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

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We have identified an RNA polymerase sigma factor, σ^R , 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 σ^R 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 *trxB* operon, encoding thioredoxin reductase and thioredoxin, was found to be under the direct control of σ^R . *trxB* 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 σ^R directed transcription from *trxBp1* *in vitro*, indicating that *trxBp1* is a target for σ^R . 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 σ^R 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 σ^R induces expression of the thioredoxin system in response to cytoplasmic disulfide bond formation. Upon re-establishment of normal thiol levels, σ^R activity is switched off, resulting in down-regulation of *trxB* and *sigR*. We present evidence that the σ^R 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, σ^R , which forms part of a novel redox homeostasis system in

Streptomyces coelicolor. We show that σ^R 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 σ^R system functions in the actinomycete pathogen *Mycobacterium tuberculosis*.

Results

Cloning of *S.coelicolor sigR*

An RNA polymerase σ factor of apparent M_r 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 σ^R , 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 σ^R 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 first four residues of the primary translation product (fMGVPV) were removed post-translationally (this occurred in four separate σ^R preparations). The gene sequence predicts that mature σ^R is a protein of 223 amino acids and M_r 24 830. Global similarity searches of the NCBI databases showed that σ^R is a member of the ExtraCytoplasmic Function (ECF) subfamily of σ factors (Lonetto *et al.*, 1994).

σ^R 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 σ^R -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, prototrophic 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 auto-regulation via its *p2* promoter.

To confirm that the *sigRp2* promoter is a direct target for σ^R -containing RNA polymerase holoenzyme ($E\sigma^R$), we performed *in vitro* transcription assays using reconstituted $E\sigma^R$. σ^R was overproduced in soluble form in *E.coli* and purified to near homogeneity (>95%), as described in Materials and methods. Varying amounts of σ^R were added to *S.coelicolor* core RNA polymerase to reconstitute $E\sigma^R$ 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

Fig. 1. Assignment of transcription start points for *sigR*. (A) and (B) S1 nuclease mapping of the *sigRp1* and *sigRp2* promoters, respectively, in wild-type *S.coelicolor*. RNA was isolated from mycelium grown on cellophane discs on SFM agar for 36 h. The lanes labelled S1 indicate the DNA fragment protected by the RNA transcripts initiating at *sigRp1* or *sigRp2*. Lanes labelled A, C, G and T indicate a dideoxy sequencing ladder using the same oligonucleotide that was used to make the S1 probe (SP5). The strongest band was chosen as the transcription start point. (C) Sequence of the *sigR* upstream region indicating transcription start points, putative -10 and -35 promoter sequences (underlined), and the 5' end of *sigR*.

the reconstituted $E\sigma^R$ holoenzyme produced a specific transcript of the predicted size (300 nt), showing that *sigRp2* is recognized by $E\sigma^R$ in the absence of other transcriptional activators. On the other hand *sigRp2* was not recognized by holoenzyme containing the major sigma factor in *Streptomyces*, σ^{HrdB} , indicating that this interaction is specific (data not shown).

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 O_2^- (superoxide) radical (Hassan and Fridovich, 1979). *Streptomyces coelicolor* produces two kinds of superoxide dismutases which disproportionate superoxide radicals to H_2O_2 (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

Fig. 2. Transcription from the *sigRp2* promoter depends on σ^R . (A) Mapping of the *sigRp1* and *sigRp2* promoters in M600 (*sigR*⁺) and J2139 (*sigR*). RNA was isolated from mycelium grown on cellophane discs on SFM agar for 36 h. The size markers (M) were a ³²P-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–*Sa*I *sigR* fragment using *S.coelicolor* total RNA polymerase (Holo), *S.coelicolor* core RNA polymerase (Core) or core enzyme plus increasing amounts of σ^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 σ^R -dependent promoters. Comparison of the sequences of the *S.coelicolor sigRp2* and *trxBp1* promoters with that of *hrdDp2* (Buttner *et al.*, 1990), previously shown to be recognized by $E\sigma^R$ (Kang *et al.*, 1997). A consensus for *S.coelicolor* σ^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. Transcription start points are in bold.

Fig. 3. Sensitivity of the *sigR* mutant to diamide. Lawns of M600 (*sigR*⁺) and J2139 (*sigR*) 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 *sigR* mutant strains before and after diamide treatment. M600 (*sigR*⁺) and J2139 (*sigR*) 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.

thioredoxin systems (Holmgren, 1979; Liochev *et al.*, 1994). Thus, menadione and plumbagin may also cause increased levels of disulfide bond formation.

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

To see whether the *sigR* mutant had a lowered capacity to reduce disulfide bonds, we measured the level of disulfide reductase activity in J2139 (*sigR*) and M600 (*sigR*⁺) 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 *sigR* 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).

σ^R directs transcription from the *p1* promoter of the *trxBA* operon in vivo and in vitro

The decrease in protein disulfide reductase activity seen in the *sigR* mutant suggested that the thioredoxin system

Fig. 5. Transcription from the *trxBp1* promoter depends on σ^R . (A) Mapping the *trxBp1* and *trxBp2* promoters in M600 (*sigR*⁺) and J2139 (*sigR*). RNA was isolated from mycelium grown on cellophane discs on SFM agar for 36 h. Lanes labelled A, C, G and T indicate a dideoxy sequencing ladder using the same oligonucleotide that was used to make the S1 probe (TRX1). (B) *In vitro* transcription from the *trxBp1* promoter by $E\sigma^R$. Transcripts were generated from a 220 bp *XhoI* *trxB* promoter fragment using *E. coli* core RNA polymerase or core enzyme plus σ^R . The expected size of a run-off transcript initiating at *trxBp1* is 112 nt. The size markers (M) were a ³²P-end-labelled *HinfI* digest of ϕ X174. (C) Sequence of the *trxB* upstream region indicating transcription start points and putative -10 and -35 promoter sequences (underlined). The *trxB* initiation codon is marked.

of *Streptomyces* is under the control of σ^R . The *trxB* operons of *S. clavuligerus* and *S. coelicolor* have been cloned and the sequence of the region upstream of the *S. clavuligerus* operon has been determined (Cohen *et al.*, 1993; accession No. Z21946). Inspection of this sequence revealed a candidate σ^R -dependent promoter. Accordingly, we sequenced the corresponding region of the *S. coelicolor* operon and found a very similar sequence (Figures 2C and 5C). To determine whether this sequence was indeed a target for $E\sigma^R$, we compared transcription of the *trxB* operon in the *sigR* mutant and its parental strain M600. In M600, two closely spaced promoters were identified, which initiate transcription 64–65 bp (*trxBp1*, corresponding to the putative promoter identified by sequence inspection) and 71 bp (*trxBp2*) upstream of the *trxB* GTG start codon (Figure 5A). However, in the *sigR* mutant J2139, transcription from the downstream promoter, *trxBp1*, was reduced by >95% (Figure 5A). *In vitro* transcription using reconstituted σ^R holoenzyme confirmed that the *trxBp1* promoter is a direct target for $E\sigma^R$ (Figure 5B). The identity of the transcript seen with core RNA polymerase alone is not clear, but it may indicate initiation at *trxBp2*, perhaps by a contaminating holoenzyme form.

The *trxBp1* 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 *trxB* operon is induced in response to thiol oxidation. The levels of the two *trxB* 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

Fig. 6. Induction of the *trxB* promoters by diamide. M600 (*sigR*⁺) and J2139 (*sigR*) were grown in NMMP liquid medium to mid-late exponential phase (~10 h) and exposed to 0.5 mM diamide. RNA was extracted at 10 min intervals over 1 h. S1 nuclease protection assays were carried out as described in the Materials and methods. Control experiments using an RNA dilution series were performed to ensure that hybridizations were carried out in conditions of probe excess.

Fig. 7. Induction of the *sigR* promoters by diamide. The same RNA samples used to examine diamide induction of *trxB* transcription in M600 (*sigR*⁺) and J2139 (*sigR*) (Figure 6) were used to examine the response of the *sigR* promoters. S1 nuclease protection assays were carried out as described in the Materials and methods.

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 *trxBp1* transcript levels by diamide suggested that $E\sigma^R$ 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, 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 *pI* promoter of the *trxB* operon is a direct target for $E\sigma^R$ 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, σ^R may also play an important role in the biosynthesis of penicillin and cephalosporin antibiotics in β -lactam-producing streptomycetes.

The identification of the *trxB* genes as a target for $E\sigma^R$, and the observed induction of *trxB* 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 σ^R activity, leading to increased production of thioredoxin and thioredoxin reductase, as well as σ^R itself. Upon re-establishment of normal reduced thiol levels by the thioredoxin system, σ^R activity is switched off, resulting in down-regulation of *trxB*. The model clearly lacks important component(s) to explain how σ^R activity responds to thiol oxidation, since σ^R itself contains no cysteines. The

Fig. 8. A model for homeostasis of the thioredoxin system in *S.coelicolor*. Exposure of *S.coelicolor* hyphae to oxidative stress causes intracellular disulfide bond formation (thiol oxidation), which induces σ^R activity, leading to increased expression of thioredoxin and thioredoxin reductase. Upon re-establishment of normal thiol levels, σ^R activity is switched off by an unknown mechanism, resulting in down-regulation of *trxB*. *sigR* expression is subject to positive autoregulation via transcription of the *sigRp2* promoter.

positive autoregulation of *sigR* via transcription of the *sigRp2* promoter by $E\sigma^R$ itself implies that there must be a negative regulatory element within the σ^R system, to prevent an endless spiral of *sigR* 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 σ^R 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 σ^R 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 σ^R 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 σ^R is not confined to the genus *Streptomyces*. From its genome sequence, there is a σ^R -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 σ^R 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

2C) that has striking similarity to the σ^R -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 (Wieleś *et al.*, 1997), σ^R 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⁺; Chakraborty and Bibb, 1997) or M145 (SCP1⁺, SCP2⁺; Hopwood *et al.*, 1985) grown on SFM agar (Hobbs *et al.*, 1989) or in YEME or NMMP liquid media as described by Hopwood *et al.* (1989). For transformation of *S.coelicolor*, unmethylated DNA was isolated from *E.coli* ET12567 (MacNeil *et al.*, 1992). Overexpression was carried out using *E.coli* BL21 λ DE3/pLysS (Studier and Moffat, 1986).

Purification of *S.coelicolor* RNA polymerase and N-terminal sequence analysis of the σ^R protein

S.coelicolor strain M145 was grown in YEME medium containing 34% sucrose and 5 mM MgCl₂ under vigorous aeration to OD₆₀₀ = 0.6–0.8, and σ^R -containing RNA polymerase holoenzyme was purified from ~50 g of mycelium by Polymyxin P precipitation of cleared lysates, salt extraction, ammonium sulfate fractionation, 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 σ^R were pooled and subjected to electrophoresis on a 7.5% polyacrylamide-SDS gel and electroblotted for 30 min at 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 σ^R polypeptide. The sequence of the first 22 residues of σ^R 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, G550, designed from the sequence of the first 22 N-terminal residues of σ^R was:

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5'-ACC-GGC-ACC-GAC-GCC-GGC-ACC-GAG-CAC-GGC-CAG-
      G G G G G G G
GCC-GAG-CAG-CCC-GAG-GGC-CGC-GGC-ACC-GCC-GCC-3'
      G G G G G G G
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G550 was radiolabelled using [γ -³²P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase and used to probe an ordered cosmid library of *S.coelicolor* chromosomal DNA (Redenbach *et al.*, 1996). After washing at 60°C in 0.2× SSC, 0.1% SDS, strong signals were obtained with overlapping cosmids 3B6 and 6F2. The *sigR* gene was subcloned from cosmid 3B6 on a 4.3 kb *Bgl*II–*Bam*HI fragment. The nucleotide sequence of *sigR* was determined and has been deposited in the DDBJ/EMBL/GenBank databases (accession No. AJ010320).

Creation of a *sigR* deletion mutant

A *sigR* null mutant allele was constructed by deleting a 303 bp *Sal*I fragment between two in-frame *Sal*I sites internal to *sigR*. The mutant allele was isolated on a 2.5 kb *Xho*I–*Sal*I fragment and cloned into the suicide delivery plasmid pDH5 (Hillemann *et al.*, 1991) cut with *Bam*HI, to create pIJ5969. pIJ5969 was introduced into protoplasts of *S.coelicolor* M600 by PEG-mediated transformation (Hopwood *et al.*, 1985). Resistance to thiostrepton, conferred by the vector's *tsr* gene, was used to select transformants in which integration had occurred via a single cross-over between either of the flanking DNA sequences and their chromosomal counterparts. A single isolate with the expected chromosomal structure (as judged by Southern hybridization) was then allowed to undergo one round of sporulation in the absence of thiostrepton and 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 σ^R

The *sigR* ORF was amplified by PCR using mutagenic primers based on the 5' end (SigRN; 5'-GGTGGGTCATATGACTGGGACCG-3') and the 3' end (SigRC; 5'-CGTCTCGTGCGGATCCCGCAGCTC-3') which introduced an *Nde*I site that overlapped the start codon and a *Bam*HI cloning site downstream from the stop codon. The resulting 715 bp PCR fragment was cloned into pET21b (Novagen) following digestion with *Nde*I and *Bam*HI, 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 NH₂MTGTD, to agree with the experimentally determined N-terminal sequence of σ^R purified from *S.coelicolor*.

Fresh *E.coli* BL21 λ DE3/pLysS 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 isopropylthio- 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, 10 mM MgCl₂, 1 mM PMSF, 0.15 M NaCl] containing 250 μ g/ml lysozyme and incubated on ice for 20 min. After gentle sonication, sodium deoxycholate (NaDOC) was added to a final concentration of 0.1% (w/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 4°C, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol) at 50 ml/h. σ^R 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 σ^R holoenzyme, varying amounts of σ^R (0.38–6.0 pmol) were incubated with RNA polymerase core enzyme (1.5 pmol) at 30°C for 15 min. *E.coli* core RNA polymerase was purchased from Epicentre Technologies. For *sigR*p2, a 1.04 kb *Xho*I–*Sal*I fragment that includes the 5' end of *sigR* and upstream sequences was used as template. For *trxBp1*, a 220 bp *Xho*I fragment isolated from pIJ5970 (pBluescript SK⁺ containing a 0.75 kb *Apa*I fragment that includes the 5' end of *trxB* and upstream sequences) was used as a template.

RNA isolation and S1 nuclease 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 *Xho*I–*Sal*I fragment that includes the 5' end of *sigR* and upstream sequences] using a 5' end-labelled oligonucleotide primer internal to *sigR* (SP5; 5'-TCGACTCCGCGCCCGTACC-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; GAGCCGATGATGATCAGTTTCGGACGTCG) 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 (Fujix 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 ~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 (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 appearance of TNB

at 412 nm. Protein concentration was determined using a Bio-Rad protein assay kit.

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