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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′-VSYCNVVMHNKVKDGMGB-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 (*olmRI*), 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 defence 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

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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, SCO0320ext), a monooxygenase (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 *olm* gene cluster contains two activator genes, *olmRI* and *olmRII*, 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

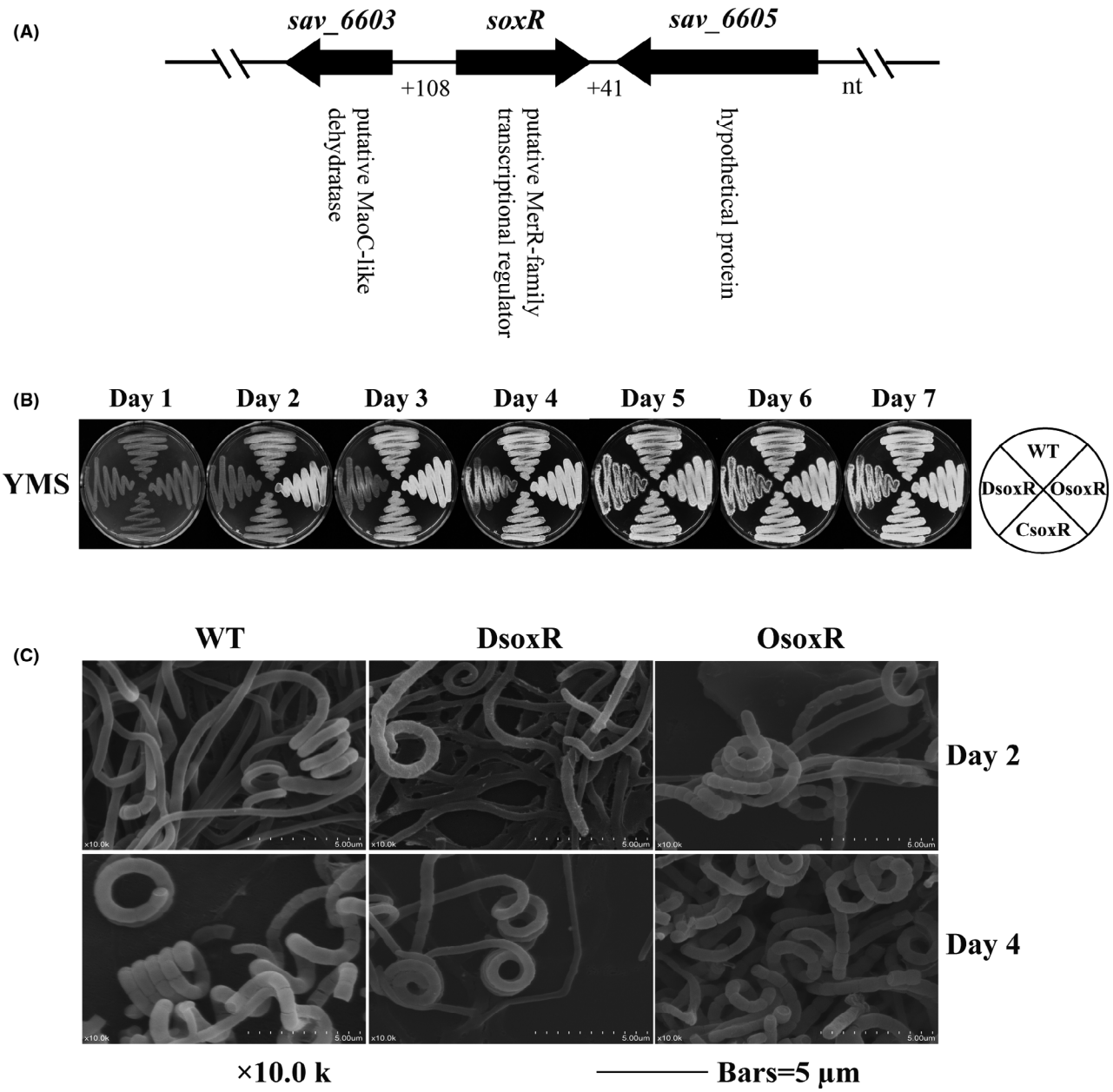

Fig. 1. Effect of SoxR on *S. avermitilis* development.
A. Schematic diagram of *soxR* and its neighbouring genes in *S. avermitilis* chromosome.
B. Phenotypes of WT strain, *soxR* deletion mutant (DsoxR), complemented strain (CsoxR) and overexpression strain (OsoxR) grown on YMS plates at 28°C.
C. SEM images showing development of WT, DsoxR and OsoxR grown on YMS for 2 or 4 days.

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

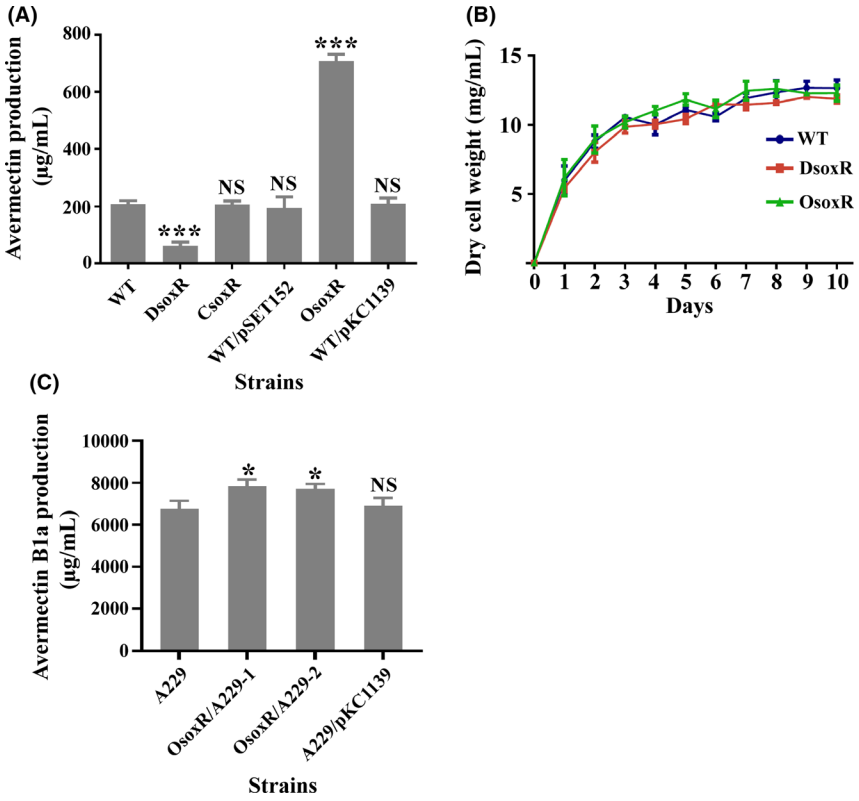

Fig. 2. Effects of SoxR on avermectin production and cell growth in *S. avermitilis*.
A. Comparative avermectin yield in WT, DsoxR, CsoxR, OsoxR and control strains (WT/pKC1139, WT/pSET152) cultured in FM-I for 10 days.
B. Growth curves of WT, DsoxR and OsoxR cultured in soluble FM-II.
C. Avermectin 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.

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 *aveBVIII*) 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 His_6-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. His_6-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*.

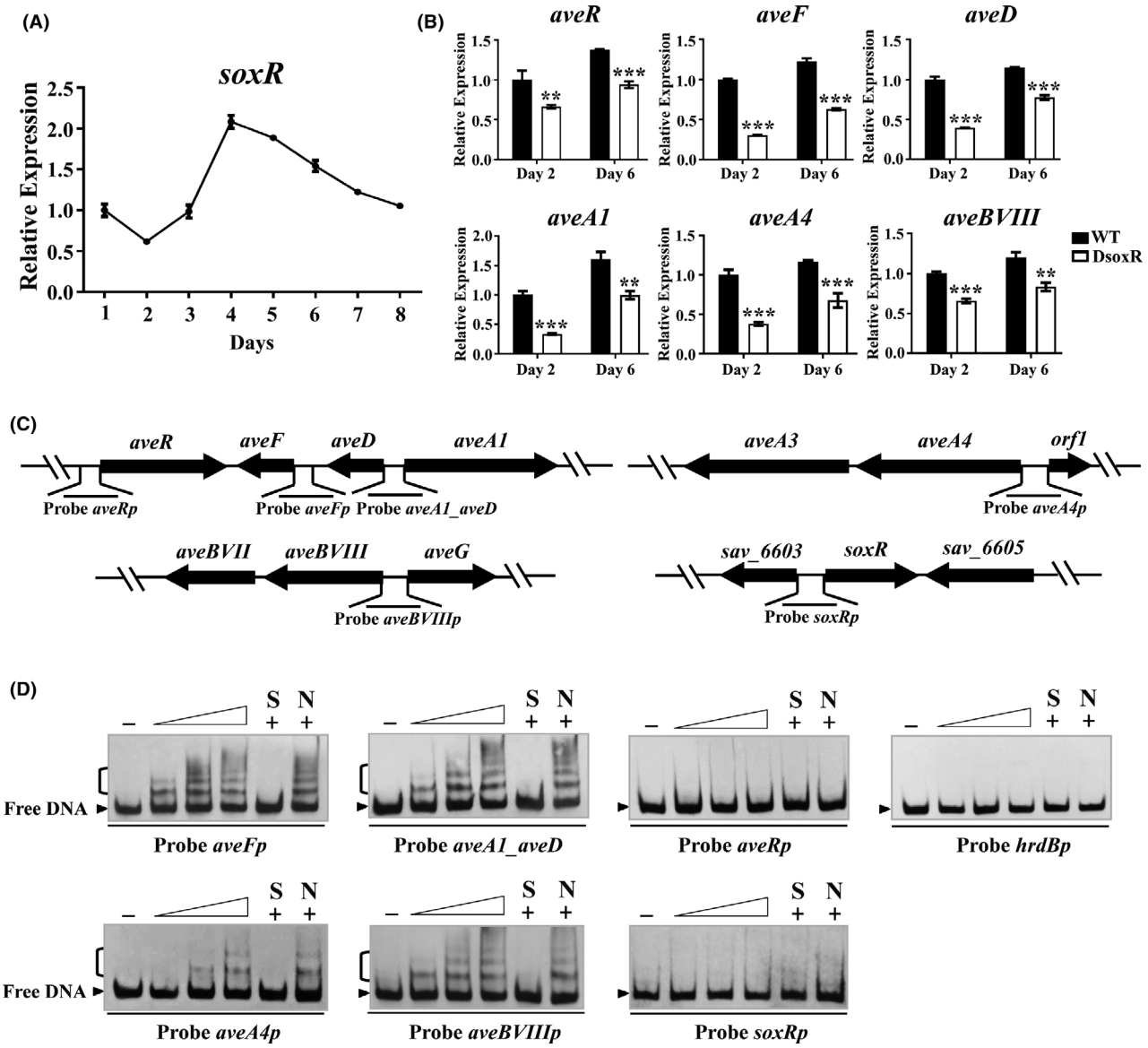

Fig. 3. Direct activation of *ave* structural genes by SoxR.
A. RT-qPCR analysis of transcriptional profile of *soxR* in WT grown in FM-I. Transcription level of *soxR* on day 1 was defined as 1.
B. RT-qPCR analysis of *ave* genes in WT and DsoxR grown in FM-I. For each gene, value was calculated relative to WT level on day 2, defined as 1. $^{**}P < 0.01$; $^{***}P < 0.001$ (*t*-test). Error bars (panels A, B): SD for three replicates.
C. Schematic diagram of promoter probes for EMSAs. Probe *aveRp*: 527-bp fragment, positions −478 to +49 relative to *aveR* TSC. Probe *aveFp*: 396-bp fragment, positions −426 to −31 relative to *aveF* TSC. Probe *aveA1_aveD*: 333-bp fragment covering *aveA1-aveD* intergenic region. Probe *aveA4p*: 274-bp fragment, positions −270 to +4 relative to *aveA4* TSC. Probe *aveBVIIIp*: 276-bp fragment, positions −259 to +17 relative to *aveBVIII* TSC. Probe *soxRp*: 239-bp fragment, positions −140 to +99 relative to *soxR* TSC.
D. EMSAs of interactions of His_6-SoxR with indicated probes. Negative control probe: *hrdBp* (262-bp fragment corresponding to upstream region of *hrdB*, which encodes major σ factor HrdB). Each lane contained 0.15 nM labelled probe. Lanes 2 to 4 contained His_6-SoxR concentrations 100, 200, and 400 nM. 400 nM His_6-SoxR was used for competition experiments (lanes +). Lanes –: EMSAs without His_6-SoxR. Lanes N and S: competition assays with ~500-fold unlabelled nonspecific probe *hrdBp* (N) and specific probe (S). Arrowhead: free probe. Bracket: SoxR-DNA complex (bound DNA).

To evaluate possible direct regulation by SoxR of its own gene, we designed promoter probe *soxRp* (Fig. 3C) and used it for EMSAs. His_6-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

EMSAs 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_7218p*; *i.e*., 200 nM His_6-SoxR retarded signals strongly for labelled *sav_7218p*, but to a much lower degree for labelled *sav_3956p*, *sav_4018p* and *sav_5665p* (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_7218p*. 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 (CCTCAAGATTGCTTGAGG) 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_7218p*-1m (Fig. 5C). His_6-SoxR bound to 50-nt WT probe *sav_7218p*-1 containing intact 18-nt palindromic sequence, but not to *sav_7218p*-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_7218p*, the seven SoxR-binding promoter regions mentioned above (*aveFp*, *aveA1_aveD*, *aveA4p*, *aveBVIIIp*, *sav_3956p*, *sav_4018p*, *sav_5665p*) 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, VSYCNVVMHNKVKDGMGB (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 $\geq$ 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*)

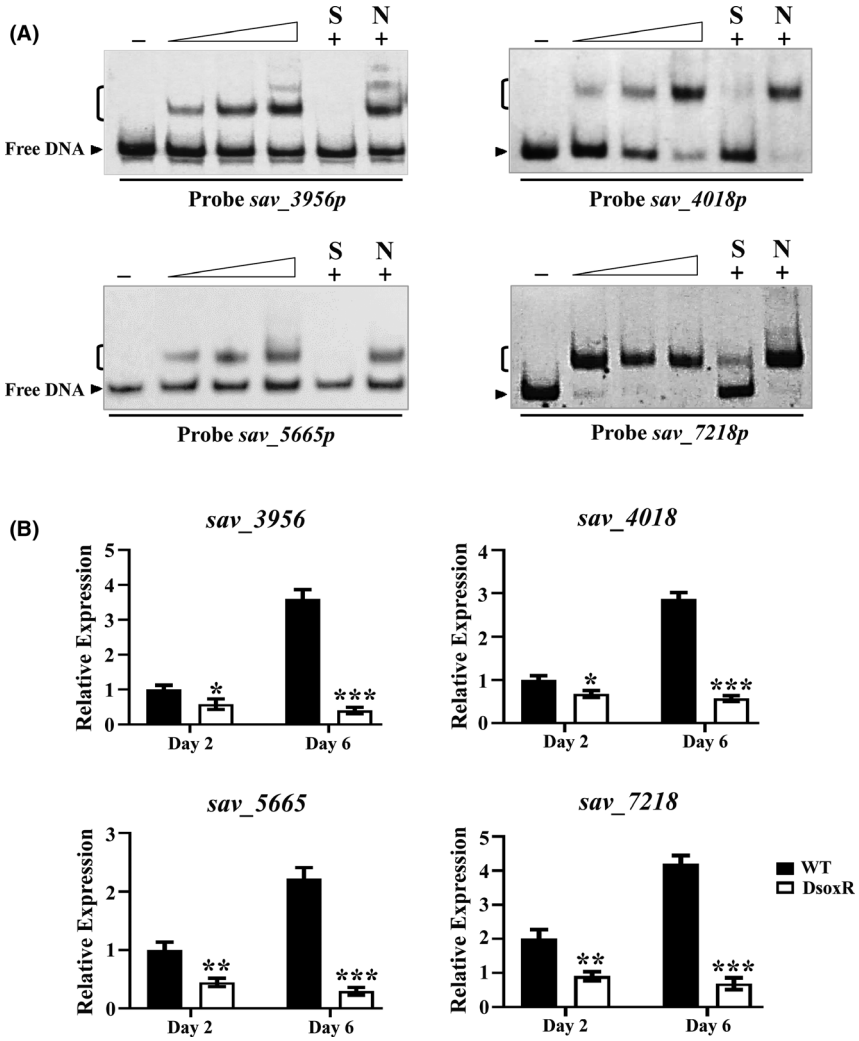

Fig. 4. Identification of SoxR target genes *sav_3956*, *sav_4018*, *sav_5665* and *sav_7218*.
A. EMSAs of His_6-SoxR with promoter probes *sav_3956p*, *sav_4018p*, *sav_5665p* and *sav_7218p*. Lanes 2 to 4 contained His_6-SoxR concentrations 50, 100 and 200 nM. 200 nM His_6-SoxR was used for competition assays (lanes +). Lane notations (–, N, S) as in Fig. 3D.
B. RT-qPCR analysis of *sav_3956*, *sav_4018*, *sav_5665* and *sav_7218* in WT and DsoxR grown in FM-I. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (*t*-test). Error bars: SD for three replicates.

encoding cell division protein FtsQ required for sporulation septation (McCormick and Losick, 1996). We performed EMSAs on these two developmental genes. Because *ftsH* and *ftsQ* are not key *Streptomyces* developmental genes, we also selected three conserved key developmental genes (*amfC*, *whiB*, *ftsZ*) that are not included in Table S1 for EMSA evaluation. *amfC* (*sav_4026*) encoding aerial mycelium-associated protein AmfC (Yonekawa *et al*., 1999), *whiB* (*sav_5042*) encoding sporulation regulator WhiB (Davis and Chater, 1992) and *ftsZ* (*sav_6124*) encoding tubulin homolog FtsZ that forms cell division scaffold (Willemse *et al*., 2011). His_6-SoxR bound specifically to promoter regions of *amfC*, *whiB*, *ftsH* and *ftsZ*, but not to that of *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 on 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 *amfC*, *whiB*, *ftsH* and *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

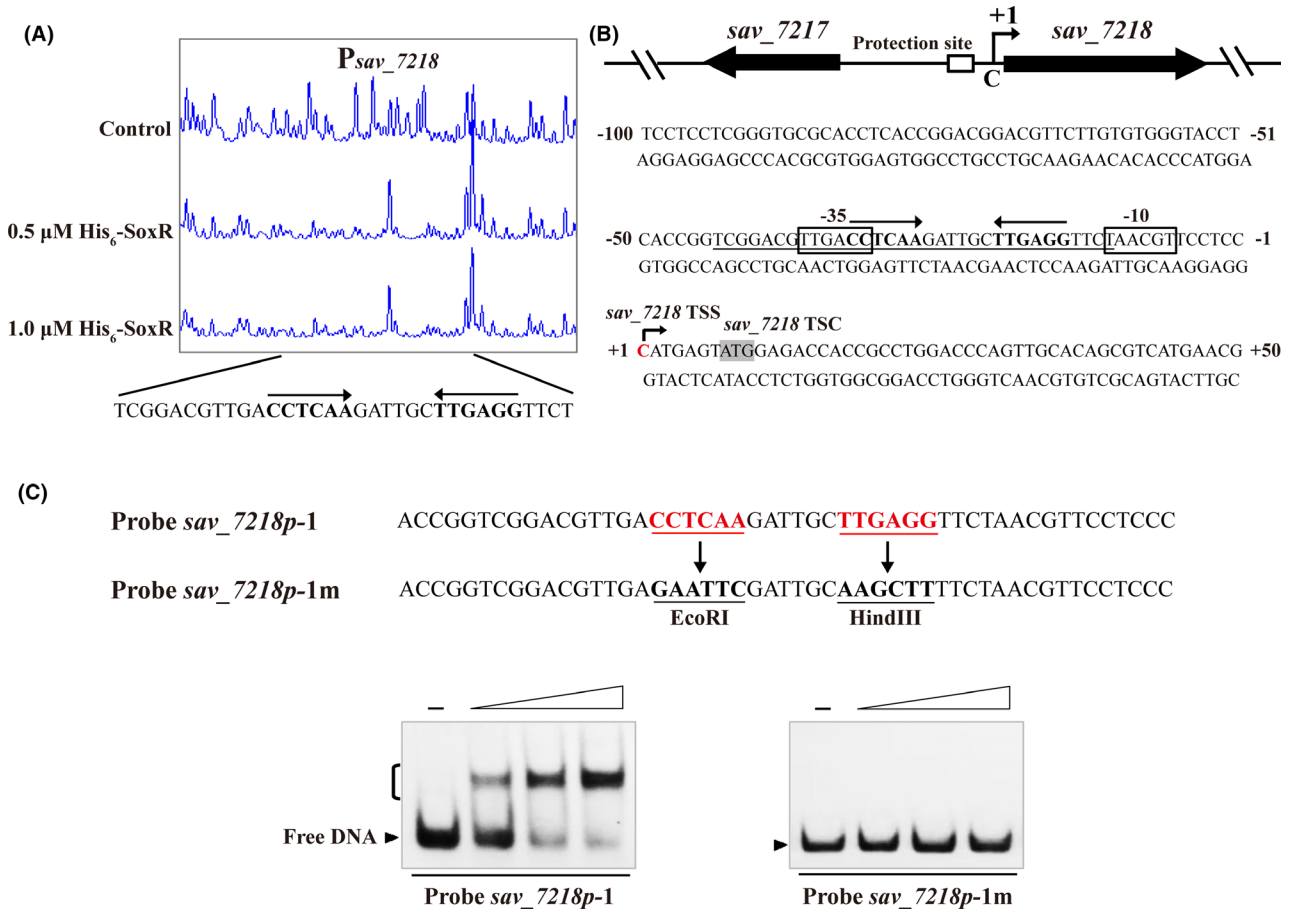

Fig. 5. Determination of SoxR-binding site.
A. DNase I footprinting assay of SoxR on *sav_7218* promoter region. Protection patterns were acquired with increasing His_6-SoxR concentrations, and reaction without His_6-SoxR was used as control.
B. Nucleotide sequences of *sav_7218* promoter region and SoxR-binding site. Numbers: distance (nt) from *sav_7218* TSS. Shading: *sav_7218* TSC. Bent arrow: *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 *sav_7218p*-1 and its mutated probe *sav_7218p*-1m. Inverted repeats in probe *sav_7218p*-1 were replaced with EcoRI and HindIII sites to produce mutated probe *sav_7218p*-1m. Lanes 2 to 4 contained His_6-SoxR concentrations 50, 100 and 200 nM.

sigma WhiG) (Gallagher *et al.*, 2020). During sporulation, WhiB functions cooperatively with its partner WhiA to activate expression of sporulation genes, including *ftsZ* (Bush *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 *et al.*, 2011). The molecular function of FtsH in *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 *olmRI*, encoding a LuxR-family cluster-situated activator essential for oligomycin biosynthesis (Yu *et al.*, 2012). EMSA revealed specific binding of probe *olmRIp* by His_6-SoxR (Fig. 6D). Transcription level of *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 *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 (*metB*, *sav_1623*, *gloA*, *plcA*, *nirB*, *thiG*, *ndh2*), transport (*smoE*) or regulatory function (*sig57*,

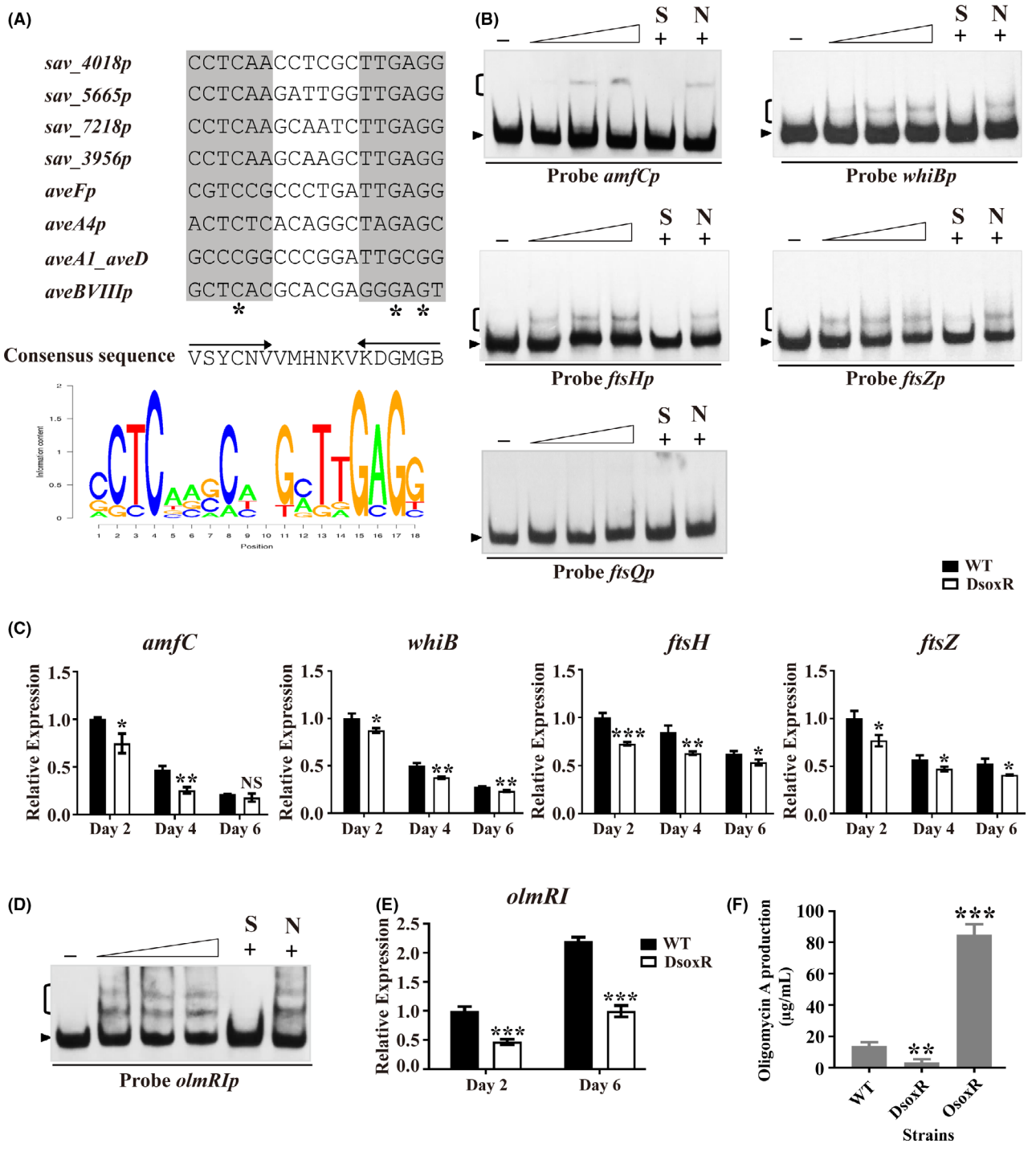

Fig. 6. Identification of SoxR target genes associated with development and oligomycin A production.
A. Analysis of consensus SoxR-binding sequence by PREDetector program. Asterisks: consensus bases. Arrows: conserved 6-nt inverted repeats.
B. EMSAs of His_6-SoxR with promoter regions of five development-associated 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 His_6-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).

sav_7278) (Table S1). His_6-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 *amfC*, *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 H_2O_2 (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 H_2O_2 and *tert*-butyl hydroperoxide (*t*BHP, 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), *trxA*5 (~2-fold), *trxB1* (~2.5-fold), *trxB2* (~1.8-fold), *mshB* (~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), *trxA*6 (~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. His_6-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

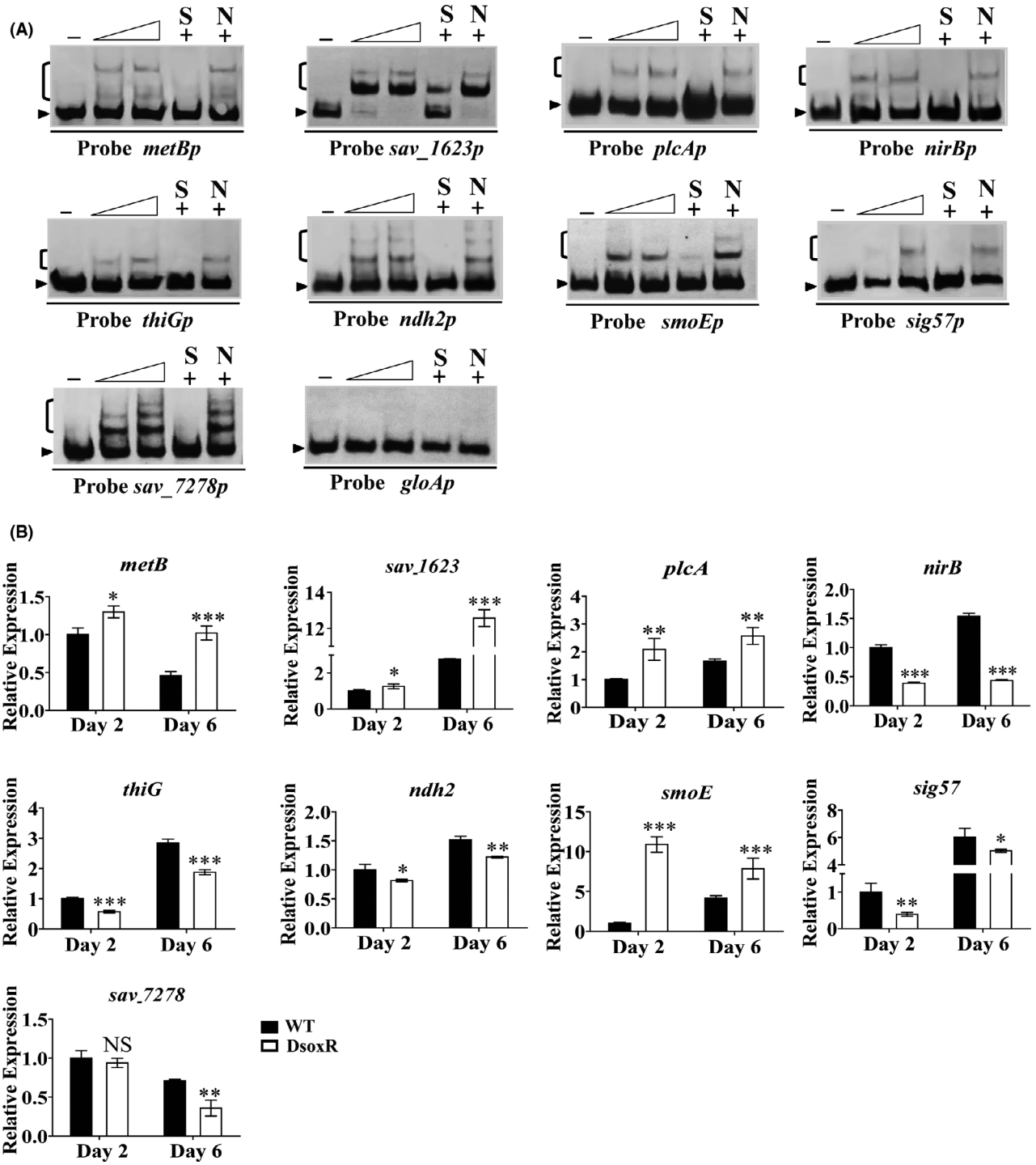

Fig. 7. Identification of SoxR target genes associated with primary metabolism, transport, and regulatory function.
A. EMSAs of His_6-SoxR with promoter regions of 10 predicted target genes. Lanes 2 and 3 contained His_6-SoxR concentrations 200 and 400 nM. Lane notations (+, –, N, S) as in Fig. 3D.
B. RT-qPCR analysis of *metB*, *sav_1623*, *plcA*, *nirB*, *thiG*, *ndh2*, *smoE*, *sig57* and *sav_7278* in WT and DsoxR grown in FM-I. NS, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (*t*-test). Error bars: SD for three replicates.

different classes of binding sites. However, DNase I footprinting assays did not reveal SoxR-binding sites on promoter regions of any of the target *trx* and *msh* genes or three developmental genes (*amfC*, *whiB*, *ftsZ*), most likely because of low DNA-binding activity of SoxR on these targets.

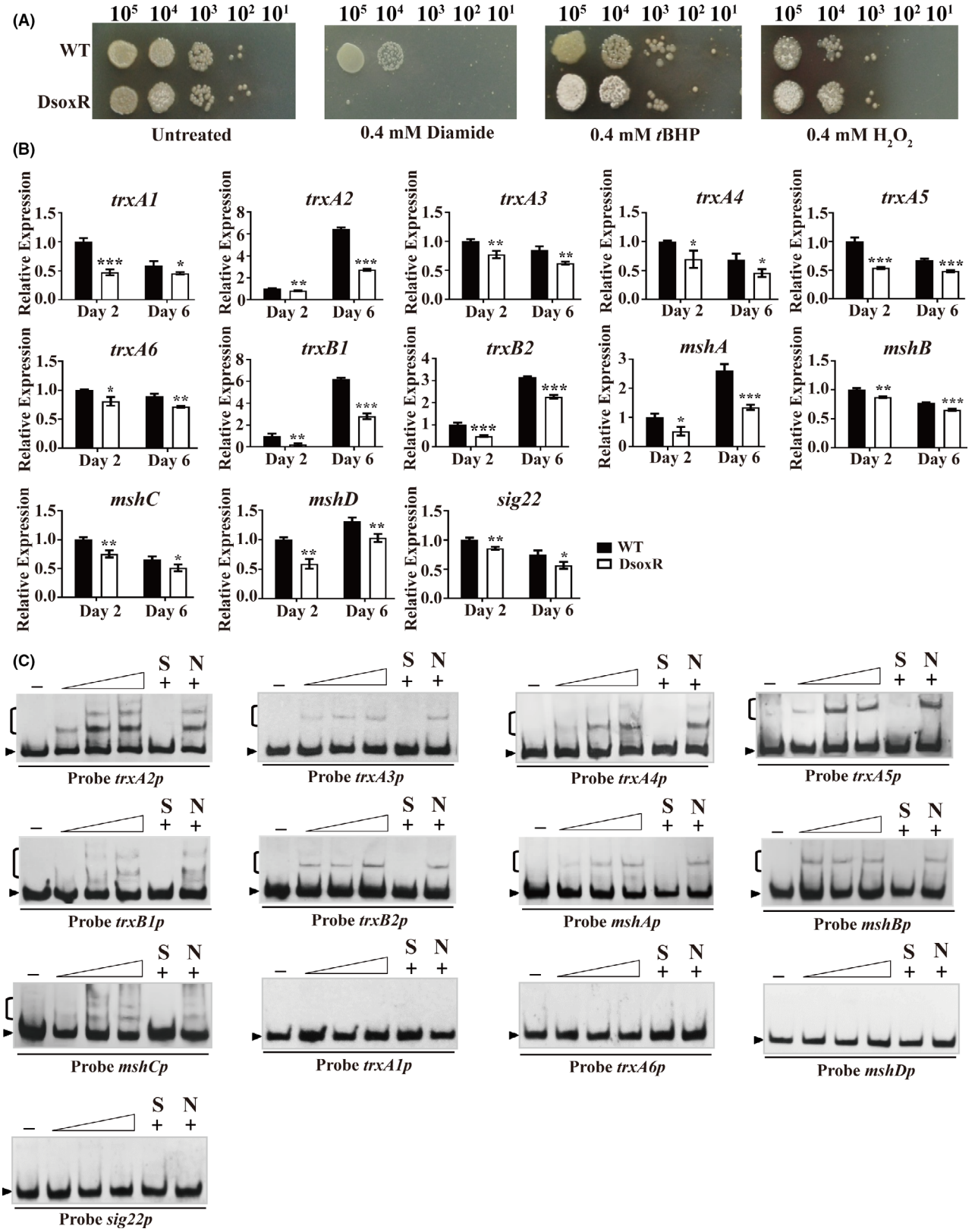

Fig. 8. Identification of SoxR target genes related to thiol-oxidative stress.
A. Sensitivity of WT and DsoxR to oxidative stress conditions. Serial dilutions of spores were spotted on YMS plates containing 0.4 mM diamide, *t*BHP or H_2O_2 and incubated for 3 days at 28°C.
B. RT-qPCR analysis of thiol-oxidative stress-related genes in WT and DsoxR grown in FM-I. For each gene, transcription level was expressed relative to WT value on day 2, assigned as 1. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (*t*-test). Error bars: SD for three replicates.
C. EMSAs of His_6-SoxR with indicated promoter probes. Lane notations as in Fig. 3D.

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 *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 *trxA1*, *trxA6*, *mshD* and regulatory genes *aveR* and *sig22* via yet-unknown mechanisms. SoxR also targets seven genes (*sav_3956*, *sav_4018*, *sav_5665*, *sav_7218*, *smoE*, *sig57*, *sav_7278*) whose functions are unclear. The regulatory roles of SoxR in *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 *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 *S. avermitilis* and *E. coli* were described previously (Liu *et al*., 2015). YMS (Ikeda *et al*., 1988) agar was used for phenotypic observation of *S. avermitilis* mutants. Insoluble fermentation medium FM-I (Jiang *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 *et al*., 2011) was used to cultivate mycelia for analysis of *S. avermitilis* biomass. Avermectin production in FM-II is lower than that in FM-I. YBP (Ou *et al*., 2009) agar was used for characterization of *S. coelicolor* phenotype.

Gene disruption, complementation and overexpression

For in-frame gene deletion of *soxR*, a 451-bp 5′-flanking region (positions −400 to +51 relative to *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 *et al*., 1992), generating *soxR* deletion plasmid pDsoxR, which was transformed into WT protoplasts. *soxR* deletion mutant was screened as reported previously (Yang *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, only WT DNA generated a 270-bp band. We thus

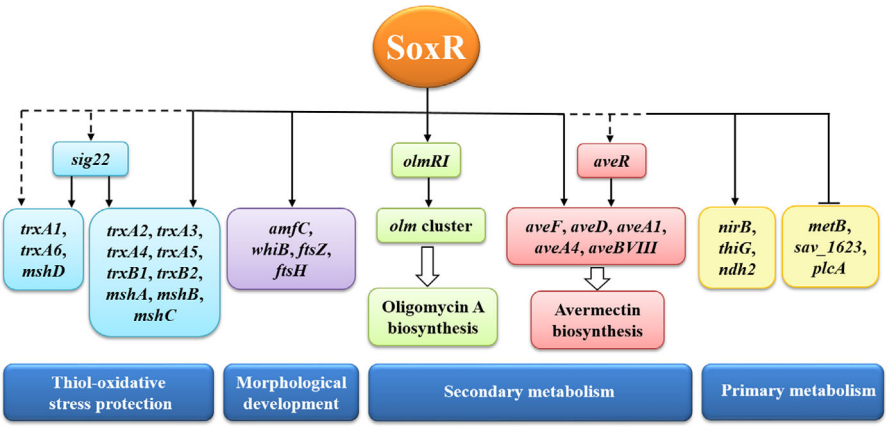

Fig. 9. Proposed model of regulatory role of SoxR in primary metabolism, secondary metabolism, development and thiol-oxidative stress response in *S. avermitilis*. Solid-line arrows: direct activation. Dashed-line arrows: indirect activation. Bar: direct repression. Hollow arrows: antibiotic biosynthesis.

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*p* (*Streptomyces* strong constitutive promoter). The 848-bp EcoRI/XbaI fragment containing *ermE*p* 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 OsoxR and OsoxR/A229 respectively. pKC-erm-soxR was introduced into *S. coelicolor* M145 to obtain OsoxR/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 OsoxR 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 ground 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 His_6-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/XhoI and ligated into pET-28a (+) to generate pET28-soxR for production of N-terminal His_6-tagged SoxR recombinant protein. pET28-soxR was transformed into *E. coli* BL21 (DE3), and His_6-SoxR production was induced by treatment with 0.4 mM IPTG for 8 h at 16°C. Bacteria containing His_6-SoxR were collected, resuspended in lysis buffer (Luo *et al*., 2014), sonicated on ice and centrifuged. Soluble His_6-SoxR in supernatant was purified on 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* (nonspecific 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 His_6-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 His_6-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 OsoxR/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.

