σR, an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in *Streptomyces coelicolor A3(2)*

Mark S.B. Paget1,2,3, Ju-Gyeong Kang4, Jung-Hye Roe4 and Mark J. Buttner1,2

1John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, 2School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK and 3Department of Microbiology, College of Natural Sciences and Research Centre for Molecular Microbiology, Seoul National University, Seoul 151–742, Korea

3Corresponding author

e-mail: pagetm@bbsrc.ac.uk

We have identified an RNA polymerase sigma factor, σR, that is part of a system that senses and responds to thiol oxidation in the Gram-positive, antibiotic-producing bacterium *Streptomyces coelicolor A3(2)*. Deletion of the gene (sigR) encoding σR caused sensitivity to the thiol-specific oxidant diamide and to the redox cycling compounds menadione and plumbagin. This correlated with reduced levels of disulfide reductase activity and an inability to induce this activity on exposure to diamide. The *trxBA* operon, encoding thioredoxin reductase and thioredoxin, was found to be under the direct control of σR. *trxBA* is transcribed from two promoters, *trxBp1* and *trxBp2*, separated by 5–6 bp. *trxBp1* is transiently induced at least 50-fold in response to diamide treatment in a sigR-dependent manner. Purified σR directed transcription from *trxBp1 in vitro*, indicating that *trxBp1* is a target for σR. Transcription of sigR itself initiates at two promoters, *sigRp1* and *sigRp2*, which are separated by 173 bp. The *sigRp2* transcript was undetectable in a sigR-null mutant, and purified σR could direct transcription from *sigRp2 in vitro*, indicating that sigR is positively autoregulated. Transcription from *sigRp2* was also transiently induced (70-fold) following treatment with diamide. We propose a model in which σR induces expression of the thioredoxin system in response to cytoplasmic disulfide bond formation. Upon re-establishment of normal thiol levels, σR activity is switched off, resulting in down-regulation of *trxBA* and sigR. We present evidence that the σR system also functions in the actinomycete pathogen *Mycobacterium tuberculosis*.

**Keywords:** disulfide bond formation/oxidative stress/ sigma factors/streptomyces/thioredoxin

**Introduction**

In bacteria the cytoplasm is maintained as a reducing environment. As well as preventing oxidative inactivation of active site thiols, this prevents inappropriate disulfide bridge formation in cytoplasmic proteins. In *Escherichia coli*, the major thiol-disulfide redox buffer is the cysteine-containing tripeptide glutathione, which is present at intracellular concentrations of ~5 mM, 98–99.5% of it in its reduced form (Hwang et al., 1992). In *vitro*, these concentrations of reduced glutathione can inhibit disulfide bond formation in many proteins (Hwang et al., 1992). The cytoplasm also contains four small thiol-disulfide oxidoreductases (thioredoxin and glutaredoxins 1–3), each of which may reduce disulfides in other proteins with the corresponding oxidation of a redox active Cys–X–X–Cys motif. The oxidized form of thioredoxin is reduced by thioredoxin reductase + NADPH, whereas the glutaredoxins are reduced by glutathione, which is in turn reduced by glutathione reductase + NADPH. Analyses of mutants defective in the thioredoxin and/or the glutaredoxin pathway have revealed that both pathways play important roles in maintaining cytoplasmic protein thiols in the reduced state (Derman et al., 1993; Prinz et al., 1997).

Glutathione is not made by actinomycetes, including streptomycetes and mycobacteria, which instead contain a low-molecular-weight sugar-containing monothiol called mycothiol (Newton et al., 1996). There is no published information about the presence of glutaredoxin-like proteins in streptomycetes, but a disulfide reductase system with broad substrate specificity has been characterized biochemically and shown to consist of thioredoxin and thioredoxin reductase (Aharonowitz et al., 1993; Cohen et al., 1993). The thioredoxin system is of particular interest in β-lactam-producing streptomycetes because it may play a role in maintaining supplies of the reduced substrate for isopenicillin-N-synthase, δ-(L-α-amino-adipyl)-L-cysteinyl-D-valine (ACV) (Aharonowitz et al., 1993), the key precursor of most β-lactam antibiotics.

The levels of thioredoxin, glutaredoxin 1 and glutathione are balanced in both *E. coli* and yeast, such that deficiency or overproduction of one component promotes the opposite effect on the levels of the others (Miranda-Vizuete et al., 1996; Muller, 1996). Thus, for example, an *E. coli* mutant deficient in glutathione biosynthesis has a 5.5-fold elevated level of glutaredoxin 1 (Miranda-Vizuete et al., 1996). However, relatively little is understood about the underlying mechanisms that control this redox poise. Recently it has emerged that OxyR, a positive regulator of the oxidative stress response of *E. coli* (Storz et al., 1990; Jamieson and Storz, 1997), controls induction of at least two components of the glutaredoxin system, glutaredoxin 1 (Tao, 1997; Zheng et al., 1998) and glutathione reductase (Christman et al., 1985), in response to the reversible formation of an intramolecular disulfide bond in OxyR itself (Zheng et al., 1998). Importantly, glutaredoxin 1, in the presence of glutathione, can catalyse reduction of OxyR thereby inactivating it, providing a homeostasis loop for its regulation.

Here we identify an RNA polymerase sigma factor, σR, which forms part of a novel redox homeostasis system in
**Materials and methods.** Varying amounts of purified to near homogeneity (H11022 consensus sequence for of the GTG in vitro performed in vivo and in vitro) total RNA polymerase purified from S.coelicolor promoter template. As shown in Figure 2B, both sigRp2 methods.

Comparison of the predicted and experimentally determined N-terminal amino acid sequences showed complete agreement, with the exception of a glycine residue at position 21 which was tentatively assigned alanine by protein sequencing. This comparison also showed that the position 21 which was tentatively assigned alanine by agreement, with the exception of a glycine residue at position 21 which was tentatively assigned alanine by protein sequencing. This comparison also showed that the four first residues of the primary translation product (fMGPV) were removed post-translationally (this occurred in four separate seqR preparations). The gene sequence predicts that mature seqR is a protein of 223 amino acids (22 N-terminal amino acids: NH₂-TGTDAGTEHGQAEQ-Edman degradation and yielded the sequence of the first NTMGVPV) were removed post-translationally (this occurred in four separate seqR preparations). The gene sequence predicts that mature seqR is a protein of 223 amino acids (22 N-terminal amino acids: NH₂-TGTDAGTEHGQAEQ-Edman degradation and yielded the sequence of the first 300 nt), showing that seqRp2 is recognized by EσR in the absence of other transcriptional activators. On the other hand seqRp2 was not recognized by holoenzyme containing the major sigma factor in Streptomyces, seqRmut, indicating that this interaction is specific (data not shown).

**Results**

**Cloning of S.coelicolor sigR**

An RNA polymerase σ factor of apparent M, 31 000 has been identified previously in S.coelicolor total RNA polymerase (Kang et al., 1997; M.S.B.Paget and M.J.Buttner, unpublished). The purified σ factor, designated σR, was subjected to sequential N-terminal Edman degradation and yielded the sequence of the first 22 N-terminal amino acids: NH₂-TGTDAGTEHGQAEQ-PEGRGT(A)(A). This was used to design a degenerate 66-mer oligonucleotide which was used to clone the σR structural gene, sigR, as described in Materials and methods.

Comparison of the predicted and experimentally determined N-terminal amino acid sequences showed complete agreement, with the exception of a glycine residue at position 21 which was tentatively assigned alanine by protein sequencing. This comparison also showed that the four first residues of the primary translation product (fMGPV) were removed post-translationally (this occurred in four separate σR preparations). The gene sequence predicts that mature σR is a protein of 223 amino acids and M, 24 830. Global similarity searches of the NCBI databases showed that σR is a member of the ExtraCytoplasmic Function (ECF) subfamily of σ factors (Lonetto et al., 1994).

**σR directs transcription of its own structural gene in vivo and in vitro**

We examined transcription of sigR using S1 nuclease mapping. Two promoters were identified, initiating transcription 41 bp (sigRp1) and 215 bp (sigRp2) upstream of the GTG sigR start codon (Figure 1). The sigRp2 promoter sequence showed clear similarity to the proposed consensus sequence for σR-dependent promoters (Figure 2C; Kang et al., 1997). To determine whether sigRp2 depends on sigR in vivo, we constructed an in-frame sigR internal deletion mutation in the plasmid-free, protrophic M600 background, creating J2139, as described in Materials and methods. This deletion removed 303 bp of DNA encoding all of region 2, including the RNA polymerase core binding and –10 recognition domains. The sigR mutation abolished transcription from sigRp2 (Figure 2A), showing that sigR is under positive autoregulation via its p2 promoter.

To confirm that the sigRp2 promoter is a direct target for σR-containing RNA polymerase holoenzyme (EσR), we performed in vitro transcription assays using reconstituted EσR. σR was overproduced in soluble form in E.coli and purified to near homogeneity (>95%), as described in Materials and methods. Varying amounts of σR were added to S.coelicolor core RNA polymerase to reconstitute EσR holoenzyme, and incubated in the presence of a sigRp2 promoter template. As shown in Figure 2B, both total RNA polymerase purified from S.coelicolor and

**σR directs transcription of its own structural gene in vivo and in vitro**

We examined transcription of sigR using S1 nuclease mapping. Two promoters were identified, initiating transcription 41 bp (sigRp1) and 215 bp (sigRp2) upstream of the GTG sigR start codon (Figure 1). The sigRp2 promoter sequence showed clear similarity to the proposed consensus sequence for σR-dependent promoters (Figure 2C; Kang et al., 1997). To determine whether sigRp2 depends on sigR in vivo, we constructed an in-frame sigR internal deletion mutation in the plasmid-free, protrophic M600 background, creating J2139, as described in Materials and methods. This deletion removed 303 bp of DNA encoding all of region 2, including the RNA polymerase core binding and –10 recognition domains. The sigR mutation abolished transcription from sigRp2 (Figure 2A), showing that sigR is under positive autoregulation via its p2 promoter.

To confirm that the sigRp2 promoter is a direct target for σR-containing RNA polymerase holoenzyme (EσR), we performed in vitro transcription assays using reconstituted EσR. σR was overproduced in soluble form in E.coli and purified to near homogeneity (>95%), as described in Materials and methods. Varying amounts of σR were added to S.coelicolor core RNA polymerase to reconstitute EσR holoenzyme, and incubated in the presence of a sigRp2 promoter template. As shown in Figure 2B, both total RNA polymerase purified from S.coelicolor and

**The sigR null mutant is sensitive to menadione, plumbagin and diamide**

The sigR mutant was unaffected in growth, morphological differentiation and antibiotic production on all commonly used growth media, but was found to be significantly more sensitive to the redox-cycling compounds menadione and plumbagin. Redox cycling compounds are thought to exert their toxic effects mainly by the intracellular formation of the toxic O₂⁻ (superoxide) radical (Hassan and Fridovich, 1979). Streptomyces coelicolor produces two kinds of superoxide dismutases which disproportionate superoxide radicals to H₂O₂ (Kim et al., 1996). However, the level of these superoxide dismutases did not change in the sigR mutant, which suggests that its sensitivity to menadione and plumbagin did not result from increased accumulation of superoxide.

The sigR null mutant was also significantly more sensitive to the thiol-specific oxidizing agent diamide (Figure 3). Diamide causes the toxic formation of cytoplasmic disulfides in low-molecular-weight thiols and in proteins (Kosower and Kosower, 1995). The increased sensitivity of the sigR mutant to diamide might reflect a lowered thiol-buffer status and/or an inability to re-establish normal thiol levels. Redox cycling compounds could also exert their toxic effects by draining electrons from reductive pathways such as the ferredoxin and

**Thioredoxin system regulation in Streptomyces**

Streptomyces coelicolor. We show that σR is required for wild-type levels of resistance to oxidative stress and that it activates expression of the thioredoxin system in response to diamide-induced thiol oxidation. We also present evidence that the σR system functions in the actinomycete pathogen Mycobacterium tuberculosis.
Fig. 2. Transcription from the sigRp2 promoter depends on $\sigma^R$.
(A) Mapping of the sigRp1 and sigRp2 promoters in M600 (sig$R^+$) and J2139 (sig$R$). RNA was isolated from mycelium grown on cellophane discs on SFM agar for 36 h. The size markers (M) were a $32^P$-end-labelled $Hpa$II digest of pBR322. (B) In vitro transcription from the sigRp2 promoter by E$\sigma^R$. Transcripts were generated from a 1.04 kb $Xho$I–$Sal$I sig$R$ fragment using $S$.coelicolor total RNA polymerase (Holo), $S$.coelicolor core RNA polymerase (Core) or core enzyme plus increasing amounts of $\sigma^R$ (0.38, 0.75, 1.5, 3.0 and 6.0 pmol for lanes 3–7) as described in the text. The amount of core enzyme was fixed at 1.5 pmol per reaction. The expected size of a run-off transcript initiating at sigRp2 is 300 nt. (C) Sequence alignment of $\sigma^R$-dependent promoters. Comparison of the sequences of the $S$.coelicolor sigRp2 and trxBP1 promoters with that of hld$Bp2$ (Buttner et al., 1990), previously shown to be recognized by E$\sigma^R$ (Kang et al., 1997). A consensus for $S$.coelicolor $\sigma^R$-dependent promoters, and a putative trxB promoter of $M$.tuberculosis are presented. Putative –10 and –35 sequences are underlined, and bases matching the consensus sequence are in bold.

Fig. 3. Sensitivity of the sig$R$ mutant to diamide. Lawns of M600 (sig$R^+$) and J2139 (sig$R$) were generated by overlaying Difco Nutrient Agar plates with soft Nutrient Agar containing fresh spores. Immediately after plating, paper discs soaked in 100 mM or 1 M diamide were added, and plates were photographed after 24 h incubation at 30°C.

Fig. 4. Disulfide reductase activity in wild-type and sig$R$ mutant strains before and after diamide treatment. M600 (sig$R^+$) and J2139 (sig$R$) were grown in NMMP liquid medium (Hopwood et al., 1985) to late exponential phase (~11 h) and exposed to 0.5 mM diamide for 1 h where appropriate. Cell extracts were prepared by sonication followed by high-speed centrifugation (100 000 g), and disulfide reductase activity was measured as described by Holmgren (1979) using either (A) insulin or (B) DTNB as substrate. Reduction of insulin was followed by recording consumption of NADPH (340 nm), and reduction of DTNB was followed by recording the appearance of TNB (412 nm). Standard deviations are indicated.

Disulfide reductase activity in a sig$R$ null mutant is lower than in wild-type $S$.coelicolor and is not induced by diamide

To see whether the sig$R$ mutant had a lowered capacity to reduce disulfide bonds, we measured the level of disulfide reductase activity in J2139 (sig$R$) and M600 (sig$R^+$) using two different substrates: insulin, which contains two interchain disulfide bonds, and a synthetic disulfide-containing substrate, DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] (Holmgren, 1977, 1979). The basal level of insulin disulfide reductase activity was 3-fold lower in the sig$R$ mutant than in M600 (Figure 4A). We also found that in M600 insulin disulfide reductase activity was induced 3.3-fold in response to treatment with 0.5 mM diamide for 1 h, whereas the disulfide reductase activity of J2139 showed no significant response to diamide (Figure 4A). Assays using DTNB as substrate gave a similar profile (Figure 4B). In $Streptomyces clavuligerus$, almost all of the insulin and DTNB reductase activity corresponds to the thioredoxin–thioredoxin reductase couple (Aharonowitz et al., 1993).

$\sigma^R$ directlys transcription from the p1 promoter of the trxBA operon in vivo and in vitro

The decrease in protein disulfide reductase activity seen in the sig$R$ mutant suggested that the thioredoxin system...
The Thioredoxin system regulation in *Streptomyces*

**The sigR promoter is induced by diamide**

Given that the *trxBp1* promoter is under σ^R^ control, and that protein disulfide reductase activity is induced by diamide treatment in a sigR-dependent manner, we tested whether transcription of the *trx*BA operon is induced in response to thiol oxidation. The levels of the two *trx*BA transcripts were examined in the *sigR^+^* strain M600 and the *sigR* mutant J2139 at 10 min intervals following diamide treatment. In M600 the level of the *trxBp1* transcript was dramatically increased (~50-fold) after 10 min exposure to 0.5 mM diamide, but fell back to uninduced levels after 40–50 min (Figure 6). In J2139 there was no transient induction of *trxBp1*, but a small steady increase which, after 1 h, was 4-fold above uninduced levels. In the wild type, the level of the *trxBp2* transcript also showed a transient increase (3-fold) in response to diamide, but this was not seen in the *sigR* mutant.

**The sigRp2 promoter is induced by diamide**

The induction of *trx*BA transcript levels by diamide suggested that Eσ^R^ activity increased in response to thiol oxidation. To see whether thiol oxidation also induced transcription of *sigR* from the σ^R^ dependent *sigRp2* promoter, we examined *sigR* transcription following treatment with diamide, using the same RNA samples used to investigate *trxBp1* induction. As with *trxBp1*, the level of the *sigRp2* transcript increased strongly (~70-fold) in the wild type after 10 min, then fell back to uninduced levels after 40–50 min (Figure 7). However, in contrast to *trxBp1*, no residual *sigRp2* activity was detected in the *sigR* mutant upon extended incubation with diamide. The level of the *sigRp1* transcript was not significantly affected by diamide.
Discussion

We have shown that the thiol-specific oxidant diamide induces expression of a disulfide reductase system in wild-type *S.coelicolor*, but that little or no induction is seen in a *sigR* null mutant. Consistent with these observations, the *p1* promoter of the *trxBA* operon is a direct target for EσR and the promoter is dramatically induced by diamide in a *sigR*-dependent manner. In *E.coli*, disruption of both the thioredoxin and glutaredoxin pathways causes increased sensitivity to diamide, but disruption of either pathway individually does not significantly affect diamide sensitivity (Prinz et al., 1997). The sensitivity of the *sigR* mutant to diamide may mean that the thioredoxin system plays a critical role in disulfide bond reduction in *Streptomyces*. In this context it is interesting to note that in another Gram-positive bacterium, *Bacillus subtilis*, thioredoxin is an essential protein (Scharf et al., 1998).

The *sigR* mutant is also sensitive to the redox cycling compounds menadione and plumbagin. Because the levels of superoxide dismutases are unaffected in the *sigR* mutant, this phenotype could reflect a compromised ability to cope with the drainage of electrons from disulfide reductase pathways. Redox cycling compounds are reduced enzymatically by oxido-reductases using electrons from NADPH, and then undergo autooxidation to produce O$_2$$^-$. In *E.coli*, thioredoxin reductase can reduce redox cycling compounds including menadione (Holmgren, 1979). We have found that menadione reductase (diaphorase) activity is inducible by diamide in wild-type *S.coelicolor*, but not in the *sigR* mutant (data not shown). It is therefore likely that the thioredoxin reductase of *S.coelicolor* can reduce menadione and plumbagin, and that in the presence of these compounds reducing power is drawn away from the thioredoxin pathway, resulting in increased disulfide bond formation in the cytoplasm. Alternatively, or in addition, O$_2$$^-$ may induce disulfide bond formation directly (Halliwell and Gutteridge, 1989).

As well as catalysing the reduction of protein disulfide bonds, *in vitro*, the thioredoxin system of streptomycetes can reduce the oxidized disulfide form of the tripeptide β-lactam precursor ACV (Aharonowitz et al., 1993; Cohen et al., 1993). Bis-ACV is not a substrate for isopenicillin -N-synthase, and it has been proposed that the thioredoxin system plays a role in maintaining the intracellular level of reduced ACV, thereby supporting the rate of synthesis of isopenicillin N (Aharonowitz et al., 1993). Thus, σR may also play an important role in the biosynthesis of penicillin and cephalosporin antibiotics in β-lactam-producing streptomycetes.

The identification of the *trxBA* genes as a target for EσR, and the observed induction of *trxBA* transcription by diamide-induced thiol oxidation, provide components for a potential autoregulatory loop, as shown in Figure 8. Exposure of *S.coelicolor* hyphae to oxidative stress causes intracellular thiol oxidation, which induces σR activity, leading to increased production of thioredoxin and thioredoxin reductase, as well as σR itself. Upon re-establishment of normal reduced thiol levels by the thioredoxin system, σR activity is switched off, resulting in down-regulation of *trxBA*. The model clearly lacks important component(s) to explain how σR activity responds to thiol oxidation, since σR itself contains no cysteines. The positive autoregulation of σR via transcription of the *sigRp2* promoter by EσR itself implies that there must be a negative regulatory element within the σR system, to prevent an endless spiral of σR expression. Recent experiments have revealed that this negative regulatory element is provided by an anti-sigma factor whose activity is directly modulated by thiol oxidation and reduction (J.-G.Kang, M.S.B.Paget, M.J.Buttner and J.-H.Roe, in preparation).

There is an interesting comparison to be made between σR and OxyR of *E.coli*. Both proteins positively regulate transcription in response to thiol oxidation and both activate expression of disulfide reductase pathways: the thioredoxin pathway in the case of σR and the glutaredoxin pathway in the case of OxyR. However, while the activity of OxyR is modulated directly by reversible disulfide bond formation, the activity of σR is modulated indirectly via an anti-sigma factor. Also, while OxyR is thought to have evolved to sense peroxides and is also required for resistance to peroxides, the *sigR* mutant is no more sensitive than the wild type to hydrogen peroxide (data not shown).

Although the *trxBp1* promoter is highly dependent on *sigR*, there is a low level of transcription from the promoter in the *sigR* null mutant that is still inducible by diamide (Figure 6). However, since the kinetics of induction are very different from those in the wild-type strain, it is not clear whether transcription is induced in response to thiol oxidation or to some other related stress. We intend to identify the sigma factor responsible for this residual transcription and determine whether, under other growth conditions, it plays a more significant role in expression of the thioredoxin system.

Finally, we note that σR is not confined to the genus *Streptomyces*. From its genome sequence, there is a σR-like sigma factor (68% identity; Rv3223c; Cole et al., 1998) in the actinomycete pathogen *M.tuberculosis*, and there is circumstantial evidence that it may play a similar role to σR in *S.coelicolor*. As in *Streptomyces*, the thioredoxin reductase and thioredoxin genes of *M.tuberculosis* are adjacent and probably co-transcribed, and there is a sequence upstream of the operon (*Mt trxBp1*; Figure 8).
2C) that has striking similarity to the σR-dependent trxBp1 promoter of S. coelicolor. Given that overexpression of the hybrid thioredoxin-reductase–thioredoxin gene of Mycobacterium leprae in the non-pathogen Mycobacterium smegmatis increases its resistance to oxygen-dependent killing within human mononuclear phagocytes (Wielies et al., 1997), σR probably plays a role in the resistance of mycobacteria to oxidative stress.

Materials and methods

Bacterial strains and culture conditions

All experiments were performed using S. coelicolor A3(2) strains M600 (SCP1–, SCP2–; Chakraburtty and Bibb, 1997) or M145 (SCP1–, SCP2–; sequence analysis of the

increases its resistance to oxygen-dependent
eating within human mononuclear phagocytes (Wieles et al., 1997).

cloning and nucleotide sequencing of the sigR gene

S. coelicolor strain M145 was grown in YEME medium containing 34% sucrose and 5 mM MgCl₂ under vigorous aeration to OD₆₀₀

A₃(2) strains M600

of the

OTGAGCTATAGCTGACAG-G σ

σ

S. coelicolor, E. coli

DNA-cellulose affinity chromatography and Superose 6 gel filtration FPLC, as described previously (Buttner et al., 1988; Brown et al., 1992). The fractions of RNA polymerase eluted from the Superose 6 gel filtration column that contained peak levels of σR were pooled and subjected to electrophoresis on a 7.5% polyacrylamide–SDS gel and electrophoresis was then separated by 0.5 A onto ‘Trans-Blot’ (Trans-Blot Transfer Medium polyvinylidene difluoride (PVDF) protein sequencing membrane; Bio-Rad) in 10 mM cyclohexylaminopropane sulfonic acid (CAPS) pH 11.0, 10% (v/v) aqueous methanol. The membrane was subsequently stained and treated as described (Brown et al., 1992) in order to visualize the 31 kDa σR-σppolyepitope. The sequence of the first 22 residues of σR was determined by sequential N-terminal Edman degradation using a gas-phase sequencer.

Cloning and nucleotide sequencing of the sigR gene

The 16384-fold degenerate 66-mer oligonucleotide, 5G50, designed from the sequence of the first 22 N-terminal residues of σR was:

G G G G G G

G G G G G G

G G G G G G

G G G G G G

G550 was radiolabelled using [3²P²P]ATP (3000 Ci/mmole) and T4 polynucleotide kinase and used to probe an ordered cosmid library of S. coelicolor cosmids DNA (Redenbach et al., 1996). After washing at 60°C in 0.2× SSC, 0.1% SDS, strong signals were obtained with overlapping cosmid 3B6 and 6F2. The sigR gene was cloned from cosmid 3B6 on a 4.3 kb BglI–BamHI fragment. The nucleotide sequence of sigR was determined and has been deposited in the DDBJ/EMBL/ GenBank databases (accession No. AJ013020).

Creation of a sigR deletion mutant

A sigR null mutant allele was constructed by deleting a 303 bp SalI fragment between two in-frame SalI sites internal to sigR. The mutant allele was isolated on a 2.5 kb XhoI–SalI fragment and cloned into the suicide delivery plasmid pDH5 (Hilleman et al., 1991) cut with BamHI, to create pIJ5967. pIJ5967 was introduced into protoplasts of S. coelicolor M600 by PEG-mediated transformation (Hopwood et al., 1985). Resistance to thiostrepton, conferred by the vector’s trxB gene, was selected and used to construct transformants. The resulting spores were plated to form single colonies. Thiostrepton-sensitive isolates, in which the delivery plasmid had presumably been lost, were analysed by Southern hybridization to see if they had the wild-type or the null allele of sigR. One sigR isolate was designated J2139.

Overproduction and purification of σR

The sigR ORF was amplified by PCR using mutagenic primers based on the 5′ end (SigR/5′-GCCGATGATGATCACGTTTCGGACGTCG-3′) and the 3′ end (SigR/3′-GGCCGATGATGATCACGTTTCGGACGTCG-3′) which introduced an NdeI site that overlapped the start codon and a BamHI cloning site downstream from the stop codon. The resulting 715 bp fragment was cloned into PET21b (Novagen) following digestion with NdeI and BamHI, yielding PET21b-sigR. The sequence of the cloned sigR gene in the PET21b vector was confirmed by DNA sequencing. PET21b-sigR was designed to yield a primary translation product starting with NhtMTGTGTD, to agree with the experimentally determined N-terminal sequence of σR purified from S. coelicolor.

Fresh E. coli BL21DE3/pLYS5 transformants carrying PET21b–sigR were inoculated in L broth containing chloramphenicol and grown, with shaking at 37°C, to an A₆₀₀ of 0.5–0.8. Following induction with 0.4 mM isopropyl-β-D-galactoside (IPTG) for 3 h, cells were harvested. Cell pellets were resuspended in lysis buffer [20 mM Tris–HCl pH 7.9, 10 % (v/v) glycerol, 5 mM EDTA, 0.1 mM DTT] and incubated on ice for 20 min. After gentle sonication, sodium deoxycholate (NaDOC) was added to a final concentration of 0.1% (v/v), and the solution was mixed well and incubated at 4°C for 10 min before centrifugation (10 000 g for 10 min). The supernatant was loaded directly onto a 60 ml Q-Sepharose CL 6B column. Proteins were eluted using a gradient of 0.2–1.0 M NaCl in TGED buffer (10 mM Tris–HCl pH 7.9 at 30°C, 0.1 mM EDTA, 0.1 % (v/v) glycerol) at 50 ml/h. σR was eluted at 0.3 M NaCl as a sharp peak. Further purification was achieved through Superose 12 and Mono-Q columns on FPLC.

In vitro transcription assay

In vitro run-off transcription assays were performed using the conditions of Buttner et al. (1988), with slight modifications as described by Kang et al. (1997). To reconstitute E. coli σR-dependent holohexamer, varying amounts of σR (0.38–6.0 pmol) were incubated with RNA polymerase core enzyme (0.5 pmol) at 30°C for 30 min. E. coli core RNA polymerase was purchased from Epicentre Technologies. For sigRp2, 1.04 kb XhoI–SalI fragment that includes the 5′ end of sigR and upstream sequences was used as template. For trxBp1, a 220 bp XhoI fragment isolated from pIJ5970 (pBluescript SK+ containing a 0.75 kb Apal fragment that includes the 5′ end of trxB and upstream sequences) was used as a template.

RNA isolation and S1 nuclelease protection analysis

RNA was isolated from mycelium scraped from cellophane discs on SFM agar plates after 36 h, or from NMMP liquid cultures grown to mid-late exponential phase (for ~10 h), essentially as described by Hopwood et al. (1985). However, rather than exhaustive phenol/chloroform extraction and DNase treatment, RNA was separated from contaminating DNA and protein by CsCl gradient centrifugation following an initial phenol/chloroform extraction, as described by Hindle and Smith (1994). RNA was isolated from SFM agar plates as described above, except that, after addition of phenol/chloroform, the mixture was heated at 65°C for 10 min prior to vortex mixing. The sigR promoter region was mapped using a 1.1 kb probe generated by PCR from pIJ5965 [pMT3000 (Paget et al., 1994) containing a 1.04 kb XhoI–SalI fragment that includes the 5′ end of sigR and upstream sequences] using a 5′ end-labelled oligonucleotide primer internal to sigR (SP5; 5′–TCGACTCGCCGGCGGCTAACC–3′) and the universal sequencing primer. The trxB promoter region was mapped using a 0.7 kb probe generated by PCR from pIJ5970 using a 5′ end-labelled oligonucleotide primer internal to trxB (TRX1:GAGCCGATGATGATCACGTTTCGGACGTCG) and the universal sequencing primer. Primers were labelled and S1 nuclease protection assays were set up as described by Jones et al. (1997). The S1 signals were quantified with a PhosphorImager (Fuji BAS1000). Experiments were carried out twice on independent RNA samples.

Disulfide reductase assays

Strains were grown in NMMP liquid medium to late exponential phase (for ~10 h) and exposed to 0.5 mM diamide for 1 h where appropriate. Cell extracts were prepared by sonication followed by high-speed centrifugation (100 000 g), and disulfide reductase activity was measured as described by Holmgren (1977, 1979) using either insulin or DTNB as substrate. Insulin disulfide reductase activity was measured by recording the consumption of NADPH at 340 nm. DTNB reductase activity was measured by following the increase in absorbance of TNB.
References


