The σ^R regulon of *Streptomyces coelicolor* A3(2) reveals a key role in protein quality control during disulphide stress

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Diamide is an artificial disulphide-generating electrophile that mimics an oxidative shift in the cellular thiol–disulphide redox state (disulphide stress). The Gram-positive bacterium *Streptomyces coelicolor* senses and responds to disulphide stress through the σ^R –RsrA system, which comprises an extracytoplasmic function (ECF) sigma factor and a redox-active anti-sigma factor. Known targets that aid in the protection and recovery from disulphide stress include the thioredoxin system and genes involved in producing the major thiol buffer mycothiol. Here we determine the global response to diamide in wild-type and *sigR* mutant backgrounds to understand the role of σ^R in this response and to reveal additional regulatory pathways that allow cells to cope with disulphide stress. In addition to thiol oxidation, diamide was found to cause protein misfolding and aggregation, which elicited the induction of the HspR heat-shock regulon. Although this response is σ^R -independent, σ^R does directly control Clp and Lon ATP-dependent AAA(+) proteases, which may partly explain the reduced ability of a *sigR* mutant to resolubilize protein aggregates. σ^R also controls *msrA* and *msrB* methionine sulfoxide reductase genes, implying that σ^R –RsrA is responsible for the maintenance of both cysteine and methionine residues during oxidative stress. This work shows that the σ^R –RsrA system plays a more significant role in protein quality control than previously realized, and emphasizes the importance of controlling the cellular thiol–disulphide redox balance.

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INTRODUCTION

Stable disulphide bonds do not form inside the bacterial or eukaryotic cytoplasm, although transient disulphides might be involved in the cytoplasmic folding of some mammalian proteins (Li *et al.*, 2002) and in the enzymic activity of some enzymes such as ribonucleotide reductase, while a disulphide bond is crucial for the activation of superoxide dismutase during oxidative stress (Furukawa *et al.*, 2004). Nonetheless, during oxidative stress, protein cysteine thiols can be susceptible to oxidation, resulting in disulphide bonds (disulphide stress) or other oxidation states, such as sulphenic acid (Paget & Buttner, 2003). Organisms adopt

two major strategies to ensure that susceptible cellular thiols are maintained in a reduced state. Firstly, protection is achieved through the use of millimolar levels of thiol buffers (e.g. glutathione) and enzymes such as catalase and alkyl hydroperoxide reductase that catalyse the degradation of oxidants. Secondly, organisms increase the expression of repair enzymes such as thioredoxin and glutaredoxin that can reduce unwanted disulphide bonds. However, functional genomic approaches are revealing that organisms in fact mount a broad response to this stress (Gasch *et al.*, 2000; Leichert *et al.*, 2003; Manganeli *et al.*, 2002).

In the antibiotic-producing bacterium *Streptomyces coelicolor*, the cellular thiol–disulphide redox balance is sensed and controlled by the σ^R –RsrA system (Kang *et al.*, 1999; Paget *et al.*, 1998). σ^R is an extracytoplasmic function (ECF) sigma factor that is continually synthesized, but held inactive by the anti-sigma factor RsrA. During disulphide stress, RsrA forms an intramolecular disulphide bond involving two of the ligands that coordinate zinc in the reduced protein (Bae *et al.*, 2004; Li *et al.*, 2003; Paget *et al.*, 2001a; Zdanowski *et al.*, 2006). This causes a structural change in RsrA, which results in the release of σ^R , the association of σ^R with RNA polymerase, and the activation of the σ^R regulon, including the thioredoxin system and

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Abbreviations: gDNA, genomic DNA; QT, quality threshold.

The microarray data discussed in this paper together with detailed methods are available from ArrayExpress under accession number E-MAXD-57.

A supplementary figure, showing an alignment of known and predicted σ^R promoter sequences in *S. coelicolor*, and two supplementary tables, listing primers used to generate probes for S1 nuclease mapping and genes downregulated by diamide, are available with the online version of this paper.

several other putative thiol–disulphide oxidoreductases. In addition, σ^R controls the expression of several genes thought to be involved in the synthesis of low-molecular-mass thiols such as cysteine (*cysM*) and coenzyme A (*coaE*), and *sigR* mutants contain fourfold lower levels of the major cytoplasmic thiol buffer mycothiol (Paget *et al.*, 2001b; Park & Roe, 2008). In support of a key role in controlling cellular thiol–disulphide redox balance, *sigR* null mutants are particularly sensitive to the artificial thiol-specific oxidant diamide.

In eukaryotes, diamide is known to cause extensive protein aggregation (Freeman *et al.*, 1995). All organisms continually monitor the quality of their proteins and can remove potentially harmful aggregates that form during certain stresses such as heat shock or pathophysiological stress (Wickner *et al.*, 1999). In *Escherichia coli*, normal protein folding pathways act synergistically with refolding and degradative pathways to prevent the accumulation of protein aggregates. Molecular chaperones, such as the DnaK machine, transiently hold folding proteins, preventing their aggregation, and in addition can act with the AAA+ chaperone ClpB to disaggregate protein aggregates (Goloubinoff *et al.*, 1999). The Clp and Lon AAA+ proteases play particularly important roles in degrading protein aggregates (Schmidt *et al.*, 2009). Clp protease holoenzyme consists of a proteolytic core of 14 ClpP subunits associated with hexamers of ATPase subunits, either ClpX or ClpA [or ClpC in *Streptomyces* spp. and *Bacillus* spp. (Porankiewicz *et al.*, 1999)]. The Lon protease contains the proteolytic and ATPase activities on the same polypeptide chain. In *E. coli*, the heat-shock sigma factor σ^{32} plays a key role in regulating the expression of both chaperone and protease pathways in response to protein misfolding (Zhao *et al.*, 2005). However, σ^{32} is absent from Gram-positive bacteria, which instead use entirely different mechanisms to control ATP-dependent protease and chaperone gene expression.

In *S. coelicolor* and in the closely related *Streptomyces lividans*, three regulatory systems govern the expression of DnaK chaperones and ATP-dependent proteases. HspR, in conjunction with DnaK, which acts as a corepressor, controls the expression of the *dnaK–grpE–dnaJ–hspR* operon, *clpB* and *lon* (Bucca *et al.*, 2000, 2003; Sobczyk *et al.*, 2002). It is proposed that the involvement of DnaK as a co-repressor provides a feedback mechanism that links DnaK expression to its cellular demand by folding pathways (Bucca *et al.*, 2000). ATP-dependent protease expression is controlled by two paralogous positive regulators, PopR and ClgR. PopR was first described in *S. lividans* and controls the expression of the *clpP3–clpP4* operon, which encodes two of the five *clpP* genes in this organism (Viala *et al.*, 2000). However, the *clpP3–clpP4* operon is not heat-inducible in *S. lividans* and is only expressed when the major *clpP1–clpP2* operon is inactivated. The *clpP1–clpP2* operon, which is also not induced by heat-shock, is controlled by ClgR (Bellier & Mazodier, 2004). In addition, ClgR controls the expression of *clpC1*

(one of three *clpC* genes) and, along with HspR (see above), the expression of *lon*. In other words, unlike the case in enteric bacteria, the expression of chaperones and ATP-dependent proteases is largely uncoupled, which allows their independent expression.

Here we monitor the global transcriptional response to diamide treatment in order to extend our knowledge of the σ^R regulon in *S. coelicolor* and demonstrate an intimate relationship with the ClgR regulon. We also show that diamide-induced disulphide stress causes protein aggregation, which leads to the induction of the HspR regulon, including the DnaK and ClpB chaperones. This extends the complex network of regulators that maintain the quality of cellular proteins in *Streptomyces*.

METHODS

Bacterial strains and growth conditions. *Streptomyces coelicolor* strains used were wild-type M600 (Kieser *et al.*, 2000) and its Δ *sigR* derivative J2139 (Paget *et al.*, 1998) or wild-type M145 (Kieser *et al.*, 2000). Strains were grown at 30 °C to mid to late exponential phase in new minimal medium with phosphate (NMMP) plus glucose liquid medium (Kieser *et al.*, 2000) with shaking at 300 r.p.m. prior to stress treatment. To induce disulphide stress, diamide (0.5 mM final concentration) was added to cultures and samples were taken at regular intervals (see figure legends) by centrifugation at 4000 g for 1 min (Paget *et al.*, 1998). In some experiments, following diamide treatment, mycelium was collected by centrifugation (4000 g, 1 min), then resuspended in fresh medium, and the time-course initiated.

Microarray studies. RNA was extracted using standard CsCl or Kirby mix methods (Kieser *et al.*, 2000) and the RNA further purified using an RNA purification kit (Qiagen). Methods to label RNA or genomic DNA (gDNA) are based on those developed by the University of Surrey Microarray Group and are described in detail in the submission to ArrayExpress. Briefly, total RNA and total gDNA from M600 were labelled with Cy3-dCTP and Cy5-dCTP, respectively, using random primers and reverse transcriptase (Superscript II) or Klenow fragment. Following purification, the labelled cDNA and gDNA were co-hybridized on PCR-product-spotted *S. coelicolor* arrays (University of Surrey, print runs SC8 and ScP28). Following hybridization and washing, slides were scanned using either Axon 4000B or Affymetrix 428 scanners, and the 16-bit TIFF image obtained was analysed using BlueFuse 2.0 (BlueGnome) and GeneSpring GX 7.3.1 (Agilent Technologies). Following further filtering based on expression level, samples were normalized by first dividing the Cy3 (cDNA) channel by the Cy5 (gDNA) channel (per spot), then each measurement was divided by the median of all measurements in that sample (per chip). Specific samples were normalized to the time 0 measurements to obtain fold inductions. A volcano plot identified genes that were twofold upregulated or downregulated ($P < 0.05$, Student's *t* test) at the 20 min time point. These genes were divided into three classes: downregulated, *sigR*-independent upregulated and *sigR*-dependent upregulated. Quality threshold (QT) clustering using a Pearson correlation score of 0.8 and minimum cluster size of 4 was applied to identify differences in expression profile.

Analysis of RsrA redox state. A Δ *sigR-rsrA* allele, which contains an in-frame 303 bp deletion in *sigR*, was amplified by PCR and fused to a 3 × FLAG-tag at the final codon of *rsrA*, then subcloned into the integrative plasmid pSETΩ (O'Connor *et al.*, 2002). The promoter *ermEp** was then cloned upstream of the *sigR* ORF between the P1

and P2 promoters to give pSX166. In this construct, *ΔsigR* and *rsrA* are constitutively expressed and the wild-type translation initiation signals are intact. pSX166 was introduced by conjugation into *S. coelicolor* strains, and exconjugants were grown in NMMP plus glucose to mid-exponential phase prior to treatment with 0.5 mM diamide (final concentration). Mycelium (1.5 ml) was sampled at regular intervals by brief centrifugation, then resuspended in ice-cold 20 % TCA and sonicated three times for 10 s at full power. Proteins were collected by centrifugation (16 000 g) for 10 min and the pellet was washed with acetone. Dried pellets were resuspended in denaturing iodoacetamide labelling buffer (100 mM iodoacetamide, 6 M urea, 200 mM Tris/HCl, pH 7.2, 10 mM EDTA, 0.5 % SDS) and incubated at 25 °C for 25 min followed by non-reducing SDS-PAGE. RsrA-FLAG was detected by Western analysis using an anti-FLAG antibody (Sigma). Data shown was obtained using strains S121 (*sigR*⁺) and J2139 (*ΔsigR*). S121 is a *zur* deletion mutant (Owen *et al.*, 2007) and displays essentially identical RsrA redox kinetics to M145 (data not shown).

Protein aggregation assay. Aggregated proteins were isolated using a slight modification of a procedure described elsewhere (Tomoyasu *et al.*, 2001). An 8 ml sample of culture was harvested by brief centrifugation (6000 g, 2 min), and the pellet washed briefly with buffer A (10 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA, 20 % sucrose), prior to freezing on dry ice. Subsequent steps were carried out at 0–4 °C. Pellets were resuspended in 100 µl buffer A containing 5 mg lysozyme ml⁻¹, left on ice for 30 min, and then 1.3 ml buffer B (10 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA) was added. Lysis of mycelium was completed by sonication (six times 10 s, Braun Labsonic M), and cell wall material and unlysed cells were removed by low-speed centrifugation (1000 g, 15 min). Aggregated proteins along with membranes were recovered from the supernatant by high-speed centrifugation (16 500 g, 20 min). Aggregated proteins were subsequently purified from membrane proteins by repeated washes with buffer B plus 10 % Nonidet P40, as described elsewhere (Tomoyasu *et al.*, 2001). Aggregated protein was resuspended in 50 µl buffer B and analysed by SDS-PAGE.

S1 nuclease mapping and *in vitro* transcription assay. Probes for S1 nuclease mapping were generated by PCR using 5' end-labelled primers internal to the gene of interest, unlabelled primers positioned 300–500 nt upstream, and M600 chromosomal DNA as template (Supplementary Table S1). Primers were labelled and S1 nuclease protection assays were set up using 20 or 30 µg RNA, as described by Kieser *et al.* (2000). *In vitro* transcription assays were carried out using *S. coelicolor* RNA polymerase holoenzyme, purified σ^R and RsrA, as described previously (Buttner *et al.*, 1988; Newell *et al.*, 2006). Briefly, RNA polymerase (40 nM) was added either alone or in combination with 50 pmol σ^R and/or 100 pmol RsrA and ~0.3 pmol template. Reactions were initiated by the addition of nucleotide mix (0.5 mM ATP, GTP and CTP and 10 µM UTP, including 5 µCi (185 kBq) [α -³²P]UTP [>800 Ci mmol⁻¹ ($>29\,600$ GBq mmol⁻¹) (GE Healthcare)], and incubated for 5 min before the addition of heparin (0.5 mg ml⁻¹) and further incubation for 10 min. Reactions were halted by addition of an equal volume of loading dye [80 % (w/v) formamide, 0.01 % (w/v) xylene cyanol, 0.01 % (w/v) bromophenol blue], then separated on denaturing 6 % polyacrylamide gels.

RESULTS AND DISCUSSION

Microarray analysis of the diamide stimulon

To investigate the *S. coelicolor* diamide stimulon and assess the role of σ^R in this response, J2139 (*ΔsigR*) and its parent M600 (*sigR*⁺) were grown in NMMP liquid medium to

mid to late exponential phase then treated with 0.5 mM diamide, as previously described (Paget *et al.*, 2001b). RNA was extracted at 20 min intervals up to 1 h, and global changes in mRNA level were determined by co-hybridizing Cy3-labelled cDNA and Cy5-labelled gDNA on *S. coelicolor* whole-genome arrays. Following filtering procedures to remove bad spots and genes that were poorly expressed, a total of 6398 genes were analysed. In this work, we focused on genes that were induced or repressed at the 20 min time point by filtering for genes that were twofold upregulated or downregulated with respect to untreated cultures.

Diamide represses expression of genes involved in protein and RNA biosynthesis

A total of 73 genes exhibited greater than twofold decreased expression in M600 (*sigR*⁺) at the 20 min time point (Supplementary Table S1), largely comprising genes involved in protein synthesis (30 ribosomal protein genes, translation factors *lepA*, *fusA* and *infA*), RNA synthesis (*rpoA*, *rpoB* and *rpoC*), and central metabolism (e.g. the ATP synthase operon). QT clustering of these genes produced five clusters (58 genes classified) that differed in the response kinetics in M600 and in J2139. In most cases, the decrease in gene expression was transient, with levels returning to pretreated levels within 40–60 min, although for most genes there was a noticeable delay in the recovery of transcript levels for J2139 (Fig. 1a; see below). The mechanism that underlies this transient downregulation of genes involved in translation and transcription is currently unknown, but does not appear to involve ppGpp, since ppGpp levels do not rise significantly upon the addition of diamide (M. S. B. Paget and A. Hesketh, unpublished results).

sigR mutants exhibit delayed switch off of *sigR*-independent genes following diamide treatment

Following an initial filter for more than twofold induction ($P=0.05$) after 20 min in M600 (*sigR*⁺), genes were classed as independent of or dependent on *sigR* if they exhibited >1.3 -fold (Fig. 1b; 36 genes) or ≤ 1.3 -fold (Fig. 1c; 42 genes) induction in J2139 (*ΔsigR*), respectively. QT clustering of *sigR*-independent and *sigR*-dependent genes gave rise to five (25 genes clustered, Fig. 1b) and four (30 genes clustered, Fig. 1c) clusters, respectively. The *sigR*-dependent genes were combined in Table 1 with genes that failed the threshold, but which had been validated as *sigR*-dependent here or previously (Paget *et al.*, 2001b). In addition, some genes were included that did not meet the statistical threshold but which possess an upstream ideal σ^R -binding site. The resulting 77 *sigR*-dependent genes (Table 1) constitute 67 likely transcription units, of which 37 have been validated by S1 nuclease mapping here or previously (Paget *et al.*, 2001b). Of the remaining transcription units, 10 possess upstream σ^R -like promoter sequences that differ from the GGAAT(n18)GTT consensus by no more than two positions or an increase or

Fig. 1. Expression profiles for genes that are twofold repressed (a, 73 genes) or twofold induced by diamide in a *sigR*-independent (b, >1.3-fold induced in J2139, 36 genes) or *sigR*-dependent manner (c, ≤1.3-fold induced in J2139, 42 genes). Genes were filtered at the 20 min time point in M600. The y axis is a log scale of the fold-change in transcript abundance relative to time 0. The x axis is time (min) following the addition of 0.5 mM diamide.

decrease of the spacer of 1 bp (Table 1, Supplementary Fig S1). Forty genes were induced by diamide independently of *sigR* (Table 2), constituting 27 predicted transcription units. For most clusters, there was a general trend of delayed restoration of normal transcript levels in J2139, analogous to the situation for genes that are repressed by DNA (Fig. 1b). One explanation for this delay in recovery is the slower consumption of diamide in the Δ *sigR* background (Fig. 2), possibly due to the absence of *sigR*-dependent induction of NADPH-dependent thiol-disulphide oxidoreductases (see below) or the reduced level of mycothiol in this background (Paget *et al.*, 2001b; Park & Roe, 2008). In addition, the failure to induce key thiol-disulphide oxidoreductases makes it likely that Δ *sigR* mutants take longer to restore a reducing cytoplasmic environment following disulphide stress. Since a key sensor of the thiol-disulphide redox state of the cytoplasm is RsrA itself, we sought to determine the effect of deleting *sigR* on the redox state of RsrA during disulphide stress.

σ^R activity correlates with transient oxidation of RsrA

Previous *in vitro* data suggest that the transient induction of σ^R activity in response to diamide treatment is due to reversible disulphide bond-mediated inactivation of RsrA, with the concomitant expulsion of zinc (Kang *et al.*, 1999; Li *et al.*, 2003). To investigate the relationship between RsrA redox state and σ^R activity, the RsrA redox state was monitored over a diamide time-course. RsrA was engineered with a C-terminal 3 $\times$ FLAG-tag and placed under the control of the constitutive promoter *ermEp** on the

integrative plasmid pSET Ω . RsrA oxidation state was monitored using an acid-trapping approach, which involved the treatment of mycelium with TCA followed by the labelling of free thiols with iodoacetamide. In a *sigR*⁺ background, diamide treatment led to the transient accumulation of a faster-migrating RsrA-FLAG species on non-reducing SDS-PAGE gels (Fig. 3), consistent with the formation of one or more intramolecular disulphide bonds (Kang *et al.*, 1999). However, in J2139 (Δ *sigR*), following the addition of diamide, the oxidized species was present for the remainder of the time-course. This delay in the reappearance of reduced RsrA might be due to the slower rate of diamide consumption, the lower level of direct reductants of RsrA (e.g. thioredoxin or mycothiol), or a combination of both. Furthermore, this correlates with the lengthened induction of *sigR*-independent genes and suggests that in addition to RsrA, other cellular proteins might be oxidized for an extended period in J2139.

Genes induced by diamide in a *sigR*-independent manner

HspR regulon. The entire known *S. coelicolor* HspR regulon (*dnaK*–*grpE*–*dnaJ*–*hspR* operon, *clpB* and *lon*) was induced by diamide, which was confirmed using S1 nuclease mapping (Table 2, Fig. 4). Transcription initiation upstream of *dnaK* and *clpB* occurred at previously described promoters, independently of *sigR* (Bucca *et al.*, 1997, 2003). However, induced *lon* transcript levels appeared to be lower in the Δ *sigR* background, suggesting that *sigR* is involved in its regulation (Fig. 4a). In support of this, the *lon* promoter was recognized by σ^R -

Table 1. Genes induced by diamide in a *sigR*-dependent manner

TU, transcription unit: the numbering corresponds to transcription units, as predicted in ScoCyc (<http://streptomyces.org.uk/>), and the first gene in each transcription unit is marked with a #; σ^R target: + indicates that a *sigR*-dependent promoter has been confirmed by S1 nuclease mapping, +* indicates an unconfirmed putative σ^R -target promoter is located upstream of the transcription unit; WT, fold induction after 20 min in M600; $\Delta sigR$, fold induction after 20 min in J2139; Cluster indicates the QT cluster in Fig. 1. Sequences of confirmed and predicted σ^R promoters are provided in Supplementary Fig. S1.

TU	Gene ID	σ^R target	WT	$\Delta sigR$	Cluster	Gene	Function or comment
1	SCO0564		2.1	1.2		–	Hypothetical protein
2	SCO0569		–	–		<i>rpmJ2</i>	50S ribosomal protein L36
2#	SCO0570	+	–	–		<i>rpmG3</i>	50S ribosomal protein L33
3	SCO0882	+	1.0	1.2		–	Hypothetical protein
4	SCO0885	+	8.0	1.7	b4	<i>trxC</i>	Thioredoxin
5	SCO1340	+*	2.0	1.3		–	Conserved hypothetical protein
6	SCO1384	+*	2.7	0.7	c2	–	Conserved hypothetical protein (FHA domain)
7	SCO1392		2.0	0.7		–	Hypothetical protein
8	SCO1421	+	5.8	0.7	c2	<i>rbpA</i>	RNA polymerase-binding protein
9	SCO1513	+	1.1	1.5		<i>relA</i>	GTP pyrophosphokinase
10	SCO1831		2.2	1.1	c4	–	Putative oxidoreductase
11	SCO1839		2.1	1.0		–	Putative transcriptional regulator
12	SCO1869	+	0.8	0.4		–	FrnE-related thiol–disulphide oxidoreductase
13	SCO1919		2.7	0.4		–	Conserved hypothetical protein
13#	SCO1920	+*	3.1	1.0		–	NifU-related conserved hypothetical protein
14	SCO1958		2.7	1.1	c1	<i>uvrA</i>	ABC excision nuclease subunit A
15	SCO1995	+	2.1	0.8	c3	–	Hypothetical protein
16	SCO1996		1.7	1.2		<i>coaE</i>	Dephospho-CoA kinase
16#	SCO1997	+	7.1	1.1	c4	–	Conserved hypothetical protein
17	SCO2161	+	1.1	2.2		–	HesB/YadR/YfhF family
18	SCO2537	+*	2.8	1.3		–	Putative DNA-binding protein
	SCO2618		3.7	0.7	c2	<i>clpP2</i>	ATP-dependent Clp protease proteolytic subunit 2
19	SCO2619	+	3.8	1.3		<i>clpP1</i>	ATP-dependent Clp protease proteolytic subunit 1
20	SCO2634	+	16.4	1.3	c1	–	Conserved hypothetical protein with DsbA fold
21	SCO2643	+	4.4	0.9		<i>pepN</i>	Aminopeptidase N
22	SCO2849	+	2.5	1.1		–	Putative F ₄₂₀ -dependent oxidoreductase
23	SCO2910		1.9	1.1		<i>cysM</i>	Putative cysteine synthase
23	SCO2911		3.2	1.6		–	Conserved hypothetical protein
23#	SCO2912	+	2.6	0.7		–	Hypothetical protein
24	SCO3083	+	2.3	0.3	c3	–	Putative integral membrane protein
25	SCO3091		9.4	0.7	c1	<i>cfa</i>	Cyclopropane-fatty-acyl-phospholipid synthase
26	SCO3162	+	1.8	0.8		–	Putative esterase
27	SCO3187	+	1.5	1.4		–	Conserved hypothetical protein SCE22.04
28	SCO3202	+	1.8	1.3		<i>hrdD</i>	RNA polymerase sigma factor
29	SCO3206		51.7	0.9	c1	–	Putative transmembrane efflux protein
30	SCO3373	+	–	0.4		<i>clpC</i>	Putative Clp-family ATP-binding protease
31	SCO3403	+	1.6	1.2		<i>folE</i>	Putative GTP cyclohydrolase I
32	SCO3415	+	0.6	1.4		–	Putative esterase
33	SCO3764		3.7	0.7	c3	–	Putative integral membrane protein
34	SCO3889		10.2	1.0	c1	<i>trxA</i>	Thioredoxin
34#	SCO3890	+	8.5	1.2	c1	<i>trxB</i>	Thioredoxin reductase
35	SCO4039		2.6	1.0	c1	–	Hypothetical protein
36#	SCO4198		1.7	0.8		–	Putative DNA-binding protein
36	SCO4199		2.3	1.3	b4	–	Hypothetical protein
37	SCO4204	+*	2.2	0.5	c3	<i>mshA</i>	Putative glycosyltransferase involved in mycothiol biosynthesis
38	SCO4297	+*	3.3	1.3	c4	–	Putative NADH : flavin oxidoreductase
39	SCO4316		2.6	0.9	c4	–	Putative ATP/GTP-binding protein
40	SCO4501		2.1	0.9		–	Putative bifunctional protein (ketoreductase/3-oxoacyl-[acyl-carrier protein] reductase)

Table 1. cont.

TU	Gene ID	σ^R target	WT	$\Delta sigR$	Cluster	Gene	Function or comment
41	SCO4736		3.5	0.8	c1	<i>mrsA</i>	Putative phospho-sugar mutase
42	SCO4770	+	2.4	0.9		<i>guaB</i>	Inosine-5'-monophosphate dehydrogenase
43	SCO4956	+	7.9	1.4		<i>msrA</i>	Methionine sulfoxide reductase
44#	SCO4967	+*	4.7	0.9		<i>mca</i>	Mycothiol conjugate amidase
44	SCO4968		3.7	0.6	c1	–	Putative membrane protein
45	SCO5042	+*	2.2	1.1		<i>fumC</i>	Fumarate hydratase C
46	SCO5163	+	–	–		–	Hypothetical protein
47	SCO5177		2.4	1.3		–	Hypothetical protein
48	SCO5178	+	2.9	1.3		<i>moeB</i>	Putative sulphurylase
49	SCO5187	+	3.4	1.4		–	Putative glutaredoxin-like protein
50#	SCO5216	+	5.4	0.7	c1	<i>sigR</i>	RNA polymerase sigma factor
50	SCO5217		7.8	1.0	c1	<i>rsrA</i>	Anti-sigma factor
51	SCO5285	+	2.9	3.0		<i>lon</i>	ATP-dependent protease
52	SCO5359	+	1.6	0.5		<i>rpmE1</i>	50S ribosomal protein L31
53	SCO5465	+	3.8	1.1	c1	–	Conserved hypothetical protein
54	SCO5545		2.1	0.8	c4	–	Hypothetical protein
55	SCO5655		2.4	0.6	c3	–	Putative aminotransferase
56	SCO5754	+	2.1	1.6	b2	<i>cinA</i>	Conserved hypothetical protein
57	SCO5755	+	1.1	0.9		<i>clgR</i>	Transcription regulator
58	SCO5796	+	3.4	0.7	c2	<i>hflX</i>	Conserved ribosome-binding GTPase
59	SCO6061	+	5.3	0.9	c1	<i>msrB</i>	Methionine sulfoxide reductase
60	SCO6423	+*	6.1	1.4	b4	<i>lplA</i>	Putative lipote-protein ligase
61	SCO6424		2.0	0.9		–	Putative two-component system sensor protein
62	SCO6551	+	6.6	1.3	c1	–	Putative oxidoreductase
63	SCO6577		2.2	0.7		–	Conserved hypothetical protein
64	SCO6759	+	1.8	0.5		<i>phyA</i>	Putative phytoene synthase
65	SCO7117		3.5	0.9	c1	<i>phdD</i>	Putative ferredoxin reductase
66	SCO7203		2.1	1.1		–	Putative integral membrane protein
67	SCO7632	+*	4.4	0.9	c1	<i>rifO</i>	Conserved hypothetical protein

containing *S. coelicolor* RNA polymerase holoenzyme *in vitro* and was repressed by RsrA (Fig. 4b). Therefore, the *lon* promoter can be induced by disulphide stress using distinct mechanisms: σ^R -dependent control involves the oxidative inactivation of RsrA- and σ^R -independent control is likely to involve the sequestration of the HspR co-repressor DnaK to misfolded proteins (Bucca *et al.*, 2003; Sobczyk *et al.*, 2002). Interestingly, the *lon* promoter is also activated by ClgR (Bellier & Mazodier, 2004), and at least one other sigma factor must recognize the promoter *in vivo*, indicating an unusually complex integration of signals at this promoter. These data underline the importance of thiol–disulphide redox state in the correct folding of proteins. In support of this, diamide has also been shown to induce chaperone expression in *Bacillus subtilis* (Leichert *et al.*, 2003).

Ribonucleotide reductase. *S. coelicolor* encodes two ribonucleotide reductases, a class Ia co-enzyme B₁₂-independent enzyme, NrdAB, and a class II B₁₂-dependent enzyme, NrdJ (Borovok *et al.*, 2002). The *nrdB* and *nrdJ* genes were both induced by diamide (Table 2), which was surprising considering that many genes involved in transcription and protein synthesis were repressed. Transcription of each operon is controlled by

the repressor NrdR, a zinc-finger repressor (Borovok *et al.*, 2004; Grinberg *et al.*, 2006), and it is conceivable that disulphide stress causes inactivation of the protein through oxidation of the cysteine zinc ligands. Alternatively, considering that ribonucleotide reductases are reactivated by thioredoxins or related proteins in an NADPH-dependent manner, the underlying signal might be a drop in cytoplasmic dNTP levels that results from sequestration of thiol–disulphide reductase(s) and/or NADPH. Consistent with this model, in *E. coli* the expression of *nrdAB* is elevated in *trxA/grxA* mutants, implying that cellular ribonucleotide reductase activity is balanced with the levels of its hydrogen donor systems (Gallardo-Madueno *et al.*, 1998).

Riboflavin biosynthesis. The induction of the predicted *ribBMAH* operon (SCO1443–1440) might be in response to an increased demand for flavin cofactors as a consequence of the induction of flavin-dependent oxidoreductases. Although several flavin-dependent enzymes exhibited *sigR*-dependent induction (e.g. *trxB* and SCO4297), at least two flavoenzymes (SCO6834 and SCO0888) were induced by diamide in the $\Delta sigR$ background. The mechanism of induction might involve the conserved FMN-sensing RFN riboswitch element,

Table 2. Genes induced by diamide in a *sigR*-independent manner

See legend to Table 1 for details. Please note: SCO1088, SCO1437 and SCO1438 are predicted to be members of transcription units that are induced by diamide and are included for completeness.

TU	Gene ID	WT	$\Delta sigR$	Cluster	Gene	Function or comment
1	SCO0620	3.1	5.1	b5	–	Conserved hypothetical protein
2	SCO0888	10.6	14.1	b1	–	NADPH-dependent FMN reductase
3	SCO1084	2.7	1.8	b3	<i>thiX</i> (<i>trxA4</i>)	Putative thioredoxin
4	SCO1087	2.4	4.3	b5	–	Putative L-threonine aldolase
4	SCO1088	1.6	3.7		–	Putative oxidoreductase
4#	SCO1089	2.4	3.5		–	Hypothetical protein
5	SCO1235	2.0	1.9	b2	<i>ureB</i>	Urease beta subunit
6	SCO1385	3.7	2.0		–	Putative membrane protein
7	SCO1437	1.5	0.8		–	Putative membrane protein
7	SCO1438	1.6	1.1		<i>hisG</i>	ATP phosphoribosyltransferase
7	SCO1439	2.2	1.8		<i>hisE</i>	Phosphoribosyl-ATP pyrophosphatase
7	SCO1440	2.3	1.6		<i>ribH</i>	6,7-Dimethyl-8-ribityllumazine synthase
7	SCO1441	3.1	2.0		<i>ribA</i>	3,4-Dihydroxy-2-butanone 4-phosphate synthase
7	SCO1442	2.2	4.1		<i>ribM</i>	Putative integral membrane protein
7#	SCO1443	3.7	2.6		<i>rib</i>	Putative riboflavin synthase
8	SCO1773	3.1	2.7		<i>ald</i>	Putative L-alanine dehydrogenase
9	SCO1925	2.7	2.2	b2	–	Conserved hypothetical protein
10	SCO3661	4.4	3.0	b1	<i>clpB</i>	ATP-dependent protease
11	SCO3668	6.2	4.2	b1	<i>hspR</i>	Heat-shock repressor
11	SCO3669	4.8	3.7	b1	<i>dnaJ</i>	Molecular chaperone
11	SCO3670	5.0	3.0	b1	<i>grpE</i>	Molecular chaperone
11#	SCO3671	2.7	2.6		<i>dnaK</i>	Molecular chaperone
12	SCO4340	2.0	3.3		–	Putative integrase
13	SCO4586	2.1	7.3	b5	–	Putative ABC transporter IMP
14	SCO5180	2.1	1.6		<i>slpE</i>	Putative peptidase
15	SCO5225	5.3	2.0	b2	<i>nrdB</i>	Ribonucleotide-diphosphate reductase small chain
16#	SCO5437	2.8	2.0		–	Putative MerR-family transcriptional regulator
16	SCO5438	3.1	2.3	b1	<i>trxA3</i>	Thioredoxin
17	SCO5466	10.8	2.3	b4	–	Putative hydrolase
18	SCO5680	2.9	3.2	b1	<i>cdd</i>	Putative cytidine deaminase
19	SCO5804	3.7	2.8	b2	<i>nrdR</i>	Regulator of ribonucleotide reductase
20	SCO5805	2.7	2.9	b2	<i>nrdJ</i>	Ribonucleotide reductase
21	SCO5821	3.3	1.4	b3	–	Putative serine protease
22	SCO5861	4.1	1.8	b3	–	Hypothetical protein
23	SCO5884	2.1	3.2		–	Hypothetical protein
24	SCO5906	2.0	1.5		–	TetR-family transcriptional regulator
25	SCO5911	3.1	1.7	b3	–	Possible oligopeptide-binding protein
26	SCO6834	2.4	5.5	b5	<i>trxB2</i>	Putative thioredoxin reductase
27	SCO6837	3.3	13.4		<i>arsB</i>	Putative arsenic resistance membrane transport protein

which is located upstream of SCO1443, possibly through a transcription anti-termination mechanism (Vitreschak *et al.*, 2002; Winkler *et al.*, 2002).

Thiol–disulphide metabolism. A putative operon encoding a MerR homologue (SCO5437) and an alternative thioredoxin (SCO5438; *trxA3*) was induced by diamide in a *sigR*-independent manner. In *Neisseria gonorrhoeae*, the MerR homologue NmlR is responsive to diamide (Kidd *et al.*, 2005), providing a precedent for the possibility that SCO5437 is a sensor of disulphide stress and regulates this operon.

Genes induced by diamide in a *sigR*-dependent manner

Maintenance of cysteine and methionine redox state.

The microarray data corroborated the previous demonstration that σ^R controls the expression of several enzymes that maintain protein cysteines in their reduced state (*trxBA*, *trxC* and the glutaredoxin-like enzyme SCO5187). In addition, the data confirm a recent report (Park & Roe, 2008) that σ^R controls *mshA*, which catalyses the first step in mycothiol biosynthesis (Buchmeier & Fahey, 2006; Buchmeier *et al.*, 2006; Newton *et al.*, 2003), and *mca*, a mycothiol conjugate amidase that functions in detoxi-

Fig. 2. Consumption of diamide in M600 (wild type, ●) and J2139 ($\Delta sigR$, ○). Cultures were grown to $OD_{450} \sim 0.9$, then treated with diamide (0.5 mM final concentration). Diamide was monitored by measuring A_{303} .

fication of alkylating agents and antibiotics (Newton *et al.*, 2000). Surprisingly, σ^R also appears to play a key role in maintaining methionine in its reduced state. SCO4956 (*msrA*) and SCO6061 (*msrB*) are predicted to encode enzymes that reduce the epimers methionine S-sulphoxide and methionine R-sulphoxide, respectively. In the case of *msrA*, S1 nuclease mapping confirmed diamide induction from a single promoter that was completely silent in the $\Delta sigR$ mutant (data not shown). The *msrB* gene lies 14 bp downstream of *murC* (UDP-*N*-acetylmuramoyl-L-alanine ligase), and a potential σ^R target promoter (GGAAT-n19-GTT) lies in the C-terminal region of *murC*. Methionine sulphoxide reductases use electrons from thioredoxins or related proteins as part of the catalytic cycle (Ezraty *et al.*, 2005). Therefore, the increased production of methionine sulphoxide reductase during disulphide stress, when

Fig. 3. Analysis of RsrA redox state during disulphide stress. S121 (*sigR*⁺) and J2139 ($\Delta sigR$) containing pSX166 (pSETΩ::*ermEp-ΔsigR-rsrA::flag*) were grown in NMMP to mid-exponential phase, then treated with 0.5 mM diamide. At the indicated time points samples were acid-trapped using TCA, then carboxymethylated using iodoacetamide, prior to non-reducing SDS-PAGE and Western analysis. RsrA::FLAG was detected using an anti-FLAG antibody. Filled arrows indicate reduced state and open arrows indicate the faster-migrating oxidized state of RsrA.

Fig. 4. The HspR regulon is induced by diamide in a *sigR*-independent manner. (a) S1 nuclease mapping of the *dnaK*, *clpB* and *lon* promoter regions. M600 (wild-type) and J2139 ($\Delta sigR$) were grown to mid to late exponential phase and RNA was isolated before (time 0) and after the addition of diamide. Arrows indicate the protected DNA fragments that correspond to the 5' end of transcripts that initiate at *dnaK*_P, *clpB*_P and *lon*_P. Time is given in minutes. (b) *In vitro* transcription mapping the *lon* promoter. Run-off transcription reactions were performed using a ~0.6 kb *lon* promoter fragment and 40 nM *S. coelicolor* RNA polymerase holoenzyme. Additional σ^R (50 pmol) or its anti-sigma factor RsrA (100 pmol) were added as indicated.

thioredoxins might be sequestered to other oxidized proteins, might help to ensure the continued protection of methionine residues. Considering the reversibility of methionine oxidation, protein methionines have been proposed to act as endogenous antioxidants that protect macromolecules from irreversible damage (Luo & Levine, 2009). The induction of *msrA* and *msrB* aligns with this theory. In summary, an apparent key role for σ^R is to increase the cellular capacity for NADPH-dependent repair reactions involving both cysteines and methionines, and thereby restore redox homeostasis during disulphide stress.

ClgR regulon. As discussed above, σ^R controls *lon*, which is additionally controlled by ClgR. Of the other known ClgR targets (*clpP1/P2*, *clpC* and *clgR*), microarray data suggested that the *clpP1/P2* operon was also a σ^R target (Table 1). A new σ^R -dependent promoter, *clpP1*_{P2}, was identified 5 bp upstream from the previously identified promoter (Fig. 5) (de Crecy-Lagard *et al.*, 1999). This promoter is identical to that recently described by Kim *et al.* (2009).

New σ^R -dependent promoters were also detected ~80 and ~55 bp upstream from the previously described *clpC1* and

Fig. 5. Control of ClgR-regulated genes by σ^R . RNA was extracted from M600 (wild-type) and J2139 ($\Delta sigR$) as described in the legend to Fig. 4. Arrows indicate the protected DNA fragment that corresponds to the 5' end of transcripts. The newly discovered *clpP1*_{P2} appears to be completely *sigR*-dependent and is positioned ~5 bp upstream of the previously identified *clpP1*_{P1}. The newly discovered *clpC1*_{P2} appears to be partially *sigR*-dependent and is positioned ~80 bp further upstream from the previously identified *clpC1*_{P1}. The newly discovered *clgR*_{P2} appears to be completely *sigR*-dependent and is positioned ~55 bp upstream of the previously identified *clgR*_{P1}. Time following diamide treatment is indicated in minutes.

clgR promoters, respectively (Bellier & Mazodier, 2004). However, σ^R is likely to play a minor role in the regulation of *clgR*, because promoters that are regulated by ClgR but not by σ^R (e.g. *clpP1*_{P1}) appear to be unaffected by diamide. In summary, it appears that σ^R co-regulates all known ClgR targets as well as the expression of *clgR* itself. In *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*, ClgR and σ^H (a σ^R orthologue) also co-regulate *clpP1/P2*, *clpC* and *clgR* itself (Engels *et al.*, 2004, 2006; Mehra & Kaushal, 2009). This implies an intimate relationship between ClgR and σ^R (σ^H) that is conserved widely in actinomycetes and suggests a key role for σ^R (σ^H) in protein quality control.

***sigR* mutants fail to cope with protein aggregates during disulphide stress.** The discovery that the HspR regulon was induced by diamide and that σ^R controls the expression of several ATP-dependent proteases suggests that diamide causes protein misfolding and aggregation, and that σ^R might play a role in coordinating the response

to this stress. To test this we adapted a method to purify protein aggregates which relies on repeated cycles of sonication and centrifugation steps in the presence of the non-ionic detergent Nonidet P40 (Tomoyasu *et al.*, 2001). In M600, diamide caused a transient increase in protein aggregation up to 30 min, which then decreased to basal levels over the next 30 min (Fig. 6a). The kinetics of protein aggregation broadly correlated with the transient activation of σ^R activity (Fig. 1). Strikingly, in J2139 ($\Delta sigR$), following the addition of diamide, protein aggregates were present for the entire 60 min time-course (Fig. 6a). To see if the reduced rate of diamide consumption accounted for the extended period of aggregation, following diamide treatment for 10 min, the oxidant was removed by washing, then the solubilization of aggregates was monitored over time. Although the preformed aggregates were removed in the $\Delta sigR$ background, the rate of disappearance was consistently lower than in the wild-type (Fig. 6b). Thus, the inability of *sigR* mutants to deal with protein aggregates is probably due to both a reduced rate of diamide consumption, caused by lower levels of thiol buffers and thiol-disulphide oxidoreductases, and the failure to fully induce enzymes such as the Clp and Lon proteases that degrade protein aggregates. The increased persistence of aggregates in the $\Delta sigR$ background is consistent with the extended period of induction of the HspR regulon (Fig. 4a), which is directly responsive to misfolded proteins.

Fig. 6. (a) Diamide-induced protein aggregation in *S. coelicolor* M600 (wild-type) and J2139 ($\Delta sigR$). Strains were grown to mid to late exponential phase prior to the addition of 0.5 mM diamide. Protein aggregates were purified before (time 0 min) and following the addition of diamide. (b) Samples were taken prior to the addition of diamide (0), 10 min after the addition of diamide but prior to washing (+D), and at various time points after the removal of diamide by the washing of mycelia (10–50 min).

It is emphasized that the underlying cause of diamide-induced protein aggregation is not clear. Each protein might aggregate as a consequence of its individual oxidation, which may disrupt its folding pathway or folded form. Alternatively, disulphide stress might sequester the protein folding and degradation systems away from their normal cellular targets towards oxidized substrates, leading to a general increase in protein misfolding and aggregation. A third possibility is that diamide somehow inactivates a critical component of the protein folding and/or degradative pathways. For example, in *E. coli*, DnaK is inactivated by peroxides, which results in the accumulation of aggregated proteins (Winter *et al.*, 2005), although diamide did not cause this effect. Significantly, it has recently been shown that diamide inactivates the ClpXP protease in *B. subtilis* through oxidation of the conserved N-terminal zinc-binding domain of ClpX (Zhang & Zuber, 2007). Further work is required to investigate the implications of this in the *S. coelicolor* disulphide stress response.

Conclusions

Diamide generates at least two perceived stress signals: protein misfolding and aggregation, which induces the HspR regulon, and an oxidative shift in the thiol–disulphide redox balance, which inactivates RsrA, thereby inducing σ^R activity. Overall, there is limited regulatory overlap between HspR and σ^R , the exception being the *lon* promoter, which is additionally recognized by ClgR. The importance of Lon in coping with oxidative stress-induced protein aggregation has been established in mitochondria (Bota & Davies, 2002). Interestingly, it has recently been shown that *ssrA*, which encodes transfer–messenger RNA (tmRNA) and is controlled in part by *sigR*, positively controls *dnaK*, suggesting a more direct link between σ^R activity and *dnaK* expression (Barends *et al.*, 2010). In summary, σ^R plays a more significant role in protein quality control than previously realized, through the control of enzymes such as thiol–disulphide reductases, methionine sulfoxide reductase and AAA+ proteases. Indeed, the fact that disulphide stress, but not heat shock (Viala *et al.*, 2000; Viala & Mazodier, 2003), induces expression of the Clp protease system emphasizes the importance of thiol–disulphide redox balance in the maintenance of protein quality.

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