Redox-sensitive transcriptional regulator SoxR directly controls antibiotic production, development and thiol-oxidative stress response in Streptomyces avermitilis

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Summary

The redox-sensitive transcriptional regulator SoxR is conserved in bacteria. Its role in mediating protective response to various oxidative stresses in Escherichia coli and related enteric bacteria has been well established. However, functions and regulatory mechanisms of SoxR in filamentous Streptomyces, which produce half of known antibiotics, are unclear. We report here that SoxR pleiotropically regulates antibiotic production, morphological development, primary metabolism and thiol-oxidative stress response in industrially important species Streptomyces avermitilis. SoxR stimulated avermectin production by directly activating ave structural genes. Four genes (sav_3956, sav_4018, sav_5665 and sav_7218) that are homologous to targets of S. coelicolor SoxR are targeted by S. avermitilis SoxR. A consensus 18-nt SoxR-binding site, 5’-VSYCNVMHNKVKDMGMB-3’, was identified in promoter regions of sav_3956, sav_4018, sav_5665, sav_7218 and target ave genes, leading to prediction of the SoxR regulon and confirmation of 11 new targets involved in development (ftsH), oligomycin A biosynthesis (olmR), primary metabolism (metB, sav_1623, plcA, nirB, thiG, ndh2), transport (smoE) and regulatory function (sig57, sav_7278). SoxR also directly activated three key developmental genes (amfC, whiB and ftsZ) and promoted resistance of S. avermitilis to thiol-oxidative stress through activation of target trx and msh genes. Overexpression of soxR notably enhanced antibiotic production in S. avermitilis and S. coelicolor. Our findings expand our limited knowledge of SoxR and will facilitate improvement of methods for antibiotic overproduction in Streptomyces species.

Introduction

The redox-sensitive protein SoxR, one of the MerR-family transcriptional regulators (TRs), functions as a homodimer; each subunit contains an N-terminal HTH DNA-binding domain, a dimerization helix and a C-terminal [2Fe-2S] cluster-binding domain (Watanabe et al., 2008). SoxR homologs are found in a wide variety of Gram-positive and Gram-negative bacteria. SoxR in E. coli and related enteric bacteria has been extensively studied and shown to mediate resistance to oxidative stress from superoxide and nitric oxide. The [2Fe-2S] cluster is essential for SoxR activity (Hidalgo and Demple, 1994). In the absence of oxidants, SoxR is inactive and has reduced [2Fe-2S] cluster. Exposure to oxidative stress results in activation of SoxR through oxidation of [2Fe-2S] cluster. Nitric oxide also activates SoxR by nitrosylation of [2Fe-2S] cluster (Ding and Demple, 2000). Activated SoxR induces expression of soxS gene. SoxS, an AraC-family TR, in turn activates transcription of >100 genes, whose products in general restore redox homeostasis and repair cellular damage that occurs during stress (Pomposiello et al., 2001). This two-step SoxRS-based regulatory mechanism occurs only in enteric bacteria because the soxS gene is restricted to these bacteria and is the sole direct target of SoxR (Dietrich et al., 2008). In contrast to the established E. coli model, two nonenteric Pseudomonas species were found not to depend on SoxR for coping with oxidative stress; rather, SoxR directly stimulates several genes apparently involved in defense against endogenous redox-active antibiotic (Palma et al., 2005; Dietrich et al., 2006; Park et al., 2006). The novel Pseudomonas paradigm led to reconsideration of biological functions of SoxR and the mechanism of its activation in nonenteric bacteria.

The Gram-positive genus Streptomyces are soil-dwelling bacteria characterized by complex life cycles and production of a great variety of secondary metabolites, including half of all known antibiotics. Antibiotic production by Streptomyces species is usually associated with
morphological development. Coordination of these two processes involves complex regulatory networks based on both cluster-situated and pleiotropic/global regulators that respond to a variety of environmental and physiological conditions (Liu et al., 2013; Urem et al., 2016). Studies of the model species *S. coelicolor* revealed that SoxR (ScSoxR) directly controls six genes that encode an ABC transporter (SCO7008), three oxidoreductases (SCO2478, SCO4266, SCO3020ext), a monoxygenase (SCO1909) and a hypothetical protein (SCO1178) (Dela Cruz et al., 2010; Shin et al., 2011; Naseer et al., 2014). The promoter regions of identified ScSoxR target genes all contain a binding sequence similar to that of *E. coli* SoxR (EcSoxR), suggesting that the DNA-binding property of SoxR is conserved. However, none of these genes has been reported to be involved in oxidative stress response. ScSoxR is activated by the endogenous antibiotic actinorhodin (Act), and expression of its target genes requires Act production and intact [2Fe-2S] cluster (Dela Cruz et al., 2010; Shin et al., 2011). Two ScSoxR targets (sco4266, sco1909) encode products similar to Act-tailoring enzymes. These findings suggest that ScSoxR responds to endogenously produced redox-active metabolites such as Act and helps protect against their toxic effects by processing and transporting the same molecules, similarly to its suggested role in *Pseudomonas aeruginosa* (Dela Cruz et al., 2010; Shin et al., 2011). J. H. Roe’s group reported that ScSoxR responds to a narrower range of redox-active compounds (RACs) than does *P. aeruginosa* SoxR (PaSoxR) or EcSoxR, but can be activated by certain exogenously added RACs that inhibit cell growth (e.g. Act, plumbagin), and therefore may protect cells from toxic RACs (Singh et al., 2013; Lee et al., 2015; Lee et al., 2017). The mechanism of such protective effect is unknown.

Two seemingly contradictory reports have been published regarding the role of ScSoxR in *S. coelicolor* development and antibiotic production: M. Chander’s group (Dela Cruz et al., 2010) found that soxR deletion resulted in a phenotype similar to that of wild-type (WT) strain, whereas J. H. Roe’s group (Shin et al., 2011) found that a soxR deletion mutant had delayed development and reduced production of antibiotics Act and undecylprodigiosin (Red) relative to WT. Regulatory mechanisms of ScSoxR on development and antibiotic production were not addressed. To our knowledge, SoxR homologs have not been investigated in other *Streptomyces* species.

The industrially important species *S. avermitilis* is well known for producing potent anthelmintic avermectins (a series of 16-membered macrolide antibiotics), which are widely applied as pesticides or drugs in agricultural and medical fields (Ikeda and Omura, 1997). The 82-kb ave gene cluster contains 17 structural genes for avermectin biosynthesis and one regulatory gene, aveR, whose product is essential for activation of all structural genes (Kitani et al., 2009; Guo et al., 2010). *S. avermitilis* also produces oligomycin A, a 26-membered macrolide antibiotic that has antitumor and antifungal activities (Pinna et al., 1967; Lin et al., 2009). The ~100-kb oml gene cluster contains two activator genes, omlRI and omlRII, essential for oligomycin biosynthesis (Yu et al., 2012). Elucidation of the functions and regulatory mechanisms of SoxR in the important species *S. avermitilis* is highly desirable, in view of our limited knowledge about *Streptomyces* SoxR to date.

We describe here characterization of SoxR (SAV_6604) in *S. avermitilis* as a key regulator in development, antibiotic production (including avermectins and oligomycin A), primary metabolism and thiol-oxidative stress response. We identified SoxR targets involved in these physiological processes and proposed a novel strategy for increasing antibiotic yield through overexpression of soxR gene.

### Results and discussion

**SoxR positively regulates morphological development and avermectin production**

soxR (sav_6604) gene from *S. avermitilis* consists of 510 nucleotides (nt) and encodes a 169-amino-acid protein which includes a conserved N-terminal HTH DNA-binding domain homologous to MerR and a C-terminal [2Fe-2S] cluster-binding domain. Protein alignment analysis revealed 80.9, 83.7 and 83.6% identity, respectively, of SoxR with its homologs in *S. coelicolor*, *S. griseus* and *S. scabies* (Fig. S1). Divergently transcribed gene sav_6603, located 108 nt upstream of soxR, encodes a dehydratase (Fig. 1A). Divergently transcribed gene sav_6605, located 41 nt downstream of soxR, encodes an unknown protein.

To clarify the roles of SoxR in *S. avermitilis*, we constructed soxR deletion mutant DsoxR (Fig. S2), complemented strain CsoxR, and overexpression strain OsoxR, and compared their phenotypes and avermectin yields with those of WT strain. On solid YMS sporulation plates, DsoxR showed delayed differentiation and sporulation, CsoxR had phenotype similar to that of WT, and OsoxR showed accelerated formation of aerial hyphae and spores (Fig. 1B). Detailed scanning electron microscopy (SEM) examination of samples on YMS plates showed that degree of aerial hyphae separation and spore number on days 2 and 4 were low in DsoxR and high in OsoxR, relative to WT (Fig. 1C). HPLC analysis of 10-day cultures in insoluble FM-I showed that avermectin yields, relative to WT level, were ~70% lower for DsoxR, ~2.4-fold higher for OsoxR, and not significantly different for CsoxR or control strains WT/pKC1139 and

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WT/pSET152 (Fig. 2A). Time-course measurement of biomass (dry cell weight) of cultures in soluble FM-II showed that values for DsoxR and OsoxR were similar to that of WT (Fig. 2B), indicating that their altered avermectin yields were not due to changes in cell growth. These findings, taken together, demonstrate that SoxR functions as a positive regulator in *S. avermitilis* development and avermectin production.

We also overexpressed *soxR* gene in industrial strain A229 by introducing the same plasmid (pKC-erm-soxR) used for WT and evaluated the effect on avermectin yield. Yield of avermectin B1a (the most effective component) in shake-flask fermentation, relative to A229 level, was ~14-16% higher for strains OsoxR/A229-1 and OsoxR/A229-2, and not significantly different for plasmid control strain A229/pKC1139 (Fig. 2C). *soxR* overexpression therefore

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appears to be an effective strategy for further enhancing avermectin yield in industrial strains.

**SoxR directly activates transcription of ave structural genes**

The role of SoxR in control of avermectin production was further investigated by using RT-qPCR to monitor soxR transcriptional profile in FM-I fermentation culture of WT. soxR transcription level declined slightly on day 2, increased to a maximal value on day 4, then declined gradually; level on day 8 was similar to that on day 1 (Fig. 3A). SoxR evidently exerts its regulatory effect on avermectin production mainly during middle fermentation stage.

Effects of soxR deletion on expression of cluster-situated activator gene aveR and of five structural genes in the ave cluster (aveF, aveD, aveA1, aveA4 and aveB-VIII) involved in avermectin biosynthesis were assessed by RT-qPCR analysis using RNA prepared from WT and DsoxR on day 2 (exponential phase) and day 6 (stationary phase) of growth in FM-I. On both these days, transcription levels of aveR and the five structural genes were reduced in DsoxR (Fig. 3B), consistent with avermectin yield data for DsoxR, indicating that SoxR activates transcription of aveR as well as ave structural genes.

The possibility that SoxR directly regulates the above ave genes was evaluated by electrophoretic mobility shift assays (EMSAs) using soluble His6-SoxR purified from E. coli and corresponding promoter probes aveRp, aveFp, aveA1_aveD (containing bidirectional promoters), aveA4p and aveBVIIIp (Fig. 3C), with nonspecific hrdBp as negative control probe. His6-SoxR did not bind to aveRp or hrdBp, but formed complexes with probes aveFp, aveA1_aveD, aveA4p and aveBVIIIp in a dose-dependent manner (Fig. 3D). Binding specificities were confirmed by competition experiments using ~500-fold unlabelled specific probes (lanes S), which competed strongly with respective labelled probes for binding to SoxR, or hrdBp (lanes N), which had no effect on the retarded bands (Fig. 3D). These findings indicate that SoxR regulates avermectin production directly through structural genes but not through aveR.

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Fig. 2. Effects of SoxR on avermectin production and cell growth in *S. avermitilis*.  
A. Comparative avermectin yield in WT, DsoxR, CsoxR, OsoxR and control strains (WT/pKC1139, WT/pSET152) cultured in FM-I for 10 days.  
B. Growth curves of WT, DsoxR and OsoxR cultured in soluble FM-II.  
C. Avermectin yield in industrial strain A229 and its derivatives OsoxR/A229-1, OsoxR/A229-2 (soxR overexpression strains) and A229/pKC1139 (plasmid control strain) cultured in FM-I for 10 days. Statistical notations (panels A, C): NS, not significant; *P < 0.05; **P < 0.001 for comparison with WT (A) or A229 (C) (Student’s t-test). Error bars: SD for three replicates.

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To evaluate possible direct regulation by SoxR of its own gene, we designed promoter probe soxRp (Fig. 3C) and used it for EMSAs. His6-SoxR did not bind to soxRp (Fig. 3D), indicating that SoxR is not autoregulated in S. avermitilis.

Identification of four sav genes homologous to ScSoxR targets
EMSA using promoter probes of genes homologous to ScSoxR targets were performed to identify additional
SoxR targets. The characterized ScSoxR regulon includes six genes: sco7008, sco4266, sco2478, sco1909, sco1178 and sco0320ext (reannotated ORF spanning sco0319 and sco0320) (Dela Cruz et al., 2010; Shin et al., 2011; Naseer et al., 2014). Four of these genes have homologs in S. avermitilis: sco7008, sco4266, sco2478 and sco0320ext are, respectively, homologous to sav_7218, sav_3956, sav_5665 and sav_4018. Thus, the four homologous sav genes were selected for EMSA evaluation. The results showed that His$_6$-SoxR bound specifically to all four promoter probes and displayed strongest binding affinity for probe sav$_{7218}p$; i.e., 200 nM His$_6$-SoxR retarded signals strongly for labelled sav$_{7218}p$, but to a much lower degree for labelled sav$_{3956}p$, sav$_{4018}p$ and sav$_{5665}p$ (Fig. 4A). These findings demonstrate that sav$_{3956}$, sav$_{4018}$, sav$_{5665}$ and sav$_{7218}$ are all SoxR targets.

Transcription levels of sav$_{3956}$, sav$_{4018}$, sav$_{5665}$ and sav$_{7218}$ were more strongly reduced in DsoxR than in WT grown in FM-I in RT-qPCR analysis (Fig. 4B), indicating that SoxR positively regulates these four target genes.

**Determination of precise SoxR-binding site**

Identification of precise SoxR-binding sites is necessary for understanding the regulatory mechanism of SoxR on its targets. We performed a DNase I footprinting assay to identify the SoxR-binding site on the promoter region of sav$_{7218}$ (Fig. 5A), since SoxR bound most strongly to probe sav$_{7218}p$. The transcriptional start site (TSS) of S. coelicolor sco7008, a sav$_{7218}$ homolog, was described previously (Dela Cruz et al., 2010). On the basis of corresponding sco7008 TSS, we predicted sav$_{7218}$ TSS to C, 6-nt upstream of sav$_{7218}$ translational start codon (TSC) (Fig. 5B). This prediction led to putative −35 and −10 promoter sequences having a long 19-nt spacer (Fig. 5B) and showing similarity to promoter architectures of E. coli soxS and six ScSoxR target genes (Dela Cruz et al., 2010; Naseer et al., 2014).

The footprinting result revealed that SoxR protected a 33-nt region on the sav$_{7218}$ promoter, extending from −44 to −12 nt relative to sav$_{7218}$ TSS and overlapping the putative −35 and −10 regions (Fig. 5A and B). The protected site contains an 18-nt palindromic sequence (CCTCAAGATTCGTTAGG) similar to the conserved SoxR-binding sequence in S. coelicolor (cssStyle="text-decoration:underline">CCTCRA-N$_6$TYGAGG; R = A/G, Y = T/C, N = A/G/C/T) (Naseer et al., 2014), indicating similarity of SoxR-binding motif in S. avermitilis and S. coelicolor.

The importance of the 18-nt palindromic sequence in SoxR binding was tested by introducing mutations into repeat motifs to generate 50-nt mutated probe sav$_{7218}p$-1 (Fig. 5C). His$_6$-SoxR bound to 50-nt WT probe sav$_{7218}p$-1 containing intact 18-nt palindromic sequence, but not to sav$_{7218}p$-1m lacking inverted repeats (Fig. 5C), indicating that the 6-nt inverted repeats in 18-nt palindromic sequence are essential for SoxR binding.

The 18-nt SoxR-binding sites on promoter regions of sav$_{7218}$ and six ScSoxR target genes all overlap with the −35 region in a manner similar to that for the E. coli soxS promoter (Dela Cruz et al., 2010; Naseer et al., 2014). SoxR is therefore presumed to activate sav$_{7218}$ transcription analogously to EcSoxR activation process; i.e., active conformation of SoxR distorts the target promoter with 19-nt spacer, thereby reducing the spacing between −35 and −10 regions and enabling RNA polymerase to initiate transcription (Watanabe et al., 2008).

**Prediction and verification of new SoxR targets**

Elucidation of broader roles of S. avermitilis SoxR requires identification of additional SoxR target genes. In addition to sav$_{7218}$p, the seven SoxR-binding promoter regions mentioned above (aveFp, aveA1_aveD, aveA4p, aveBVIlp, sav$_{3956}$p, sav$_{4018}$p, sav$_{5665}$p) all contain an 18-nt palindromic sequence similar to ScSoxR-binding motif (Fig. 6A). The role of these seven 18-nt sequences in SoxR binding was examined by EMSAs using 50-nt probes containing either intact palindromic sequence or the mutated sequence lacking 6-nt inverted repeats. Affinity of His$_6$-SoxR for the mutated probes was abolished in comparison with corresponding WT probes (Fig. S3), confirming the importance of the inverted repeats in SoxR binding.

Analysis of the 18-nt palindromic sequences in the eight SoxR target promoter regions using PREDetector (Hiard et al., 2007) revealed a consensus binding sequence, VSYCNVHMNKVKDGMBG (V = A/T/C; S = C/G; Y = T/C; N = A/G/C/T; M = A/C; H = A/T/C; K = G/T; B = G/C/T) (Fig. 6A). PREDetector scanning of the S. avermitilis genome with the 18-nt consensus SoxR-binding sequence identified 144 putative SoxR target genes (cut-off score ≥ 7) (Table S1). Of these genes, 66 were unknown or unclassified, and the remaining 78 were assigned to 16 functional groups as defined by the KEGG S. avermitilis pathway database. Accuracy of the bioinformatic analysis was tested by selecting 13 well-annotated putative targets from various groups for EMSA confirmation.

Our phenotypic observations revealed the positive role of SoxR in S. avermitilis development. Table S1 includes two putative SoxR target genes involved in development: ftsH (sav$_{4666}$) encoding putative cell division protease homologous to E. coli FtsH and ftsQ (sav$_{6123}$).
encoding cell division protein FtsQ required for sporulation septation (McCormick and Losick, 1996). We performed EMSAs on these two developmental genes. Because \textit{ftsH} and \textit{ftsQ} are not key \textit{Streptomyces} developmental genes, we also selected three conserved key developmental genes (\textit{amfC}, \textit{whiB}, \textit{ftsZ}) that are not included in Table S1 for EMSA evaluation. \textit{amfC} (\textit{sav_4026}) encoding aerial mycelium-associated protein AmfC (Yonekawa \textit{et al}., 1999), \textit{whiB} (\textit{sav_5042}) encoding sporulation regulator WhiB (Davis and Chater, 1992) and \textit{ftsZ} (\textit{sav_6124}) encoding tubulin homolog FtsZ that forms cell division scaffold (Willemse \textit{et al}., 2011). His6-SoxR bound specifically to promoter regions of \textit{amfC}, \textit{whiB}, \textit{ftsH} and \textit{ftsZ}, but not to that of \textit{ftsQ} (Fig. 6B).

Transcription levels of the four newly identified SoxR target genes were determined by RT-qPCR using RNAs extracted from WT and DsoxR grown in YMS plates for 2 (aerial growth stage), 4 (middle stage of sporulation) or 6 (spore maturation stage) days. Expression of the four genes was downregulated in DsoxR at two or three of these time points (Fig. 6C), consistent with the differentiation phenotype of DsoxR. These findings indicate that SoxR positively regulates development by directly activating transcription of \textit{amfC}, \textit{whiB}, \textit{ftsH} and \textit{ftsZ}. For many years, the biochemical function of AmfC was unknown, but it was recently revealed to be the cognate anti-sigma factor of the sporulation-specific sigma factor WhiG, and so AmfC was renamed RsiG (regulator of

Fig. 4. Identification of SoxR target genes \textit{sav_3956}, \textit{sav_4018}, \textit{sav_5665} and \textit{sav_7218}.
A. EMSAs of His6-SoxR with promoter probes \textit{sav_3956p}, \textit{sav_4018p}, \textit{sav_5665p} and \textit{sav_7218p}. Lanes 2 to 4 contained His6-SoxR concentrations 50, 100 and 200 nM. 200 nM His6-SoxR was used for competition assays (lanes -). Lane notations (-, N, S) as in Fig. 3D.
B. RT-qPCR analysis of \textit{sav_3956}, \textit{sav_4018}, \textit{sav_5665} and \textit{sav_7218} in WT and DsoxR grown in FM-I. *\textit{P} < 0.05; **\textit{P} < 0.01; ***\textit{P} < 0.001 (t-test). Error bars: SD for three replicates.

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During sporulation, WhiB functions cooperatively with its partner WhiA to activate expression of sporulation genes, including \textit{ftsZ} (Bush \textit{et al.}, 2016). FtsZ is then recruited by SsgB to the septum site to form a ladder of 50-100 Z-rings (cell division scaffolds) in each sporogenic hypha to direct sporulation septation (Willemse \textit{et al.}, 2011). The molecular function of FtsH in \textit{Streptomyces} is unclear. In DsoxR, reduced expression of the four SoxR target developmental genes contributed to delayed formation of aerial hyphae and spores. Further studies are needed to address the possibility that other development-related SoxR target genes contribute to DsoxR phenotype.

In regard to secondary metabolism, the list of putative SoxR targets includes \textit{olmRI}, encoding a LuxR-family cluster-situated activator essential for oligomycin biosynthesis (Yu \textit{et al.}, 2012). EMSA revealed specific binding of probe \textit{olmRI} by His$_6$-SoxR (Fig. 6D). Transcription level of \textit{olmRI} was lower in DsoxR than in WT grown in FM-I (Fig. 6E). Oligomycin A yield in FM-I relative to WT value was reduced ~74% in DsoxR, but increased ~5-fold in OsoxR (Fig. 6F), consistent with transcription analysis data. These findings indicate that SoxR regulates oligomycin A production by direct activation of \textit{olmRI} transcription. SoxR plays a direct role in activating avermectin and oligomycin A production by interacting with promoter regions of structural genes and cluster-situated regulatory gene, respectively, reflecting the pleiotropic roles and diverse mechanisms of SoxR in regulation of antibiotic production.

EMSAs were also performed on 10 additional putative SoxR targets with high scores involved in primary metabolism (\textit{metB}, \textit{sav$_{1623}$}, \textit{gloA}, \textit{plcA}, \textit{nirB}, \textit{thiG}, \textit{ndh2}), transport (\textit{smoE}) or regulatory function (\textit{sig57}).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Determination of SoxR-binding site.
A. DNase I footprinting assay of SoxR on \textit{sav$_{7218}$} promoter region. Protection patterns were acquired with increasing His$_6$-SoxR concentrations, and reaction without His$_6$-SoxR was used as control.
B. Nucleotide sequences of \textit{sav$_{7218}$} promoter region and SoxR-binding site. Numbers: distance (nt) from \textit{sav$_{7218}$} TSS. Shading: \textit{sav$_{7218}$} TSC. Bent arrow: \textit{sav$_{7218}$} TSS. Boxes: probable -10 and -35 regions. Solid line: SoxR-binding site. Straight arrows: inverted repeats.
C. EMSAs using 50-nt WT probe \textit{sav$_{7218}$}p-1 and its mutated probe \textit{sav$_{7218}$}p-1m. Inverted repeats in probe \textit{sav$_{7218}$}p-1 were replaced with EcoRI and HindIII sites to produce mutated probe \textit{sav$_{7218}$}p-1m. Lanes 2 to 4 contained His$_6$-SoxR concentrations 50, 100 and 200 nM.}
\end{figure}
Fig. 6. Identification of SoxR target genes associated with development and oligomycin A production.

A. Analysis of consensus SoxR-binding sequence by PREDetector program. Asterisks: consensus bases. Arrows: conserved 6-nt inverted repeats.

B. EMSAs of His6-SoxR with promoter regions of five development-associated genes. Lane notations as in Fig. 3D.

C. RT-qPCR analysis of amfC, whiB, ftsH and ftsZ in WT and DsoxR grown on YMS.

D. EMSAs of His6-SoxR with probe olmRIp. Lane notations as in Fig. 3D.

E. RT-qPCR analysis of olmRI in WT and DsoxR grown in FM-I.

F. Oligomycin A yield in WT, DsoxR and OsoxR cultured in FM-I for 10 days. Panels C, E, F: error bars: SD for three replicates; NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001 for comparison with WT (t-test).

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sav_7278) (Table S1). His6-SoxR bound directly to promoter regions of metB, sav_1623, plcA, nirB, thiG, ndh2, smoE, sig57 and sav_7278, but not to that of gloA (Fig. 7A). RT-qPCR analysis revealed that transcription levels of nirB, thiG, ndh2, sig57 and sav_7278 were lower in DsoxR than in WT grown in FM-I, whereas those of metB, sav_1623, plcA and smoE were higher in DsoxR, indicating that SoxR displays dual activator/repressor function in these target genes (Fig. 7B).

The promoter regions of putative SoxR targets ftsQ and gloA in Table S1 were not bound by SoxR in vitro. Although we cannot explain this yet, the possibility is that such binding requires an additional protein or a specific ligand that contributes to its binding. The promoter regions of SoxR targets amIC, whiB and ftsZ do not contain the identified 18-nt consensus SoxR-binding motif, suggesting the presence of different SoxR-binding sites.

soxR overexpression promotes antibiotic production in S. coelicolor

In view of the wide distribution of SoxR, we examined possible enhancement of antibiotic production by soxR overexpression in other Streptomyces species. soxR overexpression plasmid pKC-erm-soxR was transformed into model strain S. coelicolor M145 (WT), and resulting transformant OsoxR/M145, parental strain M145 and plasmid control strain M145/pKC1139 were grown on YBP plates for phenotypic comparison. OsoxR/M145 displayed faster differentiation and earlier, higher production of Red and Act, whereas M145/pKC1139 phenotype was similar to that of M145 (Fig. S4). SoxR evidently promotes antibiotic production and development in S. coelicolor. Although the ScSoxR targets involved in antibiotic production remain to be characterized, our findings, in combination with the previous report by J. H. Roe’s group that deletion of S. coelicolor soxR gene reduced Red and Act production (Shin et al., 2011), suggest that SoxR-mediated activation of antibiotic production occurs in other Streptomyces species. A strain improvement strategy based on soxR overexpression may therefore be effective in other antibiotic-producing species.

Identification of SoxR targets involved in response to thiol-oxidative stress

M. Chander’s group observed no response of ScSoxR to the standard oxidants H₂O₂ (causing peroxidative stress), diamide (causing thiol-oxidative stress) or organic peroxides (Dela Cruz et al., 2010). On the other hand, J. H. Roe’s group reported weak activation of ScSoxR by diamide (Lee et al., 2017). We evaluated possible responses of S. avermitilis SoxR to oxidative stress by measuring the sensitivity of WT and DsoxR to various oxidants on YMS plates. Relative to WT, DsoxR showed greater sensitivity to diamide, but similar sensitivity to H₂O₂ and tert-butyl hydroperoxide (tBHP, causing organic peroxidative stress) (Fig. 8A). S. avermitilis SoxR evidently plays a role in resistance to thiol-oxidative stress.

A type of damage that frequently occurs in proteins and small molecules during thiol-oxidative stress is formation of disulfide bond between two cysteine residues. In the model organism S. coelicolor, SigR plays a key role in control of response to thiol-oxidative stress. Major SigR targets include trx genes for thioredoxin system that reduces unwanted disulfide bonds and msh genes for biosynthesis of mycothiol, the major thiol buffer that prevents disulfide bond formation (Kallifidas et al., 2010; Kim et al., 2012). S. avermitilis has eight trx genes (trxA1-A6, trxB1-B2), four msh genes (mshA, mshB, mshC, mshD) and one sigR homologous gene (sig22). RT-qPCR analysis revealed lower transcription levels of these genes in DsoxR than in WT grown in FM-I (Fig. 8B), indicating that SoxR functions as an activator of these genes.

WT and DsoxR were treated with diamide for various durations to evaluate thiol-oxidative stress responses. For WT, diamide treatment caused maximal induction of trxA1 (~5-fold), trxA3 (~4.5-fold), trxA4 (~4-fold), trxA5 (~2-fold), trxB1 (~2.5-fold), trxB2 (~1.8-fold) and sig22 (~2.3-fold) within 10 min, of mshA (~1.9-fold) within 30 min, and of trxA2 (~1.8-fold), trxA6 (~2-fold), mshC (~1.5-fold) and mshD (~2-fold) within 40 min (Fig. S5). For DsoxR, diamide had minor (for trxA1) or no effect (for other genes) on gene induction (Fig. S5). These findings indicate that SoxR helps S. avermitilis resist thiol-oxidative stress by activating trx genes, msh genes and sig22.

EMSAs were performed to detect possible interactions of SoxR with the above thiol-oxidative stress-related genes. His6-SoxR bound specifically to promoter probes trxA2p, trxA3p, trxA4p, trxA5p, trxB1p, trxB2p, mshAp, mshBp and mshCp, but did not bind to probes trxA1p, trxA6p, mshDp or sig22p (Fig. 8C). These findings indicate that SoxR directly regulates expression of genes corresponding to the former group of probes, whereas its positive regulatory effect on genes corresponding to the latter group is indirect. This is the first description of a SoxR-based mechanism for control of thiol-oxidative stress response in soxS-lacking Streptomyces species. trx and msh genes are well-conserved among Streptomyces species; the above mechanism may therefore be universal for the genus.

The promoter regions of SoxR target genes trx and msh also do not contain the identified 18-nt SoxR-binding motif, supporting the possibility that SoxR has
different classes of binding sites. However, DNase I footprinting assays did not reveal SoxR-binding sites on promoter regions of any of the target \( \text{trx} \) and \( \text{msh} \) genes or three developmental genes (\( \text{amfC} \), \( \text{whiB} \), \( \text{ftsZ} \)), most likely because of low DNA-binding activity of SoxR on these targets.

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Based on our findings, a proposed model of the SoxR-mediated regulatory network involved in primary metabolism, secondary metabolism (avermectin and oligomycin A production), development and thiol-oxidative stress response in \textit{S. avermitilis} is presented in Fig. 9. SoxR plays coordinated roles in these physiological processes through: (i) positive or negative regulatory effects on target genes; (ii) indirect activation of \textit{trxA1, trxA6, mshD} and regulatory genes \textit{aveR} and \textit{sig22} via yet-unknown mechanisms. SoxR also targets seven genes (\textit{sav}_{3956}, \textit{sav}_{4018}, \textit{sav}_{5665}, \textit{sav}_{7218}, \textit{smoE}, \textit{sig57}, \textit{sav}_{7278}) whose functions are unclear. The regulatory roles of SoxR in \textit{Streptomyces} are evidently much broader than previously recognized. Identification of additional SoxR targets will help elucidate the complex roles and biological significance of SoxR in \textit{Streptomyces} species.

**Experimental procedures**

**Strains, plasmids, primers and growth conditions**

Strains and plasmids used in this work are summarized in Table S2, and primers are listed in Table S3. Growth conditions for \textit{S. avermitilis} and \textit{E. coli} were described previously (Liu \textit{et al.}, 2015). YMS (Ikeda \textit{et al.}, 1988) agar was used for phenotypic observation of \textit{S. avermitilis} mutants. Insoluble fermentation medium FM-I (Jiang \textit{et al.}, 2011) was used for routine avermectin fermentation. Because FM-I contains insoluble yeast meal and biomass is expressed as dry cell weight, soluble fermentation medium FM-II (Jiang \textit{et al.}, 2011) was used to cultivate mycelia for analysis of \textit{S. avermitilis} biomass. Avermectin production in FM-II is lower than that in FM-I. YBP (Ou \textit{et al.}, 2009) agar was used for characterization of \textit{S. coelicolor} phenotype.

**Gene disruption, complementation and overexpression**

For in-frame gene deletion of \textit{soxR}, a 451-bp 5'-flanking region (positions −400 to +51 relative to \textit{soxR} TSC) was amplified from WT genome with primers WQ1/WQ2, and a 422-bp 3'-flanking region (positions +370 to +791) was amplified with primers WQ3/WQ4. These two fragments were digested, respectively, with EcoRI/XbaI and XbaI/HindIII and then ligated into EcoRI/HindIII-digested pKC1139 (Bierman \textit{et al.}, 1992), generating \textit{soxR} deletion plasmid pDsoxR, which was transformed into WT protoplasts. \textit{soxR} deletion mutant was screened as reported previously (Yang \textit{et al.}, 2015), confirmed by colony PCR with primers WQ5/WQ6 (flanking the exchange regions) and WQ7/WQ8 (located within the deletion region) (Fig. S2), and subjected to DNA sequencing. When primers WQ5/WQ6 were used, a 1.4-kb band appeared, whereas a 1.7-kb band was detected in WT genomic DNA. When primers WQ7/WQ8 were used, a 270-bp band. We thus
obtained soxR gene deletion mutant DsoxR, in which a 318-bp fragment within soxR ORF (positions +52 to +369 relative to TSC) was deleted (Fig. S2). The deleted portion of soxR covered the coding region for DNA-binding domain and [2Fe-2S] cluster-binding site. The remaining fragment was therefore not likely to be functional.

For complementation of DsoxR, a 790-bp PCR fragment containing soxR promoter and ORF was amplified with primers WQ9/WQ10, digested with EcoRI/XbaI and inserted into integrative plasmid pSET152 (Bierman et al., 1992) to give soxR-complemented plasmid pSET152-soxR, which was then transformed into DsoxR to obtain complemented strain CsoxR.

For overexpression of soxR, a 628-bp fragment carrying soxR ORF was amplified with primers WQ19/WQ20 and inserted into pJL117 (Li et al., 2010) to generate pJL117-soxR, in which soxR was controlled by ermE* promoter (Streptomyces strong constitutive promoter). The 848-bp EcoRI/XbaI fragment containing ermE* and soxR ORF from pJL117-soxR was ligated into pKC1139 to generate soxR overexpression plasmid pKC-erm-soxR, which was then transformed into S. avermitilis WT and industrial strain A229 to obtain soxR overexpression strains OssoR and OssoR/A229 respectively. pKC-erm-soxR was introduced into S. coelicolor M145 to obtain OssoR/M145.

Production and analysis of antibiotics

HPLC analysis of avermectin and oligomycin A yield in fermentation culture of S. avermitilis strains was performed as described previously (Luo et al., 2014).

Scanning electron microscopy (SEM)

Spores and mycelia of S. avermitilis WT, DsoxR and OssoR strains grown on YMS plates at 28°C for 2 or 4 days were observed by SEM. Specimens were prepared and examined as described previously (Sun et al., 2016).

Reverse transcription and quantitative real-time PCR (RT-qPCR) analysis

S. avermitilis mycelia grown in liquid FM-I, YEME (Kieser et al., 2000), or on YMS plates were taken at various time points for RNA extraction. Samples were grown in liquid nitrogen and suspended in TRIzol reagent (Tiangen; Beijing, China) for RNA isolation. Crude RNAs were treated with RNase-free DNase I (TaKaRa; Dalian, China) to remove genomic DNA. Reverse transcription for cDNA synthesis and subsequent real-time PCR analysis (using primers listed in Table S3) were performed as described previously (Luo et al., 2014). Transcription levels of tested genes were normalized relative to level for internal control housekeeping gene 16S rRNA. Experiments were repeated in triplicate.

Heterologous production and purification of His6-SoxR

For production of S. avermitilis SoxR in E. coli, the 600-bp fragment containing 510-bp soxR coding region was amplified with primers WQ21/WQ22 from WT genomic DNA. The obtained PCR fragment was cut out with EcoRI/Xhol and ligated into pET-28a (+) to generate pET28-soxR for production of N-terminal His6-tagged SoxR recombinant protein. pET28-soxR was transformed into E. coli BL21 (DE3), and His6-SoxR production was induced by treatment with 0.4 mM IPTG for 8 h at 16°C. Bacteria containing His6-SoxR were collected, resuspended in lysis buffer (Luo et al., 2014), sonicated on ice and centrifuged. Soluble His6-SoxR in supernatant was purified by Ni-NTA column (Qiagen; Hilden, Germany), and fractions eluted with 200 mM imidazole were dialyzed against binding buffer (for EMSAs) (Zhu et al., 2016) to eliminate imidazole. Purified protein was quantified by Bradford assay and stored at −80°C.

Electrophoretic mobility shift assays (EMSAs)

Promoter probes were obtained by PCR using corresponding primers (Table S3) and labelled with digoxigenin-1-ddUTP at 3’-terminus. EMSA conditions for binding reaction and signal detection were as described previously (Zhu et al., 2016). Specificity of SoxR/probe interaction was confirmed by adding ~500-fold excess of unlabelled hrdBp (non-specific probe) or respective specific probe to each reaction system before incubation.

DNase I footprinting

To determine SoxR-binding site on sav_7218 promoter region, a 488-bp 5′ FAM fluorescence-labelled DNA probe corresponding to the upstream region of sav_7218 was PCR-synthesized using primers AM-WQ41/WQ42, and gel-purified. DNase I footprinting assays were performed as described previously (Zianni et al., 2006; Sun et al., 2016), and data were processed with GeneMarker v. 2.2.0.

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Conflict of interest

The authors declare that they have no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Amino acid sequence alignment of *S. avermitilis* SoxR and its homologs in three *Streptomyces* species. Black line at top: HTH DNA-binding domain. Red line at top: [2Fe-2S] cluster-binding site.

**Fig. S2.** Method for *soxR* deletion (schematic). Large arrows: genes and their directions. Small arrows: positions of primers used for amplifying exchange regions and confirming gene deletion. Blocks: homologous exchange regions used for gene deletion.

**Fig. S3.** EMSAs of His6-SoxR with 50-nt WT probes and their mutated probes. EcoRI and HindIII sites were introduced into the inverted repeats in WT probes sav_3956p-1, sav_4018p-1, sav_5665p-1, aveFp-1, aveA1_aveD-1, aveA4p-1, and aveBVIIIp-1 to generate mutated probes sav_3956p-1m, sav_4018p-1m, sav_5665p-1m, aveFp-1m, aveA1_aveD-1m, aveA4p-1m, and aveBVIIIp-1m, respectively. Lanes 2 to 4 contained His6-SoxR concentrations 50, 100, and 200 nM.

**Fig. S4.** Effects of *soxR* overexpression on antibiotic production and development in *S. coelicolor*. M145, M145/pKC1139 (M145 carrying control plasmid pKC1139), and OssoXR/M145 (*soxR* overexpression strain of M145) were grown on YBP plates at 28°C and photographed at indicated times.

**Fig. S5.** Induction of thiol-oxidative stress related genes by 0.3 mM diamide in WT and DsoxR grown in YEME. Transcription level of each gene in WT before diamide addition (0 min) was assigned as 1. Error bars: SD for three replicates.

**Table S1.** Putative targets of SoxR.

**Table S2.** Strains and plasmids used in this study.

**Table S3.** Primers used in this study.

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