying network integrating stress-induced and developmental proteins is likely to involve sigma factors as well as their regulatory proteins and transcriptional activators and repressors.

Promoter recognition specificity is programmed into nine *sigB* homologues or 56 additional *S. coelicolor* sigma factors belonging to other subfamilies. Although most sigma factors have unique promoter specificities, shared hexamer recognition has been documented for *B. subtilis* σ^F and σ^G, directing the sporulation cascade, *B. subtilis* ECF-type sigma factors σ^X and σ^W and *E. coli* σ^S and σ^{70} (Sun *et al*., 1989; Sun and Setlow, 1991; Huang *et al*., 1998; Hengge-Aronis, 1999).

In the simplest model, each stress stimulon may be controlled by its own unique, stress-specific sigma factor that is highly specialized to transcribe a conserved group of promoter sequences. Experiments reported here unexpectedly suggested a more complex regulatory organization of the osmotic stress stimulon controlled by multiple sigma factors sometimes with overlapping promoter specificities. Several results provide evidence for, but do not prove, the existence of shared promoter recognition specificity within the osmotically regulated sigma factors. The primary RNAP in crude mycelial extracts that bound to *Pctc* was EsigH (Fig. 2A), and EsigH transcribed *Pctc in vitro* (Fig. 2B). In addition, a recombinant paralogous sigma factor, SigI, allowed transcription of *Pctc in vitro* (Fig. 3). SigH, SigI and SigJ are very similar in their primary sequence, particularly in the regions that contact the −10 and −35 promoter sequences (i.e. region 2.4 and 4.2). In *B. subtilis*, many SigB-transcribed genes are expressed from alternative promoters in the absence of SigB (Hecker and Volker, 1998). Thus, sigma factor redundancy in *S. coelicolor*, like promoter redundancy in *B. subtilis*, may provide back-up systems for important regulatory capabilities. If so, stress-specific stimulons in *S. coelicolor* may represent parallel linear organizations characterized by specific sensors and sigma factors. Alternatively, sigma factors with less specific, overlapping promoter recognition may serve as nodes connecting branches within an interactive stress/energy depletion/developmental regulatory network.

Such interactive nodes could also be determined by protein recognition specificities of antisigma factors. *sigH*

mutants grew and differentiated normally, presumably reflecting redundancy of sigma factor function within a flexible network. The developmental block elicited when *prsH* and *sigH* were both inactivated suggests that the antisigma factor is needed to inhibit other regulatory targets. Supporting this model is the observation that overexpression of some members of this sigma factor family leads to developmental defects (K. T. Nguyen, unpublished). It has been shown recently that inactivation of other *S. coelicolor* antisigma factors also results in sporulation defects (Gehring *et al*., 2001; Paget *et al*., 2001).

Transcriptional regulators may also play important roles in linking this network to development. BldD, a transcription regulator required for the formation of aerial hyphae (Kelemen *et al*., 2001), represses SigH accumulation in liquid cultures. This was originally indicated by the dramatically increased levels of SigH isoforms in *bldD* mutants. However, none of the *bld* mutant cultures screened (*bldJ*, *bldA*, *bldB*, *bldC*, *bldD*, *bldG*, *bldH*, *brgA* and *bldK*) showed significant alterations in SigI and SigJ/SigX levels (P. H. Viollier *et al*., unpublished data). Moreover, although efficiently revealing SigH isoforms in cultures developing in liquid (Fig. 1) or solid media (R2YE; P. H. Viollier *et al*., manuscript in preparation), immunoblots did not detect SigI or SigJ/SigX without osmotic induction. The simplest interpretation of these data is that SigI- and SigJ/SigX-dependent proteins are not required for growth or development. However, the SigH antibody may not detect changes in levels or *in vivo* activity of these minor cross-reactive bands that take place in highly localized regions of the colony experiencing osmotic shock induced by glycogen (Bruton *et al*., 1995) or lipid depolymerization.

In conclusion, the organizational paradigm established in *B. subtilis* involving post-translational regulation of a single sigma factor to control a general stress stimulon is reflected by many parallel *sigB*-like regulatory modules and specific stimulons that are growth phase regulated in *S. coelicolor*. Osmotic stress-specific induction of *Pctc* transcription, SigI, SigJ and SigX levels, and probably SigH activity, fulfil two-dimensional gel predictions of independent stress-specific sensing systems (Vohradsky *et al*., 2000). The fact that strains lacking *prsH*, the antisigma factor paralogue upstream of *sigH*, are conditionally bald and overproduce pigmented antibiotics provided further proof that stress shock regulators are also needed to control developmental genes or at least to provide stress adaptation supporting the underlying metabolic changes.

Experimental procedures

DNA techniques

Recombinant DNA techniques were performed according to the methods of Sambrook *et al*. (1989). Minipreps and recovery of DNA fragments from agarose gels were done using the Qiaprep or Qiaquick kits from Qiagen.

Plasmid constructions pF3 and pS1 are pIJ2925 (Kieser *et al*., 2000) derivatives containing the *sigH* locus on either a 3 kb *Pst*I fragment or a 6.5 kb *Bam*HI fragment respectively. The SigH-σ^{37} overexpression construct was made by cloning a filled-in 1.1 kb *Sma*I–*Bgl*I fragment isolated from pF3 into the *Sma*I site of pOK12 (Vieira and Messing, 1991). A clone was selected (pVHP230) with *sigH* in the same orientation as *lacZ*. The region of *sigH* encoding the SigH N-terminus was subsequently replaced with one containing an *Nde*I site overlapping the putative start codon. In addition, this synthetic DNA fragment simultaneously replaced the nucleotides encoding the first five amino acids with synonymous codons commonly associated with highly expressed genes in *E. coli*. Thus, a synthetic double-stranded DNA fragment obtained by hybridization of primers P3 (5′-GATCCTCATATGTCTGAACACGAACGTCACGCCGACGGCCACGCGCCGGG-3′) and P4 (5′-TGCACCCGGCGCGTGGCCGTCGGCGTGACGTTCGTGTTCAGACATATGAG-3′) was ligated into *Apa*II–*Bgl*II-cleaved pVHP230 to yield pVHP232. Subsequently, the SigH-encoding ORF was released by digestion with *Nde*I and *Bam*HI and cloned into pET11c (native SigH-σ^{37}; pVHP225) and pET15b (His6-SigH-σ^{37}; pVHP226) purchased from Novagen.

The *B. subtilis sigB* gene was amplified from *B. subtilis* ATCC 3366 genomic DNA using the Expand Long Template kit (Roche Biochemicals) and oligos SigB_low (5′-GTTTGGATCCGCTCGTTTTCTTC-3′) and SigB_up (5′-GGGGAGCGAGTTGCATATGACAC-3′). The ≈ 800 bp product was digested with *Nde*I and *Bam*HI and cloned into pET11c to give pVHP227.

pUC-ctc and pXE-ctc were made by removing the *ctc* promoter from pMI340 (Igo and Losick, 1986) using *Bam*HI and *Hin*dIII and ligating it into pUC21 (Vieira and Messing, 1991) and pIJ2839 (constructed from pXE3 by T. Clayton). pXEveg was made by amplifying P_{veg} from *B. subtilis* ATCC 3366 genomic DNA using primers veg_up (5′-CATAGAAGCTTGTCAAAA-′3) and veg_down (5′- TTTCGGGATCCACCGTT-′3) and cloning the *Bam*HI–*Hin*dIII fragment into pIJ2839.

pVHP520 was constructed by amplifying *sigI* from J1916 genomic DNA using primers SigI_new1 (5′-NNNNNNCATATGTCACCCCGGCTCGACGGATCGCGTACCCACCAA-3′) and SigI_new2 (5′-NNNNAGATCTAGGGCAAGCGTCACGCGGTGAGGATCA-3′) and the Expand Long Template kit (Roche Biochemicals). The resulting fragment was cleaved with *Nde*I and *Bgl*II and cloned into pET15b that had been cut with *Nde*I and *Bam*HI.

pVHP601 was constructed by amplifying a truncated *sigI* allele containing sequences encoding a hexahistidine tag at the C-terminus from J1916 genomic DNA using primers SigI_up (5′-NNNGAATTCGTCGACACTCCCTCCGGAACA-′3) and SigI_low (5′-NNNGAATTCAGTGGTGGTGGTGGTGGTGCTCCTCGACCGTGAGCCCCTTG-′3) using the Expand Long Template kit (Roche Biochemicals). The resulting fragment was cleaved with *Eco*RI and cloned into pKC1132 (Bierman *et al*., 1992) to yield pVHP601.

pVHP602 and pVHP609 were constructed by amplifying truncated *sigJ* alleles containing sequences encoding a hexahistidine tag at the C-terminus from J1916 genomic DNA using primer SigJ_up (5′-NNNGAATTCAGCACCA

GTACGCACGCAACA-′3) with SigJ_low (5′-NNNGAATTC AGTGGTGGTGGTGGTGGTGGGTGGTGCTGAGCATG CCTTCCC-′3) for pVHP602 and SigJ_up2 (5′-NNN GAATTCTGCCGTACATCGTCGGGGAGATCAA-′3) with SigJ_low for pVHP609 using the Expand Long Template kit (Roche Biochemicals). The resulting fragments were cleaved with *Eco*RI and cloned into pKC1132 yielding pVHP602 and pVHP609 respectively.

Plasmid pIJ904 was constructed by inserting a polylinker [isolated from pJOE814 (Altenbuchner *et al*., 1992) using *Bam*HI and *Bgl*II] into the unique *Bam*HI site of pIJ903 (Kieser *et al*., 2000).

E.coli *and* Streptomyces *cultivation, transformation and conjugation*

Streptomyces coelicolor and *E. coli* were grown as described previously (Sambrook *et al*., 1989; Puglia *et al*., 1995; Kieser *et al*., 2000). *S. coelicolor* A3(2) strains included: MT1110 (SCP1$^-$, SCP2$^-$), M145 (SCP1$^-$, SCP2$^-$) and a *sigG*$^-$ derivative (Kormanec *et al*., 1999), J1984 (M145, *sigF*) (Kelemen *et al*., 1998), M146 (M145, SCP1^{NF}), J1981 (M145, *rpoC-His6*) (Babcock *et al*., 1997), J1982 (M145, *rpoC-His6*, *rifR*) (Babcock *et al*., 1997), J1508 (*hisA1*, *uraA1*, *strA1*, Pgl$^-$, SCP1^{NF}, SCP2$^-$) (Ikeda *et al*., 1984), J1501 (*hisA1*, *uraA1*, *strA1*, Pgl$^-$, SCP1$^-$, SCP2$^-$) (Chater *et al*., 1982) and J1916 (*hisA1*, *uraA1*, *strA1*, Pgl$^-$, *esp*, SCP1$^-$, SCP2$^-$) (Kelemen *et al*., 1995). M146 was made by crossing J1508 with the M145-derived *sigG*::*tsr* mutant (conferring resistance to thiostrepton), selecting for prototrophic progeny that had lost the *tsr* resistance gene. *E. coli* XL1-Blue (Stratagene) and BL21(DE3)/pLysS (Novagen) were used as hosts for cloning and expressing proteins. When transforming *S. coelicolor*, non-methylated DNA was prepared from *E. coli* ET 12567 (Kieser *et al*., 2000) in order to circumvent the restriction barrier.

YEME, NMMP, J-medium, MG, TSB, MS (also known as SFM) and R2YE have been described previously (Doull and Vining, 1989; Hobbs *et al*., 1992; Puglia *et al*., 1995; Kieser *et al*., 2000). MR2YEM was prepared as R2YE but without sucrose and proline, and glucose was replaced by mannitol. When required, minimal media were supplemented with histidine (100 μg ml^{-1}) and uracil (15 μg ml^{-1}).

For plasmid selection in *E. coli*, LB was supplemented with ampicillin (100 μg ml^{-1}) or apramycin (50 μg ml^{-1}). For antibiotic selection in *Streptomyces*, solid R2YE medium was supplemented with thiostrepton (50 μg ml^{-1}) or apramycin (100 μg ml^{-1}). Liquid medium was supplemented with thiostrepton (5 μg ml^{-1}), apramycin (20 μg ml^{-1}) or rifampicin (150 μg ml^{-1}). All antibiotics were purchased from Sigma.

Transformations were performed according to the methods of Thompson *et al*. (1982). Conjugal transfer of DNA from *E. coli* to *S. coelicolor* was done according to Mazodier *et al*. (1989) as modified by Flett *et al*. (1997).

Ni^{2+}*-chelate affinity chromatography of* S. coelicolor *crude extracts*

Mycelia were lysed by sonication in buffer I (10 mM Tris, pH 7.9, 100 mM NaCl, 1.2 mM β-mercaptoethanol, 2.5 mM imidazole and 5% glycerol) and clarified by centrifugation at 15000 r.p.m. for 15 min. Crude extract (2 mg of protein) was incubated with 600 μl of Ni^{2+}-NTA agarose (equilibrated in buffer I) in a total volume of 2 ml with gentle agitation at 4°C for 1 h. The Ni^{2+}-NTA agarose was pelleted by centrifugation at 5000 r.p.m. for 2 min and the supernatant removed. The agarose matrix was washed seven times with 1 ml of buffer I and twice with 1 ml of buffer I supplemented with 10 mM imidazole. Retained proteins were eluted by washing the agarose once with 1 ml of buffer I containing 20 mM imidazole, 50 mM imidazole and 200 mM imidazole.

Preparation of a Pctc *DNA affinity column*

Biotinylated *Pctc* DNA fragment was generated in 200 PCRs with a universal primer (5′-GCCAGGGTTTTCCCAGTCAC GA-′3) and a biotinylated reverse primer (5′-GAGCGGATAA CAATTTCACACAGG-′3). Standard PCR conditions were used to amplify ≈10 μg of biotinylated DNA fragment per reaction using pUC-ctc as a template. Unincorporated biotinylated primers were separated from the PCR product by perfusion chromatography on a Poros HQ/F (Roche Biochemicals). After loading the pooled PCR reaction mixture onto the column that had been equilibrated in buffer TA [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% (v/v) glycerol, 50 mM NaCl], a 50 ml linear gradient from 50 mM to 1 M NaCl was applied to elute the biotinylated DNA fragment. Although the primers typically eluted at a salt concentration corresponding to 0.1–0.2 M NaCl, the biotinylated ≈ 450 bp fragment eluted near the end of the gradient. Fractions that contained the desired DNA fragment were concentrated to 4 ml by ultrafiltration (YM10, Amicon). Coupling of the biotinylated DNA fragment to streptavidin-coated magnetic particles (Roche Biochemicals) was done according to the method of Folcher *et al*. (2001).

Immunoblots

Crude extracts (20 μg) were separated on 10% SDS-polyacrylamide gels. Proteins were blotted onto polyvinylidene difluoride membrane (PVDF; Millipore) in transfer buffer (15 mM Tris, 120 mM glycine, 10% methanol). The membranes were incubated in blocking buffer (TBS; 20 mM Tris-HCl, pH 7.6, 130 mM NaCl) containing 5% non-fat dried milk and 0.1% Tween-20 for 2 h at room temperature. The SigH antibody was added to the blocking buffer at 1:5000 dilution and incubated with the membrane for 1 h. Unbound antibody was removed by four 5 min washes in TBS. The membrane was immersed into blocking buffer containing horseradish peroxidase-conjugated swine anti-rabbit IgG (Dako) at a dilution of 1:10 000, incubated for 1 h and then washed four times (5 min) in TBS. Reactive bands were visualized using chemiluminescence (ECL plus; Amersham Pharmacia).

Antibody production

A total of 2 mg of purified, recombinant untagged SigH was

used to immunize two rabbits (Eurogentec) for the production of polyclonal antibodies. The specificity of the antibodies was demonstrated in immunoblots of crude *E. coli* lysates expressing *S. coelicolor* SigH (pVHP225), *B. subtilis* SigB (pVHP227) or *S. coelicolor* SigF (pIJ5894) (Kelemen *et al*., 1998). In each case, the SigH antibody recognized proteins from hosts expressing SigH, SigB or SigF (see below) that were not detected with extracts prepared from *E. coli* containing the expression vector (pET11c). However, the titre of the serum for SigH was at least one order of magnitude higher against *S. coelicolor* SigH than against *B. subtilis* SigB or *S. coelicolor* SigF (data not shown).

N-terminal sequencing

In order to determine the N-terminal sequence of the SigH isolated from *S. coelicolor*, the purified holoenzyme (10 μg) was separated on 10% SDS-PAGE and blotted onto a PVDF Immobilon membrane (Millipore) in 10 mM CAPS (3-(cyclohexyl-amino)-1-propanesulphonic acid) buffer (pH 11.0) containing 10% methanol. N-terminal Edman degradation was performed by T. Murakami (Meiji Seika).

Production and purification of recombinant SigH (SigH-σ^{37}), His6-SigH (His6-SigH-σ^{37}) and His6-SigI (His6-σ^{40}) in E. coli

Escherichia coli BL21(DE3)/pLysS cultures containing plasmids for the production of sigma factors (pVHP225, native SigH; pVHP226, His6-SigH; pVHP520, His6-SigI) were grown overnight in LB supplemented with ampicillin (100 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}) at room temperature. Cultures were diluted 50-fold into 2 l of the same medium and grown to an OD_{600} of 0.4 at room temperature. Synthesis of the recombinant proteins was induced by the addition of IPTG to a final concentration of 0.4 mM for 3 h. The cells were harvested by centrifugation, washed once in TBS and frozen at −70°C.

His6-SigH and His6-SigI were purified by metal chelate affinity chromatography using a Ni^{2+}-NTA matrix (Qiagen). Briefly, cells were lysed by sonication in buffer I, and particulate matter was removed by centrifugation. The supernatant was loaded onto 6 ml of Ni^{2+}-NTA resin and washed extensively with buffer I containing 0.5 M NaCl, buffer I containing 15 mM imidazole and finally eluted in buffer I supplemented with 200 mM imidazole.

Untagged recombinant SigH was purified to homogeneity using a combination of ion exchange, heparin and gel filtration chromatography. *E. coli* protein extract containing SigH was prepared as described above, except that cells were resuspended in TGED (20 mM Tris-HCl, pH 7.9, 0.2 mM DTT, 0.1 mM EDTA and 10% glycerol) containing Complete protease inhibitors (Roche Biochemicals) before sonication. After the cells had been disrupted, cell debris was removed by centrifugation (Sorvall). The supernatant was fractionated by DEAE Sepharose FF (XK26/50; Amersham Pharmacia) ion exchange chromatography using a linear NaCl gradient ranging from 0.1 to 1 M in TGED. Fractions containing the recombinant protein were pooled, diluted and separated further by Q Sepharose FF (XK26/20; Amersham Pharmacia) ion exchange chromatography using the same gradient. The sample was then applied to a heparin–Sepharose Cl-6B FF column (XK16/20; Amersham Pharmacia), and retained proteins were eluted using a linear NaCl gradient ranging from 0.1 mM to 1 M. The final gel filtration chromatography purification step (Superdex 200, XK26/50; Amersham Pharmacia) yielded a preparation of SigH that was >99% pure, as determined by high-performance liquid chromatography (HPLC) analysis.

Construction and analysis of an S. coelicolor sigH *null mutant and* prsH-sigH *deletion mutants*

The constructions described below were used to generate mutated alleles shown in Fig. 8A.

A *sigH* mutant allele was constructed *in vitro* by inserting an apramycin resistance gene (Blondelet Rouault *et al*., 1997) into *sigH* at a unique *Pfl*MI restriction site within pF3 (see below). This mutant allele was cloned into the suicide vector pDH5 (Hillemann *et al*., 1991), containing the thiostrepton resistance gene (*tsr*). The resulting plasmid was used to transform *S. coelicolor* J1508, selecting for thiostrepton- and apramycin-resistant progeny that had integrated the mutated *sigH* allele by homologous recombination at the *sigH* locus. Thiostrepton-sensitive, apramycin-resistant isolates were identified after three successive rounds of sporulation allowing for the loss of the vector sequences by a second recombination event. In the resulting strain (J2100), the wild-type *sigH* gene was replaced by the *sigH*::*aacC4* allele. A genetic cross between J2100 and M146 was used to isolate recombinant apramycin-resistant prototrophs, one of which was designated BZ10.

The 6.5 kb *Bam*HI fragment carrying the *sigH* locus was cloned from pS1 into pPM925. The resulting plasmid was digested with *Xho*I, liberating a 1.1 kb fragment harbouring the 3′ end of *prsH* and most of the *sigH* ORF, and religated to give pVHP290. The deleted *prsH-sigH* allele was cloned into pIJ2581 (Kieser *et al*., 2000) using *Bam*HI to generate pVHP313. The apramycin resistance gene was inserted as a *Sma*I fragment into *Xho*I-restricted pVHP313 that had been blunt-ended using T4 DNA polymerase. The resulting plasmid carrying the *prsH-sigH* allele in which the *Xho*I fragment was replaced with the *aacC4* gene was named pVHP340. This allele was cloned onto pSET151 using *Bam*HI, yielding pVHP341. pVHP340 and pVHP341 were used to transform J1916 and MT1110 protoplasts. Apramycin-resistant transformants that were sensitive to thiostrepton were presumed to result from a double cross-over event. PCR analysis and immunoblots were used to confirm the *prsH-sigH* deletion. These strains were designated PV1000 (J1916 host) and PV1001 (MT1110 host).

The 6.5 kb fragment harbouring the *sigH* locus was cloned from pS1 into the *Bam*HI site of pACYC184. The resulting plasmid, pHPV372, was cut with *Pml*I to remove the *prsH-sigH* fragment and replace it with the apramycin resistance gene that was isolated on a *Sma*I fragment (pVHP374). The mutated allele was cloned onto pSET151 using *Bam*HI, yielding pVHP375. pVHP375 DNA was used to transform J1916 and MT1110 protoplasts to apramycin resistance. Transformants were selected that were sensitive to thiostrepton, indicating a double cross-over. PCR analysis and immunoblots

were used to confirm the *prsH-sigH* deletion. These strains were designated PV1002 (J1916 host) or PV1003 (MT1110 host).

prsH was cloned on an ≈ 630 bp *Sap*I fragment from pS1 into the *Sma*I site of pOK12, yielding pVHP387. The *prsH* fragment was removed by cleavage of pVHP387 with *Bam*HI and *Bgl*II and cloned into the low-copy-number shuttle vector pIJ904. The resulting plasmid, pVHP391, was used in complementation studies.

Purification of SigH from S. coelicolor *crude extracts*

Streptomyces coelicolor MT1110 mycelium (200 g wet weight) grown to late exponential phase in a fermenter containing 50 l of YEME was lysed by sonication in 600 ml of RNAP lysis buffer (Buttner and Brown, 1985) containing Complete™ 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 100 000 *g*.

RNAP was enriched for *Pctc* transcribing activity as described by Buttner and Brown (1985). Briefly, RNAP was fractionated using heparin affinity, MonoQ ion exchange chromatography and Superose-6 gel filtration and assayed for *in vitro* transcription on the *ctc* promoter. Active fractions were pooled, dialysed against TA buffer [10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% (v/v) Triton X-100 and 10% (v/v) glycerol] supplemented with 0.1 M NaCl and concentrated by ultrafiltration (YM10; Amicon). The sample was then incubated with the *Pctc* affinity matrix for 1 h at room temperature and for an additional hour at 4°C. Non-specific binding was eliminated by washing the matrix extensively in the loading buffer and, thereafter, with 4 ml of TA buffer containing 100 mM NaCl and 100 μg ml^{-1} competitor DNA [poly-(dI–dC).poly-(dI–dC)]. Retained proteins were eluted by washing the matrix with buffer TA containing 0.25 M, 0.5 M and 1 M NaCl.

In vitro *transcription*

Run-off transcription reactions were performed with [α-^{32}P]-CTP (Amersham Pharmacia), as described by Buttner and Brown (1985). *In vitro* transcription on the *ctc* or *dagA* promoters used a 340 bp *Eco*RI–*Bam*HI fragment of pUC-ctc or a 590 bp *Sma*I–*Ava*II fragment of pIJ2027 (Angell *et al*., 1994) respectively. Sequencing ladders were generated using a T7 sequencing kit (Pharmacia), M13mp18 template, universal primer and [α-^{32}P]-dATP (Amersham Pharmacia). Transcripts were electrophoresed on 6% polyacrylamide–6 M urea gels alongside the sequencing reactions and analysed by autoradiography.

Core RNA polymerases from S. coelicolor *and* E. coli

Streptomyces coelicolor RNAP was isolated based on the technique described by Buttner and Brown (1985). After ammonium sulphate precipitation, extracts were separated on heparin–Sepharose Cl-6B (Amersham Pharmacia) (instead of DNA cellulose), Superose 6 (Amersham Pharmacia) gel filtration chromatography and MonoQ (HR10/10, Amersham Pharmacia) ion exchange chromatography. The trailing fractions that eluted from the MonoQ column at an NaCl concentration of 0.5–0.6 M were dialysed and concentrated by ultrafiltration (YM10; Amicon). This fraction was not active in *in vitro* transcription assays using the *dagA* promoter or the *ctc* promoter, and was thus used as core RNAP.

Escherichia coli core RNAP was purchased from Epicentre Technologies.

XylE enzyme assay

Catechol dioxygenase activities were calculated as the rate of change in OD at 375 nm, per minute, and expressed as specific activity (milliunits mg^{-1} protein) (Ingram *et al*., 1989).

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