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 stimulus 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*, 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 *prsH* 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.

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; Süssstrunk 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 (Süssstrunk 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; Süssstrunk 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^h^, but also involving σ^70^ and σ^38^; 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 stimuli 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 stimuli 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 osmoregulation 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 osmo-sensitive (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), H2O2 (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 (Pveg). In vitro, Pveg 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 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 H2O2 (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.

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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 \( \text{sigH} \) 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-\( \sigma_{52} \); 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-\( \sigma^{37} \) and His6-SigH-\( \sigma^{37} \) (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 core-enzymes were able to retard the migration of a radiolabelled DNA fragment in the presence of high amounts (10 \( \mu \)g) of competitor DNA [poly-(dl–dC).poly-(dl–dC); data not shown]. Neither SigH nor \( E. \ coli \) core enzyme 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 \( \text{sigH} \) 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-\( \sigma_{52} \); P. H. Viollier et al., manuscript in preparation).

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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-α37 and SigH-α52 reached maximum levels just before entry into stationary phase (Fig. 1B). However, although SigH-α52 was present throughout growth, only small amounts of SigH-α37 accumulated in the early phases. The migration of SigH-α52 and SigH-α37 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-α52 and SigH-α37. 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-α52 and SigH-α37 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 Ni2+-chelate affinity chromatography. Crude extracts prepared from sucrose- or salt-induced J1981 and its isogenic parent (M145) were incubated with a Ni2+-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-α52 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-α37 and SigH-α52, two SigH-like cross-reacting proteins were detected (P40 and P44).](image-url)

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SigH-like proteins induced by osmotic shock are RNA polymerase subunits. S. coelicolor J1981 (poeC-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 Ni2+-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.

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 Ni2+-chelate resin, and retained proteins were 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. The 46 kDa protein in crude extracts bound to a Ni2+-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 tag fusion to SigJ (M145::pVHP602 and J1501::pVHP602) as described above for P44 (sigI). Salt induction of M145::pVHP602 (expressing SigJ-His6) led to the induction of M145::pVHP602 (sigI-His6) as likely candidates. 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.
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 Ni²⁺-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 Ptc transcription in vitro**

In order to test whether SigI contributed to the residual activity of Ptc in the absence of SigH, it was assayed for its ability to transcribe Ptc in vitro. When combined with core RNAP from *S. coelicolor*, both His-tagged SigH and SigI reconstituted similarly active RNAP holoenzymes that transcribed Ptc 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 Ptc 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-α52 or SigH-α37 proteins in any of these media during the first 60 min; very small amounts of SigH-α37 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 of the SigH operon were replaced with the apramycin resistance cassette. A deletion of SigH-α32 and SigH-α37 was constructed in strain J1981 (Fig. 7, lane y). A deletion of SigH-α44 and SigH-α60 was constructed in strain J1982 (Fig. 7, lane z). The deletion of SigH-α32 and SigH-α37 was constructed in strain J1981 (Fig. 7, lane y). A deletion of SigH-α44 and SigH-α60 was constructed in strain J1982 (Fig. 7, lane z).
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 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 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).
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^32 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 Ensing, 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^32, 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.

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Osmotic stress response systems in S. coelicolor

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 stimulus, 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^70 and σ^37, directing the sporulation cascade, B. subtilis ECF-type sigma factors σ^6 and σ^35 and E. coli σ^70 and σ^37 (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 \textit{prsH} and \textit{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 \textit{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 transcriptional 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 \textit{bldD} mutants. However, none of the \textit{bld} mutant cultures screened (\textit{bldJ}, \textit{bldA}, \textit{bldB}, \textit{bldC}, \textit{bldD}, \textit{bldG}, \textit{bldH}, \textit{brgA} and \textit{bldK}) showed significant alterations in SigI and SigJ/SigX levels (P. H. Viellier et al., unpublished data). Moreover, although efficiently revealing SigH isoforms in cultures developing in liquid (Fig. 1) or solid media (R2YE; P. H. Viellier 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 glycerogen (Bruton et al., 1995) or lipid depolymerization.

In conclusion, the organizational paradigm established in \textit{B. subtilis} involving post-translational regulation of a single sigma factor by a general stress stimulus is reflected by many parallel sigma-like regulatory modules and specific stimuli that are growth phase regulated in \textit{S. coelicolor}. Osmotic stress-specific induction of \textit{Pctc} transcription, SigI, SigJ and SigX levels, and probably SigH activity, fulfill two-dimensional gel predictions of independent stress-specific sensing systems (Vohradsky et al., 2000). The fact that strains lacking \textit{prsH}, the antisigma factor paralogue upstream of \textit{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 \textit{et al.} (1989). Minipreps and recovery of DNA fragments from agarose gels were done using the Qiaprep or Qiiaquick kits from Qiagen.

Plasmid constructions pf3 and ps1 are pJ2925 (Kieser \textit{et al.}, 2000) derivatives containing the \textit{sigH} locus on either a 3 kb \textit{Pos} fragment or a 6.5 kb \textit{BamH}I fragment respectively. The SigH-\textit{O} overexpression construct was made by cloning a filled-in 1.1 kb \textit{SmaI}–\textit{BglI} fragment isolated from pf3 into the \textit{SmaI} site of p0K12 (Vieira and Messing, 1991). A clone was selected (pVHP230) with \textit{sigH} in the same orientation as \textit{lacZ}. The region of \textit{sigH} encoding the SigH N-terminus was subsequently replaced with one containing an \textit{NdeI} 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 \textit{E. coli}. Thus, a synthetic double-stranded DNA fragment obtained by hybridization of primers P3 (5'-GATCCTCATATGCTGACACACCCGCAGGCGGGG-3') and P4 (5'-TGCACCCTGGGCTGGGCGGCGGCGCTGTGACAGTTTTTGTTTTCATGAGGACG-3) was ligated into ApaI–\textit{BglI}-cleaved vPvHP230 to yield pVHP232. Subsequently, the SigH-encoding ORF was released by digestion with \textit{NdeI} and \textit{BamH}I and cloned into pET11c (native SigH-\textit{O}'; pVHP225) and pET15b (His6-SigH-\textit{O}'; pVHP226) purchased from Novagen.

The \textit{B. subtilis} \textit{sigB} gene was amplified from \textit{B. subtilis} ATCC 3366 genomic DNA using the Expand Long Template kit (Roche Biochemicals) and oligos \textit{BldB} low (5'-GGTGCTCCTCGACCGTGAGCCCCTTG-3') and P4 (5'-TGCACCCTGGGCTGGGCGGCGGCGCTGTGACAGTTTTTGTTTTCATGAGGACG-3) was ligated into ApaI–\textit{BglI}-cleaved vPvHP230 to yield pVHP232. Subsequently, the SigH-encoding ORF was released by digestion with \textit{NdeI} and \textit{BamH}I 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 \textit{BamH}I and HindIII and ligating it into pUC21 (Vieira and Messing, 1991) and pJ2839 (constructed from pX3 by T. Clayton). pXEv was made by amplifying \textit{Pev} from \textit{B. subtilis} ATCC 3366 genomic DNA using primers \textit{veg}\textit{down} (5'- TTTTGGATCCATCCACCCTTG'TT-3') and cloning the \textit{BamH}I–HindIII fragment into pJ2839.

pVHP520 was constructed by amplifying \textit{sigl} from J916 genomic DNA using primers \textit{sigl}\textit{new}1 (5'-NNGNNNCCATATGTACCGGACGCCTGACTGCCACCACCA-3') and \textit{Sigl}\textit{new}2 (5'-NNGNGATTCAGGGCAACGCCTGACGCCGTTAACGCA-3') and cloning the \textit{BamH}I–HindIII fragment into pJ2839.

pVHP601 was constructed by amplifying a truncated \textit{sigl} allele containing sequences encoding a hexahistidine tag at the C-terminus from J916 genomic DNA using primers \textit{Sigl}\textit{up} (5'-NNGGGATATCTCGCAGACTCCTCCCCGCAGGACA-3') and \textit{Sigl}\textit{low} (5'-NNGGGATATCTCGCAGACTCCTCCCCGCAGGACA-3') and \textit{BglI} and \textit{HindIII} and cloned into pET15b that had been cut with \textit{NdeI} and \textit{BamH}I.

pVHP602 and pVHP609 were constructed by amplifying truncated \textit{sigl} alleles containing sequences encoding a hexahistidine tag at the C-terminus from J916 genomic DNA using primer \textit{SigJ}\textit{up} (5'-NNGGAATTCAGGCACCCCAA-3').
GTACGACCGCAACA–3′ with SigJ_low (5′-NNNGAATTC AGTGGTGTTGCTTGCTGGGTGAGCATG CTTCCCGG-3′) for pVHP602 and SigJ_up2 (5′-NNNG GAATTCGCGTACATCGTGCGGGAGATCA-3′) with SigJ_low for pVHP609 using the Expand Long Template kit (Roche Biochemicals). The resulting fragments were cleaved with EcoRI and cloned into pKC1132 yielding pVHP602 and pVHP609 respectively.

Plasmid pJ904 was constructed by inserting a polylinker [isolated from pJOE814 (Altenbuchner et al., 1992) using BamHI and BglII] into the unique BamHI site of plasmid pJ903 (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., 1992; Puglia et al., 1995; Kieser et al., 1995; Kieser et al., 1992) and J1916 (hisA1, uraA1, strA1, Pgl1, SCP1–, SCP2–) (Ikeda et al., 1984), J1501 (hisA1, uraA1, strA1, Pgl1, SCP1–, SCP2–) (Chater et al., 1982) and J1916 (hisA1, uraA1, strA1, Pgl1, esp, SCP1–, SCP2–) (Kelemen et al., 1995). M146 was made by crossing J1508 with the M145-derived sigG–tsr mutant (confering 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; Hobs et al., 1992; Puglia et al., 1995; Kieser et al., 2000). MR2YE 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).

Ni2+-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 Ni2+-NTA agarose (equilibrated in buffer I) in a total volume of 2 ml with gentle agitation at 4°C for 1 h. The Ni2+-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 PtcC DNA affinity column

Biotinylated PtcC DNA fragment was generated in 200 PCR with a universal primer (5′-GCCAGGTTTCCAGGTAC (GA–3′) and a biotinylated reverse primer (5′-GAGCGTAAACATTGGACGAG–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 MgCl2, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 50 mM NaCl), a 50 µl 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

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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 holoezyme (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-\(\sigma^7\)), His6-SigH (His6-SigH-\(\sigma^7\)) and His6-Sigl (His6-\(\sigma^{40}\)) 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-Sigl) 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. 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 (Blaudont Rouault et al., 1997) into sigH at a unique PMI restriction site within pF3 (see below). This mutant allele was cloned into the suicide vector pH5 (Hilleman 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 sporation 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 BamHI fragment carrying the sigH locus was cloned from pS1 into pPM925. The resulting plasmid was digested with XhoI, liberating a 1.1 kb fragment harboung the 3’ end of prsH and most of the sigH ORF, and religated to give pVHP290. The deleted prsH-sigH allele was cloned into pIL2581 (Kieser et al., 2000) using BamHI to generate pVHP313. The apramycin resistance gene was inserted as a Smal fragment into Xhol-restricted pVHP313 that had been blunt-ended using T4 DNA polymerase. The resulting plasmid carrying the prsH-sigH allele in which the XhoI fragment was replaced with the aacC4 gene was named pVHP340. This allele was cloned onto pSET151 using BanHI, 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 BamHI site of pACYC184. The resulting plasmid, pHPV372, was cut with PstI to remove the prsH-sigH fragment and replace it with the apramycin resistance gene that was isolated on a Smal fragment (pVHP374). The mutated allele was cloned onto pSET151 using BanHI, 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 Sapl fragment from pS1 into the Smal site of pOK12, yielding pVHP387. The prsH fragment was removed by cleavage of pVHP387 with BamHI and BglII and cloned into the low-copy-number shuttle vector pJ904. 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 (Babcock et al., 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 MgCl₂, 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-(dl–dC).poly-(dl–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-³²P]-CTP (Amersham Pharmacia), as described by Buttner and Brown (1985). *In vitro* transcription on the ctc or dagA promoters used a 340 bp EcoRI–BamHI fragment of pUC-ctc or a 590 bp Smal–Avall fragment of pJ2027 (Angell et al., 1994) respectively. Sequencing ladders were generated using a T7 sequencing kit (Pharmacia). M13mp18 template, universal primer and [α-³²P]-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 Epi-centre 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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