이학박사학위논문

*Streptomyces coelicolor*에서 SigR과 WblC에 의한 스트레스 반응

Stress Responses Regulated by SigR and WblC in *Streptomyces coelicolor*

2017년 2월

서울대학교 대학원

생명과학부

유 지 선

Stress Responses Regulated by SigR and WblC in *Streptomyces coelicolor*

by

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A Thesis Submitted in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

February, 2017

School of Biological Sciences

The Graduate School

Seoul National University

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지도교수 노정혜

이 논문을 이학박사 학위논문으로 제출함

2017년 2월

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생명과학부

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ABSTRACT

Stress Responses Regulated by SigR and WblC in *Streptomyces coelicolor*

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Cells modulate gene expressions to adapt environmental changes. Among the regulators that respond to environmental changes, SigR is one of the most abundant sigma factor in *Streptomyces coelicolor* and governs the thiol-oxidative stress response. RsrA, a redox-sensitive anti-sigma of SigR, senses redox changes through zinc coordinating histidine-cysteine residues and modulates SigR activity.

In order to better understand the physiological function of SigR, chromatin immunoprecipitation combined with sequence and transcript analyses were conducted, revealing the 108 SigR target promoters. In addition to reported genes for thiol homeostasis, protein degradation and ribosome modulation, 64 additional operons were identified suggesting new functions of this global regulator. By analyzing the newly found target genes, it was demonstrated that SigR is required to maintain the level and activity of the housekeeping sigma factor HrdB during thiol-

oxidative stress, and to protect cells against ultraviolet and thiol-reactive damages, by regulating UvrA.

Next I found that antibiotics induce SigR and its regulon via a redox-independent pathway, leading to antibiotic resistance. The amplification of response to thiol-oxidative stress is achieved by producing an unstable isoform of SigR called SigR′ which is degraded in minutes. Unlike this transient response amplification mediated by unstable SigR', antibiotics induce stable SigR eliciting a prolonged response. Among the antibiotics, the *sigR*p1 transcripts is induced by sub-minimal inhibitory concentration of translation-inhibiting antibiotics, resulting in increased synthesis of the stable SigR. I also found that the increased expression of SigR by antibiotics was mediated by WblC/WhiB7, a DNA-binding protein similar to WhiB. WblC has three transcriptional start sites and *wblC* transcripts increased in all three promoters by antibiotic treatment. The amount of WblC protein and its binding to the *sigR*p1 promoter *in vivo* increased upon antibiotic treatment. By comparing antibiotic susceptibility of mutant and wild type, it was confirmed that SigR and WblC confer resistance to translation-inhibiting antibiotics. In addition, the *sigR*-homologous genes in *Mycobacterium tuberculosis* were induced by antibiotics as well.

These findings reveal a novel antibiotic-induced resistance mechanism conserved among actinomycetes and give an example of overlap in cellular damage and defense mechanisms between thiol-oxidative and anti- translational stresses.

Keywords: Bacterial genetics, Transcription factor, Oxidative stress, Antibiotics,

Actinomycetes, *Streptomyces coelicolor*, SigR, WblC/WhiB7

Student Number: 2009-20345

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LIST OF ABBEREVIATIONS

WT	wild type
ECF	extra-cytoplasmic function
ZAS	zinc-containing anti-sigma
RNAP	RNA polymerase
TSS	transcription start site
ORF	open reading frame
ROS	reactive oxygen species
RAC	redox active compounds
REC	reactive electrophile species
RNS	reactive nitrogen species
ABX	antibiotics
MIC	minimal inhibitory concentration
OD	optical density
ChIP	chromatin immunoprecipitation
Mtb	*Mycobacterium tuberculosis*

CHAPTER I. INTRODUCTION

I-1. Biology of *Streptomyces coelicolor*

Streptomyces coelicolor, a filamentous, high G-C, gram-positive bacteria, was first dubbed *Streptothrix coelicolor* in 1908 by R. Muller after he found it on a potato (Conn, 1943). The *Streptomyces coelicolor* A3(2) strain studied in depth by David A Hopwood and sequenced by the John Innes Center and the Sanger Institute is actually taxonomically a member of the *Streptomyces violaceoruber* genus, although it retains the former name (Hopwood, 1999).

Streptomyces coelicolor, like the streptomyces genus in general, live in the soil. Streptomyces are responsible for much of the degradation of organic material in the soil as well as the earthy smell of soil. *Streptomyces coelicolor* are important bacteria because of their "adaptability to environmental stress", "source of bioactive molecules for medicine and industry", and "relation to human pathogens" (Kieser et al., 2000).

Streptomyces coelicolor has a very similar core genome to *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*, as well as some similarity to *Mycobacterium leprae* (Thompson et al., 2002). The Streptomyces genus is responsible for producing over two-thirds of all natural antibiotics in use today, as well as some immune-suppressants and anti-tumor agents. *Streptomyces coelicolor* also has a complicate life-cycle that includes differentiation into aerial mycelium and spore formation which involves complex regulation of gene expression in space and time (Hopwood, 1999).

I-2. Biological defense systems to oxidative stress

Cells are exposed to various forms of oxidative stressors such as reactive oxygen species (ROS), natural or xenobiotic redox-active compounds (RAC), or some antibiotics, which elicit the formation of ROS inside the cell. The response mechanism involves various enzymes to remove such oxidants, systems to repair and recycle damaged cell components, and to maintain optimal cell physiology (Imlay, 2008; Zuber, 2009). One form of oxidative damage that frequently occurs in proteins and small molecules is the oxidation of cysteine thiols by ROS (Kiley and Storz, 2004). Cysteine thiols can also be modified by reactive nitrogen species (RNS) and thiol-reactive electrophiles (Rudolph and Freeman, 2009).

The presence of ROS, RNS and other thiol-reactive compounds can be sensed directly through thiol-based sensor-regulators, which modulate the expression of genes encoding functions that constitute the biological stress response (Antelmann and Helmann, 2011). The best studied examples of thiol-based sensor-regulators that respond to ROS in bacteria include H_2O_2-sensing OxyR in *Escherichia coli*, organic peroxide-sensing OhrR in *Bacillus subtilis*, and the anti-sigma factor RsrA that senses thiol oxidation and modulates regulator SigR activity in *Streptomyces coelicolor* (D'Autreaux and Toledano, 2007; Li et al., 2003). In eukaryotes, thiol-based redox switches have been well exemplified in Yap1 of *Saccharomyces cerevisiae* and the mammalian Nrf2/Keap1 system (Brandes et al., 2009; Thimmulappa et al., 2002). Representative target genes of these thiol-based regulators are those that encode thiol homeostasis system such as thioredoxin (Trx), glutaredoxin (Grx) and small molecular thiol systems (regulated by OxyR, SigR/RsrA, Yap1, Nrf2/Keap1), catalases and peroxidases (by OxyR and Yap1), organic hydroperoxidase (by OhrR), detoxification of electrophiles (by SigR and

Nrf2), and some proteolytic system (by OxyR, SigR, Yap1, Nrf2) (Imlay, 2008; Thimmulappa et al., 2002).

I-3. Antibiotic stress responses in bacteria

Many actinomycetes, especially those of *Streptomyces* genus, are well recognized for undergoing complex developmental programs and producing diverse secondary metabolites. In soil environment where *streptomycetes* inhabit, thousands of bacterial species are estimated to reside in one gram of soil producing more than 10^4 bioactive small molecules (Schloss and Handelsman, 2006; Wright, 2010). In natural environment, *streptomycetes* deal with numerous growth-inhibitory antibiotics which are made by themselves (endogenous) or other organisms (exogenous). Therefore, while being major producers of antibiotics, actinomycetes are the major sources of antibiotic resistance mechanisms (Davies and Davies, 2010). The mechanisms of antibiotic resistance found in clinical pathogens derive their origin from environmental bacteria as first identified for aminoglycoside resistance in *Streptomyces* (Benveniste and Davies, 1973). Since then, various parallel examples, such as *vanHAX* gene cluster for vancomycin resistance, were reported in soil actinomycetes as well as in clinical strains (Marshall et al., 1997).

Whether living inside the human body or in natural environment, bacteria are exposed to wide concentration ranges of antibiotics. In most cases, they are exposed to non-lethal or sub-minimal inhibitory concentration (MIC) of antibiotics. Antibiotics at sub-MIC act as signals and stressors to elicit physiological and genetic changes to cope with antibiotic stress (Andersson and Hughes, 2014; Bernier and Surette, 2013; Davies et al., 2006). Antibiotic resistance phenotype is induced by

sub-inhibitory antibiotics through modulating gene expression and physiology (intrinsic resistance) or through changing genetic information via mutation or horizontal transfer of resistance genes (acquired resistance). Modulation of bacterial gene expression to enhance intrinsic resistance is mediated via hosts of regulators. Some known transcriptional regulators include RNA polymerase sigma factors such as RpoS (Gutierrez et al., 2013), a redox-sensitive regulator such as SoxR (Dietrich et al., 2008; Lee et al., 2009), or a WhiB-like factor (WblC/WhiB7 (Morris et al., 2005; Nguyen and Thompson, 2006)). Unraveling the vast array of regulatory pathways and their networks are needed to understand and control resistance mechanisms.

I-4. SigR-RsrA in *Streptomyces coelicolor*

Among regulators that respond to environmental changes, a group of alternative sigma factors called extra-cytoplasmic function (ECF) sigma factors are abundantly encoded in bacterial genomes (Mascher, 2013; Staron et al., 2009). They are also called group 4 sigma factors consisting of only σ_2 and σ_4 domains that recognize -35 and -10 regions, respectively, of cognate promoters (Helmann, 2002; Lonetto et al., 1994). In *Streptomyces coelicolor*, 50 such factors are encoded in the genome (Hahn et al., 2003). Among them, the role of only several factors has been elucidated, such as SigR (SCO5216 (Paget et al., 1998)), BldN (SCO3323 (Bibb et al., 2000)), SigU (SCO2964 (Gehring et al., 2001)), SigE (SCO3356 (Paget et al., 1999)), SigT (SCO3892 (Mao et al., 2013)) and SigQ (SCO4908 (Shu et al., 2009)).

A zinc-containing anti-sigma (ZAS) factor RsrA that binds an ECF (group 4) sigma factor SigR in *S. coelicolor* responds to diamide-induced thiol oxidation by forming disulphide bonds, releasing SigR to transcribe its target genes (Bae et al., 2004; Kang

et al., 1999; Li et al., 2003). In addition to thiol-oxidants, the presence of non-oxidative thiol-reactive compounds also induces expression of the SigR regulon, suggesting that RsrA may respond to these compounds either directly through thiolation of reactive cysteines or indirectly through changes in reduced thiol pools such as mycothiol, the functional equivalent in actinomycetes of glutathione (Park and Roe, 2008). The products of known SigR target genes include thiol-redox proteins such as thioredoxin systems (TrxBA, TrxC), the first enzyme in mycothiol synthesis (MshA), and a glutaredoxin-like protein called mycoredoxin (Mrx). They also include proteolytic components of protein quality control (PepN, SsrA, ClpP1P2, ClpX, ClpC, Lon), cysteine production (CysM), methionine reduction (MsrA, MsrB), guanine synthesis (GuaB), ribosome-associated function (RpmE, RelA), and electrophile detoxification (Mca) (Kallifidas et al., 2010). Therefore, perturbation of intracellular thiols, as sensed through RsrA, induces expression of gene products that contributes to protein quality control, detoxification of thiol-conjugative xenobiotics, and modulation of transcription and translation.

The SigR system in *S. coelicolor* is activated by thiol-reactive chemicals that oxidize or alkylate cysteine thiols (Paget et al., 1998; Park and Roe, 2008). The induction mechanism involves the inactivation of its anti-sigma factor RsrA via forming disulfide bonds, and liberating active SigR (Bae et al., 2004; Kang et al., 1999; Li et al., 2003), which then positively regulates the expression of its own gene from the SigR-dependent upstream promoter (*sigR*p2) (Fig. I-1). The positively amplified *sigR*p2-derived SigR protein contains N-terminally extended 55 more amino acid residues, and is called $\sigma^{R\text{II}}$ to distinguish it from the apparently constitutive form σ^{R} expressed from the downstream promoter (*sigR*p1) (Kim et al., 2009). A prominent difference between σ^{R} and $\sigma^{R\text{II}}$ is in their stability. Whereas σ^{R} is stable for hours, $\sigma^{R\text{II}}$ is short-lived with a half-life of ~10 min (Kim et al., 2009). The SigR regulon includes the thiol-reducing systems which contribute to

reactivating RsrA via disulfide reduction. It also includes proteases which degrade σ^{R}, thereby turning off the response within an hour (Kang et al., 1999; Kim et al., 2009). Therefore, the response of SigR-RsrA system to thiol-reactive chemical stresses is transient and is mediated by sensor RsrA and amplified σ^{R}.

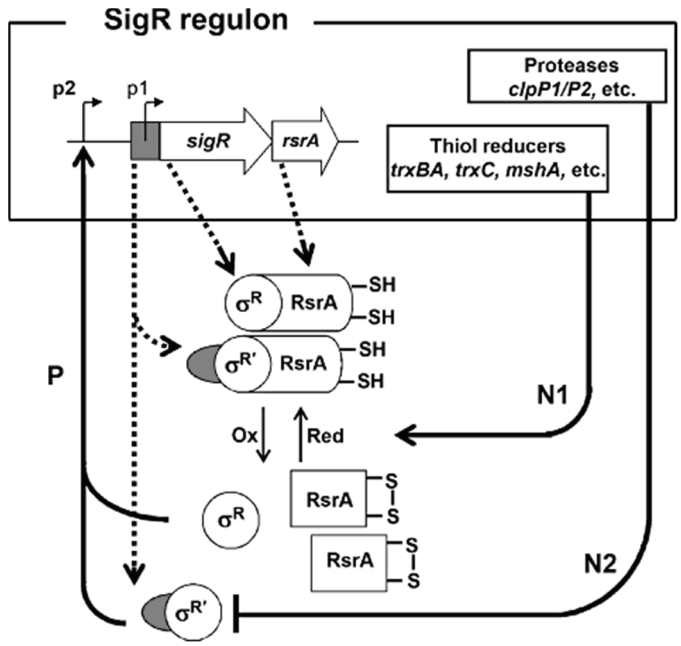


Figure I-1. The regulatory loop involving $\sigma^{R'}$ in the induction of SigR regulon.

The *sigR* coding region produces two forms of SigR product: σ^R from p1 transcript and $\sigma^{R'}$ from p2 transcript. Under reducing environment, the reduced RsrA binds σ^R and $\sigma^{R'}$ and inhibits SigR-directed transcription. Upon oxidative stress, disulphide bonds are formed in RsrA and SigR is released from the complex. The released σ^R and $\sigma^{R'}$ direct transcription of target genes that includes those for itself (from *sigR*p2). Induction of *sigR* gene by released σ^R and $\sigma^{R'}$ constitutes the initial positive amplification loop (P). The induced thiol reducers contribute to reducing RsrA which then binds both σ^R and $\sigma^{R'}$ and turns off the response. This constitutes a negative feedback loop (N1). An additional feedback regulatory loop ensures rapid turn-off of the response, by degrading over-produced $\sigma^{R'}$ by induced proteases (N2).

I-5. WhiB7/WblC in Actinomycetes

WblC is a WhiB-like protein conserved in actinomycetes (Chandra and Chater, 2014; Soliveri et al., 1993; Soliveri et al., 2000) and reported to confer resistance to antibiotics in *Mycobacterium* and *Streptomyces* (Fowler-Goldsworthy et al., 2011; Morris et al., 2005) (Fig. I-2A). WblC/WhiB7 proteins contain three functional domains such as an Fe-S cluster binding domain with four conserved cysteines, a G(V/I)WGG turn interacting with the SigA, and an AT-hook DNA binding domain (Burian et al., 2012a) (Fig. I-2C). The *whiB7* gene is known to be induced by a variety of antibiotics via autoregulation (Fig. I-2B), and WhiB7 may contribute to intrinsic resistance to antibiotics by activating antibiotic export, antibiotic inactivation and changes in thiol redox balance in mycobacteria (Burian et al., 2012b; Nguyen and Thompson, 2006).

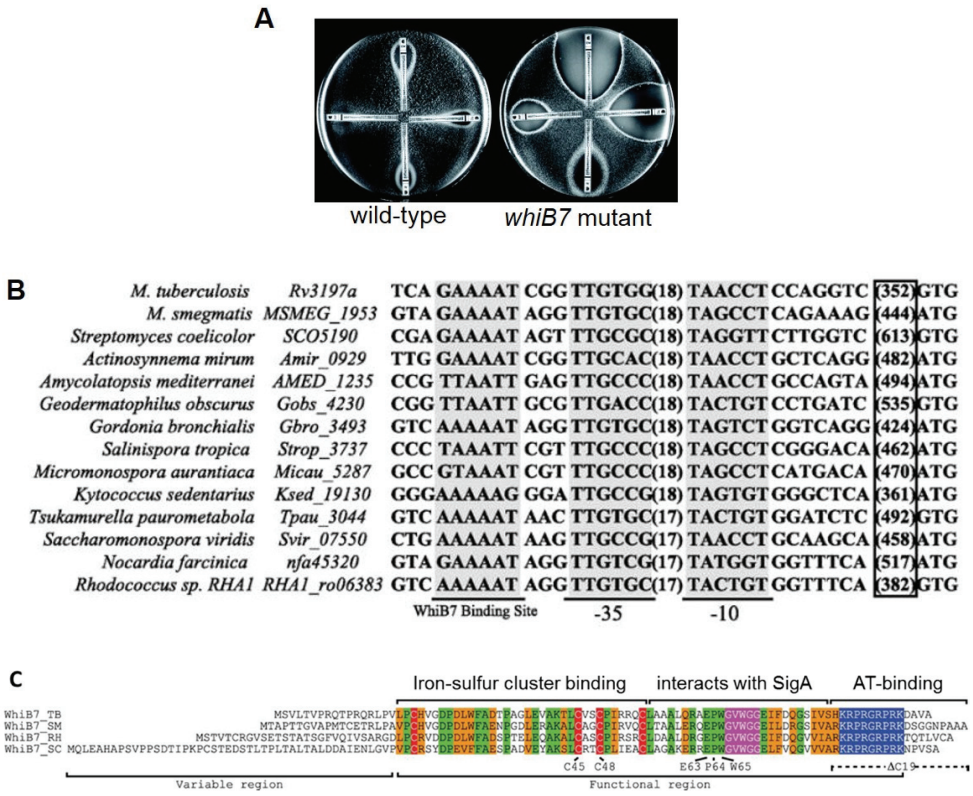


Figure I-2. WhiB7 in actinomycetes.

A. *whiB7* mutant is more susceptible to antibiotics (Morris et al., 2005).

B. The promoter sequences of *whiB7* homologous genes in representative 14 actinomycetes (Burian et al., 2012b).

C. WhiB7 contains three functional domains: iron-sulfur cluster binding domain, SigA-interacting domain, and AT-rich DNA binding domain (Burian et al., 2012a).

CHAPTER II. MATERIALS AND METHODS

II-1. Strains and plasmids

All strains and plasmids used in this study were listed in Table II-1 and Table II-2. The pSET152H plasmid and *E. coli* ET12567, a non-methylating strain containing pUZ8002 for donor functions, were used for complementation as recommended (Gust et al., 2003). *E. coli* DE3/gold strain and pET15b plasmid was used for protein over-expression.

II-2. Antibiotics and reagents

Antibiotics and reagents were obtained from Sigma-Aldrich and Duchefa biochemie. Following manufacture's instruction, the solutions of antibiotics and chemicals were prepared freshly before each treatments.

II-3. Culture conditions

Spores of wild type, Δ*sigRrsrA* (MK1) (Kim et al., 2009) and Δ*wblC* (Fowler-Goldsworthy et al., 2011) were inoculated in 100 ml YEME liquid medium containing 5 mM $MgCl_2$•$6H_2O$ and 10% sucrose, and were grown at 30°C (Kieser et al., 2000). For aeration, the 500 ml cultural baffled flasks containing 100 ml YEME liquid medium were placed in shaking incubator at 180 rpm. NA plates (0.8% nutrient broth, 2% agar powder) were used for spotting analysis. *E. coli* was grown in LB broth. *M. tuberculosis* H37Rv cells were grown at 37°C in Middlebrooks 7H9 broth supplemented with 10% OADC and with or without antibiotics.

II-4. Site-directed mutagenesis

Each residue from K33 to K47 in RsrA was replaced with alanine by site-directed mutagenesis, using alanine-scanning primers and pUC19-*sigRrsrA*, according to the protocol provided by GENEART® site-directed mutagenesis system (Invitrogen). The mutated *rsrA* genes were confirmed by sequencing. The HindIII/BamHI fragments from the resulting plasmids were cloned into the EcoRV site of pSET152H, and the final recombinant plasmids were introduced into the Δ*sigR-rsrA* mutant of *S. coelicolor* through conjugation. The desired ex-conjugants were selected and confirmed by DNA sequencing.

II-5. Spotting assay

NA plates containing various antibiotics (20 μg/ml ampicillin, 1 μg/ml norfloxacin, 2 μg/ml rifampicin, 10 μg/ml chloramphenicol, 2 μg/ml erythromycin, 10 μg/ml lincomycin or 2 μg/ml tetracycline) were used to monitor sensitivity. An equal number of spores of wild type and mutant *S. coelicolor* strains were serially diluted by 10-fold and spotted on antibiotic-containing NA plates using a 48-pin replica plater (Sigma). The spotted plates were incubated at 30°C for up to 3 days before taking photos.

II-6. Kirby-mix RNA extraction

The *S. coelicolor* cells grown to OD_{600} of 0.3~0.4 in YEME were treated with various chemicals and harvested by centrifugation at 12000 rpm for 4 min at 4℃. Harvested cells were disrupted with sonicator in Kirby-mix using Q500 sonicator (QSonica) using a 3 mm tip at 20% maximum power with 5 sec pulse. RNA was

isolated through extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitation with isopropyl alcohol according to a standard procedure (Kieser et al., 2000). The isolated RNA sample was quantified with NanoDrop 2000 spectrophotometer (Thermo scientific).

II-7. Hot-acid-phenol RNA extraction

The *S. coelicolor* cells grown to OD_{600} of 0.3~0.4 in YEME were harvested by centrifugation at 12000 rpm for 4 min at 4℃. The cell pellet were re-suspended in 1 ml of 10% glycerol containing lysozyme (2 mg/ml) and incubated at 180 rpm shaking for 10 min at 37℃. The cells were harvested by centrifugation at 5000 rpm for 4 min at 4℃. The cell pellet were washed and re-suspended in 540 μl of cold AE buffer. 60 μl of 10% SDS was added and vortexed. Phenol solution was added, vortexed, and incubated for 5 min at 65℃. The mixture was cooled rapidly on ice and centrifuged at 13000 rpm for 10 min at 4℃. The upper aqueous phase (~500 μl) was transferred to a fresh tube. The RNA was precipitated by adding 50 μl of 3 M sodium acetate and 1 ml of ethanol. The sample was incubated for 20 min at -80℃ and centrifuged at 13000 rpm for 10 min at 4℃. The pellet was washed with 70% ethanol, dried, and resuspended in 50 μl of RNase free water. For removing DNA contamination from RNA samples, RQ1 RNase-Free DNase (Promega) was treated. The DNase-treated RNA samples were purified by phenol/chloroform/iso-amyl alcohol (25:24:1) extraction and ethanol precipitation.

II-8. Mycobacterial RNA preparation

For mycobacterial RNA preparation, harvested cells were resuspended in TRIzol®

Reagent (Ambion, Life Technologies, Carlsbad, CA, USA), mixed with acid-washed 425~600 glass beads (Sigma-Aldrich G8772), and lysed using a mini-bead beater (BioSpec, Bartlesville, OK, USA). Following chloroform extraction and isopropanol precipitation, the RNA pellet was resolved in RNA-free water (Ambion, Life Technologies, Carlsbad, CA, USA).

II-9. S1 nuclease mapping analysis

For each sample, 1~100 μg of total RNA was analyzed with gene-specific DNA probes labeled with γ-^{32}P-ATP. Hybridization was done at 50°C overnight, followed by S1 nuclease (Thermo) treatment. The protected DNA probes were loaded on 5% polyacrylamide gel containing 7M urea (Kim et al., 2009). Mycobacterial RNA was also analyzed by S1 nuclease protection assay as same procedure.

For enrichment of 5′ -triphosphate-RNA, the 10 μg RNA samples were treated with or without Tobacco Acid Pyrophosphatase (TAP, Epicentre). The reaction mixture was extracted with phenol/chloroform/isoamyl alcohol, and the RNA was precipitated by ethanol in the presence of glycogen. Further digestion with Terminator Exonuclease (TEX, Epicentere) was carried out and the sample was extracted with phenol/chloroform/isoamyl alcohol following ethanol precipitation in the presence of glycogen. The sample was analyzed by S1 nuclease mapping as described above.

II-10. Purification of WblC and RsrA proteins

The *rsrA* ORF and *wblC* ORF were amplified by PCR. The PCR fragments were cloned in pET15b after appropriate restriction enzymatic digestion. *E. coli* BL21(DE3)pLysS cells were transformed with pET15b-RsrA and pET-15b-WblC

and grown on LB agar plate containing ampicillin and chloramphenicol. A fresh single colony was inoculated in liquid LB containing ampicillin and chloramphenicol and grown to OD_{600} ~0.4 at 37℃. For RsrA overexpression, cells were induced by adding 1 mM isopropylthio-β-D-galactoside (IPTG) and harvested after further incubation at 37℃ for 3 hours. For WblC overexpression, cells were induced by adding 0.5 mM IPTG and harvested after incubation at 20℃ for 6 hours. The harvested cells were disrupted by sonication and purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin following the manufacture's instruction.

II-11. Western blot analysis

Harvested cells were re-suspended in lysis buffer [20 mM Tris-HCl (pH 7.9), 10% (v/v) glycerol, 5 mM EDTA, 0.1 mM DTT, 10 mM MgCl2, 1 mM PMSF, 0.15 M NaCl]. The suspension was sonicated with Q500 sonicator (QSonica), and cleared by centrifugation (Kim et al., 2012). Protein concentration in crude cell extract was determined by Bradford reagent solution (Bio-Rad) using Bovine Serum Albumin Standard (BSA) Ampules (Thermo) as a standard.

To detect HrdB, ~10 μl cell extract containing 50 μg protein was further diluted to the final concentration of 0.125 μg μl−1 with lysis buffer (up to 400 μl) that additionally contain 50 μg BSA to serve as a protein buffer. Aliquots of 8 μl containing 1 μg crude protein extract and the same amount of BSA were resolved on 10% SDS-PAGE. Immuno-detection by polyclonal rabbit antibody against HrdB protein and the anti-rabbit IgG secondary antibody at 1:5000 dilution ratio followed by ECL detection.

To detect SigR, cell extracts containing 25 μg protein were diluted to the final concentration of 0.125 μg/μl with lysis buffer (200 μl) that contains 100 μg BSA to

serve as a protein buffer. Aliquots of 8 µl containing 1 µg crude protein extract and 4 µg BSA were resolved on 15% SDS-PAGE. Immuno-detection was done by using polyclonal rabbit antibody against SigR and the anti-rabbit secondary antibody at 1:5000 dilution ratio.

For detecting WblC and RsrA, cell extracts containing 20 µg protein were resolved on 15% SDS-PAGE. Immuno-detection was done by using polyclonal rabbit antibody against WblC or RsrA and the anti-rabbit secondary antibody at 1:10000 dilution ratio. The polyclonal rabbit antibody against WblC or RsrA were developed by Abclon service using WblC-His or RsrA-His as the antigen.

II-12. Chromatin immuno-precipitation

Exponentially grown *S. coelicolor* cells (at OD_{600} of 0.3~0.4) were treated with 2 µg/ml tetracycline for 1 h, followed by fixation with 1% formaldehyde for 15 min. 125 mM glycine was subsequently added for 5 min at room temperature. Harvested cells were washed twice with cold TBS wash buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). To break cells and shear DNA, cells were sonicated in RIPA buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) with a sonicator (QSonica Q500) using a 3 mm tip at 20% maximum power, with 20 sec pulses for 8 times on ice. Following centrifugation at 13000 rpm and 4°C for 10 min to clear the cell debris, 50 µl of each supernatant was set aside for input DNA control sample.

To the cleared supernatant anti-WblC polyclonal rabbit antibody (5 µl) was added, and incubated at 4°C for 1 h, with gentle mixing by rotation. Subsequently, 20 µl protein A/G beads (Santacruz) were added and rotated overnight at 4°C. The samples were centrifuged for 1 min at 4°C and 3000 rpm and the pellets were washed once with low salt wash buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM

EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with high salt wash buffer (50 mM HEPES-KOH, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with LiCl wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate), and twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was eluted by incubation in the elution buffer (10 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, 1% SDS) at 65°C for 30 min, followed by treatment with 5 μg proteinase K and 2 μg RNaseA for 1 h at 45°C. NaCl was added to final concentration of 350 mM, and incubation continued at 65°C overnight for reverse-crosslinking. DNA was purified by phenol-chloroform-isoamyl alcohol extraction. The amount of *sigR*p1, *sigR*p2, and *rsrA*-specific DNA was quantified by qPCR (Agilent Stratagene Mx3000P), using primer sets which encompass the *sigR*p1 promoter region (from -125 to -34 nt position, relative to the *sigR* start codon), *sigR*p2 promoter region (from -311 to -186 nt position, relative to the *sigR* start codon), and *rsrA* (from +920 to +991 nt position, relative to the *sigR* start codon).

Table II-1. Strains used in this study

Strains	Genotype or description	Source or reference
***S. coelicolor* A3(2)**		
M145	Wild type (SCP1-,SCP2-)	(Hopwood et al., 1985)
MK1	M145 *sigRrsrA*::aac(3)	(Kim et al., 2009)
MK10	MK1 *attP::pSET152H-sigR-rsrA*	(Kim et al., 2009)
MK11	MK1 *attP::*pMKH1	(Kim et al., 2009)
Δ*uvrA*	M145 *uvrA*::aac(3)	(Kim et al., 2012)
Δ*wblC*	M145 *wblC*::aac(3)	(Fowler-Goldsworthy et al., 2011)
JRA33~JRA47	MK1 *attP::*pJR33A~pJR47A	This study
JRM1	MK1 *attP::*pJRM1	This study
JWM1	Δ*wblC attP::*pJWM1	This study
JWU1	Δ*wblC attP::*pJWU1	This study
JWU2	Δ*wblC attP::*pJWU2	This study
M. tuberculosis		
H37Rv	Wild type	The Korean Institution of Tuberculosis (KIT)
E. coli		
DH5α	See reference	(Hanahan, 1983)
BL21(DE3)pLysS	See reference	(Studier and Moffatt, 1986)
ET12567pUZ8002	See reference	(Gust et al., 2003)
BW25113pIJ790	See reference	(Gust et al., 2003)

SCP1, *S. coelicolor* plasmid 1; SCP2, *S. coelicolor* plasmid 2

Table II-2. Plasmids used in this study

Plasmids	Genotype or description	Source or reference
pUC18/pUC19	E. coli cloning vector	(Yanisch-Perron et al., 1985)
pGEM-Teasy	*E. coli* cloning vector	Promega
pET15b	*E. coli* overexpression vector	Novagen
pET15b-RsrA	pET15b containing PCR fragment of *rsrA*	This study
pET15b-WblC	pET15b containing PCR fragment of *wblC*	This study
pSET152	Non-replicating conjugative plasmid	(Bierman et al., 1992)
pSET152H	pSET152 containing 1.7 kb SphI fragment carrying hyg gene	(Kim et al., 2009)
pUC19*sigRrsrA*	pUC19 containing the 1.45 kb HindIII/BamHI fragment carrying *sigR* promoters (P1 and P2)	(Jung et al., 2011)
pUC19*sigRrsrA*-NdeI	pUC19 containing the 1.45 kb HindIII/BamHI fragment carrying *sigR* promoters (P1 and P2) and NdeI site at *rsrA* start codon	This study
pJR33A ~ pJR47A	pSET152H containing 1.4 kb HindIII/BamHI fragment carrying *sigR* and *rsrA* K33A~47A (RsrA alanine scanning mutants)	This study
pJRM1	pSET152H containing 1.5 kb fragment carrying *sigR* and *rsrA*-myc	This study
pJWM1	pSET152H containing 1.2 kb fragment carrying SCO5189 and *wblC*-myc	This study

pGUS	pSET152H containing glucuronidase	JIC
pJWU1	pSET152H containing 1.1 kb fragment carrying SCO5189 and *wblC*	This study
pJWU2	pSET152H containing 1.1 kb fragment carrying SCO5189 frame-shift mutant and *wblC*	This study

Table II-3. Primers used in this study

Primer	Sequences	Description
SPS	GGC GCG GGC ATG GGC CGG G	*sigR*
pUC-NdeI	TAA CTA TGC GGC ATC AGA GC	pUC
RsrAmyc-F	CGT CGG CCC CGC AGG AGT CCG GCA GCG GCT CCT TCG AGC TCG AGG AGC AGA A	RsrAmyc
myc-pSET-R	AGC TAT GAC ATG ATT ACG AAT TCG ATG GAT CCT CAT CAC AGG TCC T	myc tag
pSETsigRrsrA-F	GCG GCC GCG CGC GAT AGG AAA GGG CGC TGG AGC T	RsrAmyc
sigRrsrAmyc-R	GGA CTC CTG CGG GGC CGA CG	RsrAmyc
wblCmyc-F	CAA GAA CCC GGT TTC GGC AGG CAG C GG CTC CTT CGA GCT CGA GGA GCA GAA	WblCmyc
pSETwblC-F	GCG GCC GCG CGC GAT AGG AAG GCG TGG GGC TTG A	WblCmyc
wblCmyc-R	TGC CGA AAC CGG GTT CTT G	WblCmyc
RsrAK33A-f	GAC TGC GTG GCC TTC GAG CAC	RsrA ala
RsrAF34A-f	TGC GTG AAG GCC GAG CAC CAC	RsrA ala
RsrAE35A-f	GTG AAG TTC GCC CAC CAC TTC	RsrA ala
RsrAH36A-f	AAG TTC GAG GCC CAC TTC GAG	RsrA ala
RsrAH37A-f	TTC GAG CAC GCC TTC GAG GAG	RsrA ala
RsrAF38A-f	GAG CAC CAC GCC GAG GAG TGC	RsrA ala
RsrAE39A-f	CAC CAC TTC GCC GAG TGC TCG	RsrA ala
RsrAE40A-f	CAC TTC GAG GCC TGC TCG CCC	RsrA ala
RsrAC41A-f	TTC GAG GAG GCC TCG CCC TGC	RsrA ala
RsrAS42A-f	GAG GAG TGC GCC CCC TGC CTG	RsrA ala

RsrAP43A-f	GAG TGC TCG GCC TGC CTG GAG	RsrA ala
RsrAC44A-f	TGC TCG CCC GCC CTG GAG AAG	RsrA ala
RsrAL45A-f	TCG CCC TGC GCC GAG AAG TAC	RsrA ala
RsrAE46A-f	CCC TGC CTG GCC AAG TAC GGG	RsrA ala
RsrAK47A-f	TGC CTG GAG GCC TAC GGG CTG	RsrA ala
RsrAK33A-r	GTG CTC GAA GGC CAC GCA GTC	RsrA ala
RsrAF34A-r	GTG GTG CTC GGC CTT CAC GCA	RsrA ala
RsrAE35A-r	GAA GTG GTG GGC GAA CTT CAC	RsrA ala
RsrAH36A-r	CTC GAA GTG GGC CTC GAA CTT	RsrA ala
RsrAH37A-r	CTC CTC GAA GGC GTG CTC GAA	RsrA ala
RsrAF38A-r	GCA CTC CTC GGC GTG GTG CTC	RsrA ala
RsrAE39A-r	CGA GCA CTC GGC GAA GTG GTG	RsrA ala
RsrAE40A-r	GGG CGA GCA GGC CTC GAA GTG	RsrA ala
RsrAC41A-r	GCA GGG CGA GGC CTC CTC GAA	RsrA ala
RsrAS42A-r	CAG GCA GGG GGC GCA CTC CTC	RsrA ala
RsrAP43A-r	CTC CAG GCA GGC CGA GCA CTC	RsrA ala
RsrAC44A-r	CTT CTC CAG GGC GGG CGA GCA	RsrA ala
RsrAL45A-r	GTA CTT CTC GGC GCA GGG CGA	RsrA ala
RsrAE46A-r	CCC GTA CTT GGC CAG GCA GGG	RsrA ala
RsrAK47A-r	CAG CCC GTA GGC CTC CAG GCA	RsrA ala
Rv3223c-700f	TGG GGA ATG CAC GCT TGG GA	Mtb *sigH*
Rv3223c-496f	ATC GGC AGT GCC TGG CCG C	Mtb *sigH*
Rv3223c-295f	TCA CCC TAA CGC CCT GCT CGA	Mtb *sigH*
Rv3223c+14r	TCG ATG TCG GCC ATC TTG ATT AAC T	Mtb *sigH*
Rv3223c+63r	TGT CTC CTC AGA CGG CCC AG	Mtb *sigH*
Rv1221-421f	CAG CAA GCA CAG CAA CGC A	Mtb *sigE*

Rv1221-346F	GGC CAG GAT CAC GTC TTC AGA TA	Mtb *sigE*
Rv1221+89R	GCA CTG CAA TAA GTT GGC AAG TCG	Mtb *sigE*
Rv1221+50R	CAA AGT TGC GAT TCC GTA TTC CCA A	Mtb *sigE*
Rv3197A-497F	GCA TCG GTG CCC GCA AGC	Mtb *whiB7*
Rv3197A+20R	GGG ACT GTC AGT ACC GAC AC	Mtb *whiB7*
wblCov-nde1-F	TAT TAC ATA TGC AAC TCG AAG CGC ACG	WblCover
wblCov-bH1-R	AAT ATG GAT CCT GCG GTG TTC ATG CCG	WblCover
wblC-hd3-688F	ATA TTA AGC TTG ACG AGG ACG ACT GAT ACA AAC TCG TC	*wblC*
wblC-xbaI-688F	ATT AAT CTA GAC GAG GAC GAC TGA TAC AAA CTC G	*wblC*
wblC-eRI+443R	AAT AGA ATT CGT GGG TGG ACG GCT TCA TC	*wblC*
SCO5190-700F	GGC GTG GGG CTT GAC GAG GA	*wblC*
SCO5190+470R	GTC TTC AGG CGC GGT GCC TG	*wblC*
SCO5190-840F	TCT TCA CCG ACA AGA CGC TG	*wblC*
SCO5190-750F	AGC TCA ATC GCT ACG GGA CCG A	*wblC*
SCO5190-691F	CTT GAC GAG GAC GAC TGA TA	*wblC*
SCO5190-438R	GAT CGC TCG GCC GTC TCC CT	*wblC*
SCO5190-377R	CAT TCG TGC CGT GCG TCC G	*wblC*
SCO5190-168R	GAT CCA GCC TGA TCG TCG AT	*wblC*
SCO5190+50R	CCG CCT TCC GAC ACG ATC CC	*wblC*
uORF-frame-F	GGC GAG CCC ATG AAT CAG CAT CAA G	*wblC*
uORF-frame-R	CTT GAT GCT GAT TCA TGG GCT CGC C	*wblC*

CHAPTER III. RESULTS

III-1. Role of SigR-RsrA in thiol oxidative stress response

III-1.1. Redox acive compounds activate *sigR* transcription

To analyze whether *S. coelicolor* requires SigR for its normal growth, I compare the growth of wild type and *sigR* mutant after inoculating an equal number of spores, and *sigR* mutant shows growth retardation (Fig. III-1.1).

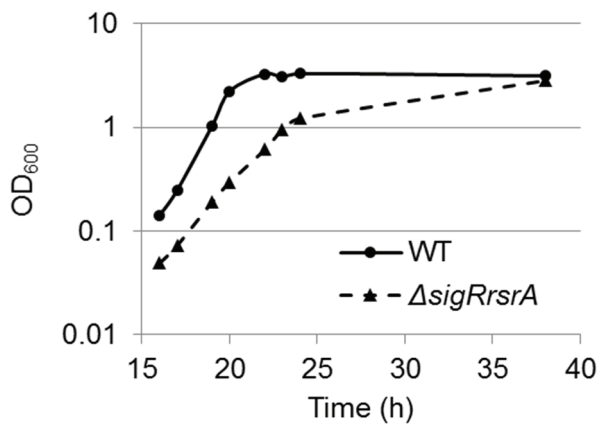


Figure III-1.1. Growth retardation of *sigRrsrA* mutant

The growth curve of Wild type (WT) and *sigRrsrA* mutant. An equal number (2 x 10^9) of spores were inoculated in liquid YEME medium and incubated for 38 hours. OD_{600} of each strains were detected and plotted in log scale.

Next I investigated whether various redox active chemicals affect SigR-RsrA system. Phenazine methosulfate (PMS) is a known redox-cycling redox-active compounds (RAC) that can produce superoxide radicals and hence peroxides (Nishikimi et al., 1972). Plumbagin and *p*-Benzoquinone (BQ) are known to be a redox-cycling RAC and reactive electrophile species (RES) (Castro et al., 2008; O'Brien, 1991). Diamide (DA) and mono-bromobimane (mBBr) are electrophiles with different propensities for reaction products. Whereas diamide rapidly forms disulfide bonds between neighboring cysteine thiols (Kosower and Kosower, 1995), monobromobimane preferentially forms S-alkylated products on cysteine thiols (Kosower et al., 1979).

Fig. III-1.2 shows *sigR* is highly induced by DA, BQ and PL and moderately induced by mBBr and PMS. Diamide exerted direct effects on RsrA through immediate disulfide bond formation. The induced SigR system turned off completely within an hour because of disulfide reduction of RsrA by thioredoxin systems and MSH (Kang et al., 1999; Park and Roe, 2008), accompanied by the removal of diamide. Among the induced SigR-target gene products, several nitroreductase candidates may function in degrading diamide, as observed for nitroreductases (AzoR1 and AzoR2) in *B. subtilis* (Antelmann et al., 2008).

BQ immediately activated the SoxR and SigR systems. Plumbagin induces the SigR system is activated in a delayed fashion. The delayed induction of the SigR target gene suggests that plumbagin may slowly shift redox homeostasis to favor disulfide formation in RsrA as an indirect consequence, rather than causing oxidation or modifying RsrA directly. Depletion of reduced MSH through S-alkylation could cause a shift in thiol redox homeostasis to favor disulfide formation in RsrA. Shutting off SigR induction at late time points suggests that thiol redox homeostasis is regained within 2 h, most likely because of the restoration of MSH and reduction

of protein thiols through the action of SigR target gene products (Kim et al., 2012; Park and Roe, 2008).

Induction of the SigR-target gene by PMS through oxidizing RsrA appeared quite inefficient. This suggests that the amount of peroxides formed by PMS is not sufficient for immediate disulfide bond formation in RsrA, considering that RsrA is relatively insensitive to hydrogen peroxide (Kang et al., 1999; Rajasekar et al., 2016).

mBBr moderately activated the SigR system in an immediate and prolonged manner. This agrees with the suggestion that mBBr activates the SigR system by depleting the MSH pool for relatively long time (up to 80 min) at 20 μM, which facilitates disulfide formation in RsrA (Park and Roe, 2008).

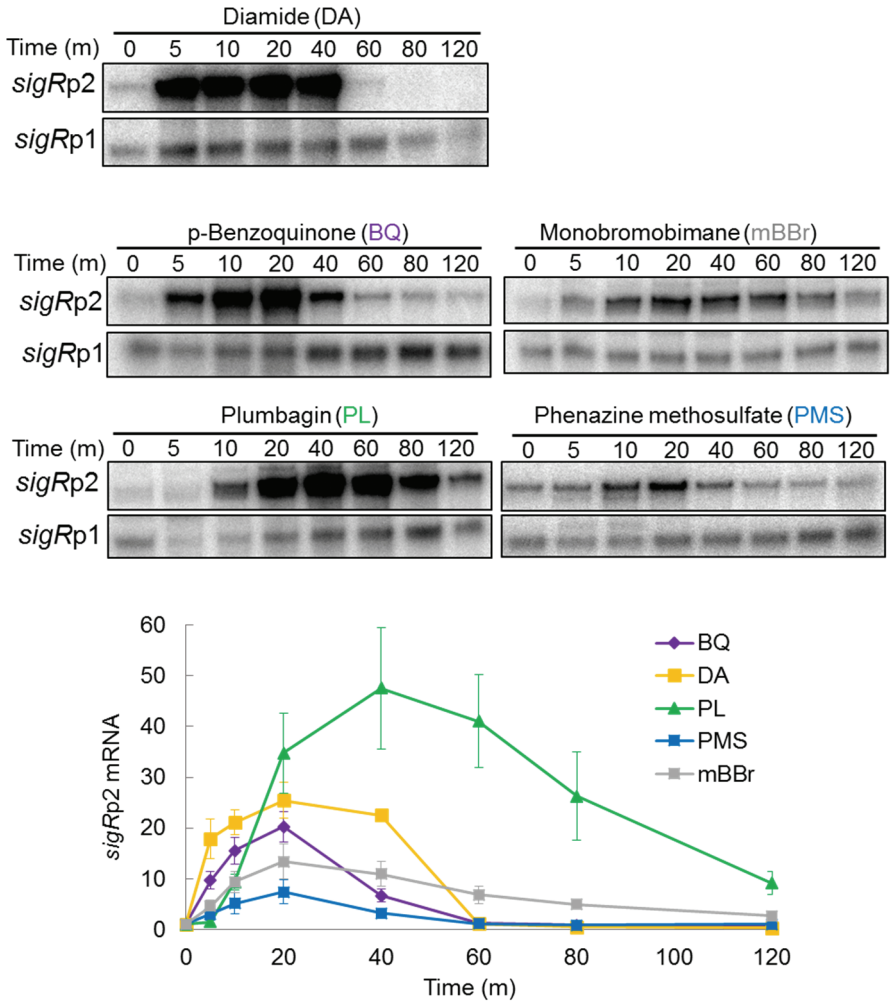


Figure III-1.2. Redox active compounds activate *sigR* transcription

Transcripts from the target genes of SigR (*sigR*p2) were monitored by S1 nuclease mapping. *Streptomyces coelicolor* M145 cells were treated with diamide (500 μM), *p*-benzoquinone (50 μM), plumbagin (20 μM), monobromobimane (20 μM) and phenazine methosulfate (50 μM) for 5 to 120 min, prior to cell lysis, to prepare RNA samples.

III-1.2. Function of the RsrA as a redox sensor

RsrA which senses the thiol oxidative stress, has seven cysteine residues and five histidine residues. Among these residues, His37-Cys41-Cys44 are important for zinc-containing. Any of mutation of these three residues makes RsrA incapable to SigR-binding, therefore the mutants show some defect to sporulation (Fig. III-1.3).

To verify cysteine/histidine residue of RsrA N-terminal are important for the capability of redox sensing, I analyzed the zinc content of RsrA N-terminal mutant proteins using 4-(2-Pyridylazo)resorcinol (PAR). Fig. III-1.4 shows C3 and H7 residues of RsrA may function as zinc containing residues *in vitro*. Next, I analyzed the *in vivo* induction of *sigR* transcription in RsrA N-terminal region mutants. Fig. III.1.5. shows that all the RsrA N-terminal region mutants are less sensitive than wild type RsrA. The results in Fig. III-1.4 and Fig. III-1.5 demonstrate that N-terminal region of RsrA sensitizes RsrA to redox changes.

To test weather RsrA increased upon thiol oxidative stress, I tagged 6xmyc to RsrA C-termianl and developed poly-clonal antibody of RsrA. RsrA were detected well in the both methods. RsrA is increased under thiol oxidative stress *in vivo* (Fig. III-1.6).

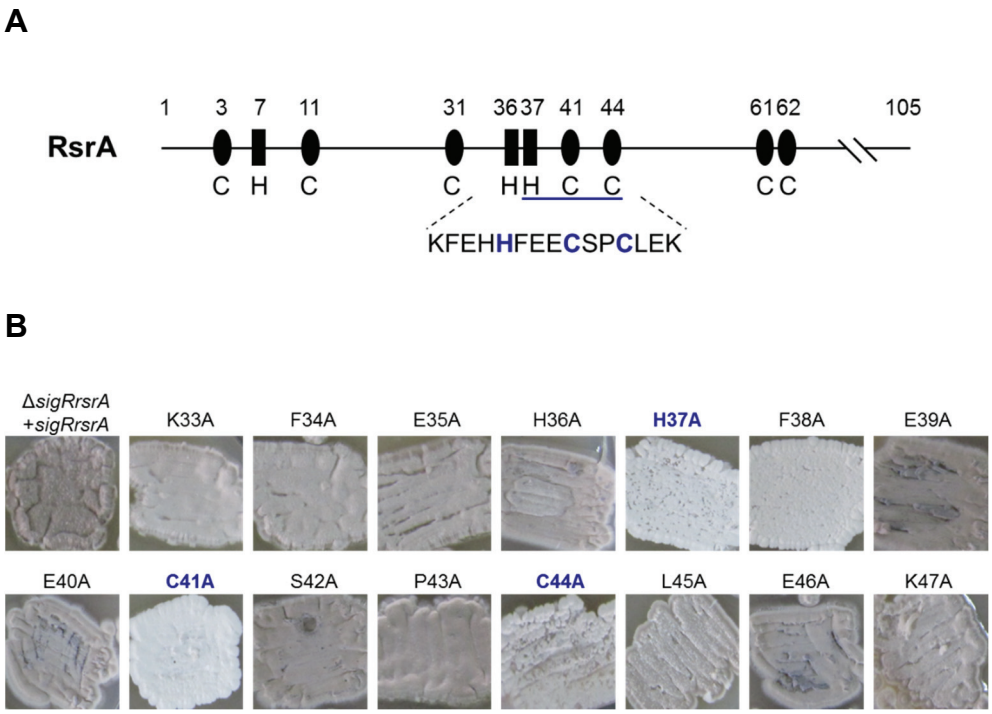


Figure III-1.3. The phenotypes of RsrA-Ala substitution mutants

A. Seven cysteine residues and histidine near cysteine residues in RsrA.

B. The spores of RsrA-alanine substitution mutants are incubated for 4 days in Mannitol-Soy flour-Agar plate and pictured.

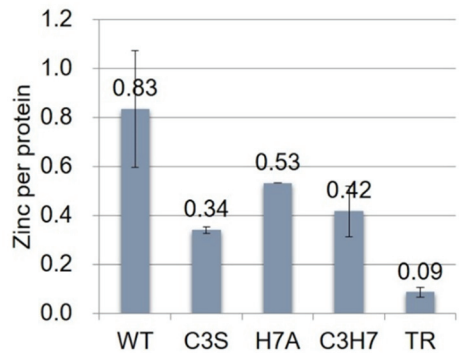
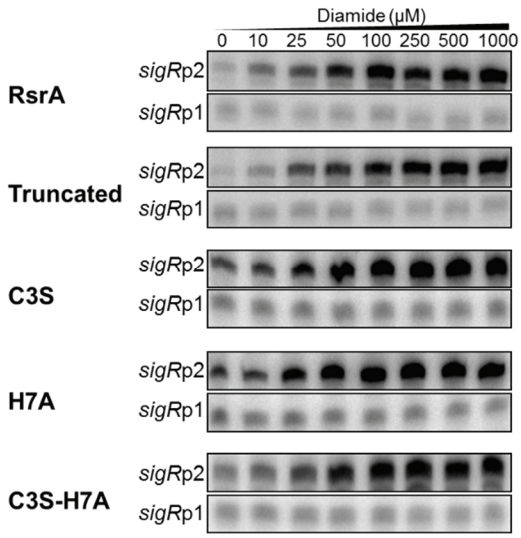


Figure III-1.4. The N-termial region of RsrA affects to zinc binding *in vitro*

The zinc content of RsrA protein was analyzed using 4-(2-Pyridylazo) resorcinol assay; WT for wild type, C3S for Cys3 to serine, H7A for His7 to alanine, C3H7 for Cys3 to serine-His 7 to alanine and TR for truncated. The data represents average from three independent experiments and error bar means standard deviation.



Type	*sigR*p2 Basal level	Conc. of Induction$_{50}$ (μM)
RsrA	1.0	**30.4**
Truncated	0.5	**73.6**
C3S	3.0	**41.2**
H7A	2.5	**34.0**
C3S-H7A	2.6	**48.4**

Figure III-1.5. The N-terminal region sensitizes RsrA to redox changes

Δ*sigRrsrA* mutant was complimented with *sigR-rsrA* (wild type RsrA, truncated RsrA, C3S RsrA, H7A RsrA and C3SH7A RsrA). Each complimented strains were treated with the serial concentration of diamide, and the *sigR* transcripts analyzed by S1 nuclease mapping (left). The basal expression level of *sigR*p2 transcripts and the diamide concentration for 50% induction were displayed in table (right).

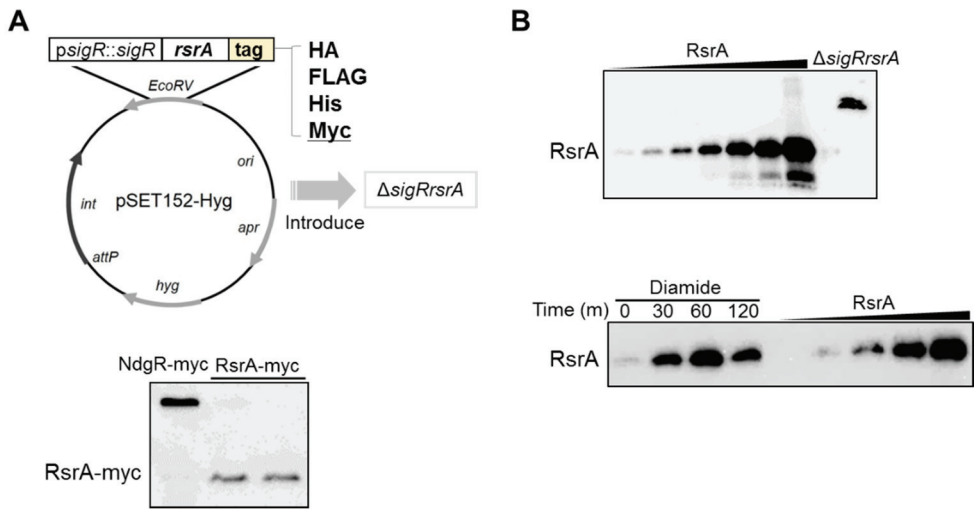


Figure III-1.6. RsrA is increased under thiol oxidative stress *in vivo*

A. Tagging strategy of x6 myc to RsrA C-terminal and western blot of RsrA-myc with αc-myc monoclonal antibody.

B. Western blot of RsrA with αRsrA-His poly-clonal rabbit antibody. Purified RsrA protein was detected with αRsrA antibody (upper). Wild type *S. coelicolor* cells treated with 0.5 mM diamide for 2 hours were detected with αRsrA antibody (lower).

III-2. Regulon of SigR in thiol oxidative stress response

III-2.1. S1 nuclease mapping confirms predicted SigR targets

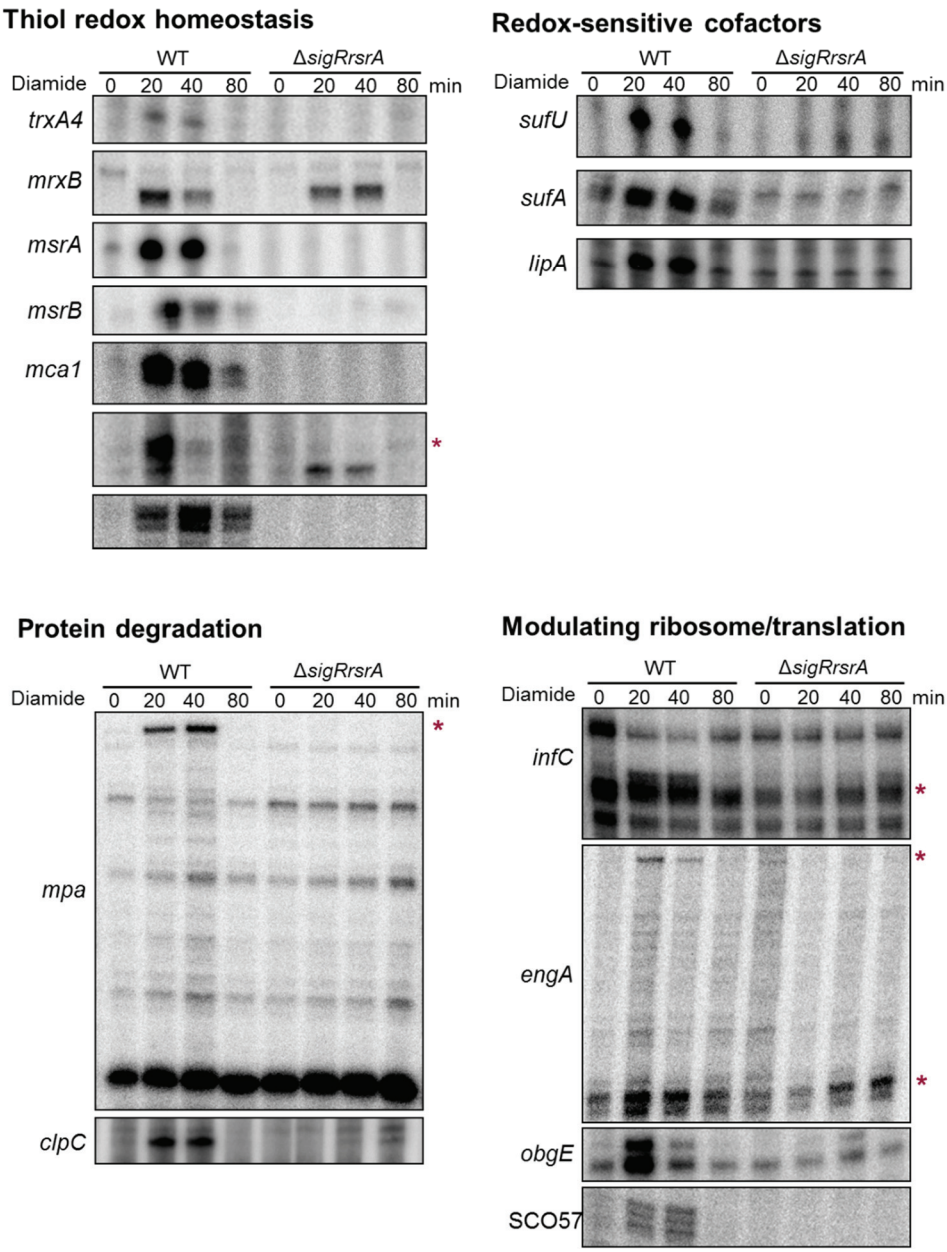
To identify SigR target sites in the *S. coelicolor* genome Kim performed a ChIP-chip experiment using anti-SigR serum with wild-type and Δ*sigR* mutant cell cultures treated with diamide to activate SigR activity. After subtracting non-specific signal, 122 regions were identified significantly enriched by the immuno-precipitation of SigR (P-value ≤ 0.05). Next, to identify more precisely putative SigR promoters, Yann searched within the enriched genomic regions for putative SigR promoters by detecting sequences that differed from the previously proposed SigR promoter (GGAAT-N18-GTT; (Paget et al., 2001) by at most 2 nucleotides with either of 2 possible spacer lengths (18 and 19 nucleotides) on either DNA strand. Only 5 of the 122 enriched regions did not contain at least one sequence matching criteria. Overall, 176 putative SigR promoter sequences were detected, and 108 of them are oriented towards annotated genes within 500 nt from the start codon. Considering operon structures (http://scocyc.streptomyces.org.uk), more than 163 genes are predicted to be under the direct control of SigR. Among these, 44 promoters have been previously reported to produce SigR-dependent transcripts by S1 mapping experiments (Kallifidas et al., 2010; Paget et al., 2001; Park and Roe, 2008) or microarray studies (Kallifidas et al., 2010). Re-examination of the transcriptional microarray data predicts SigR-dependent transcripts from a total of 62 promoters within 57 of the regions bound by SigR *in vivo* (Kallifidas et al., 2010). Therefore, ChIP-chip analysis revealed between 46 and 64 new SigR target promoters, undetectable from transcriptome profiling alone. The lack of detection in microarray alone could be due to low level of gene expression and/or additional transcription from one or more SigR-independent promoter (Kim et al., 2012).

When 108 SigR target operons were classified on the basis of known and predicted functions, many of them fall in the functional groups related with maintenance of thiol redox homeostasis and sulphur-containing amino acids (11 operons), protein folding and degradation (7 operons), and oxidoreductases (15 operons), fortifying previous proposals that the SigR regulon functions to achieve redox homeostasis and protein quality control under conditions of thiol-oxidative stress (Kallifidas et al., 2010; Paget et al., 2001; Park and Roe, 2008).

To provide additional experimental evidence for some of the newly identified SigR targets, I examined 25 selected transcripts by S1 nuclease mapping at different times after exposing *S. coelicolor* cells to the thiol oxidant diamide. I observed SigR-specific transcripts from 24 promoters whose 5′ ends match with the predicted location of the promoters. This supports the hypothesis that nearly all the SigR regulon genes produce SigR-dependent transcripts. I detected new SigR-dependent transcripts from 15 promoters whose expression has not been previously reported (SCO1084, 1600, 1648, 1758, 1936, 2194, 2254, 2595, 2763, 3296, 3442, 4797, 5705, 5820, 7784), confirming that ChIP-chip analysis detects SigR target genes with high sensitivity. Figure III.2.1 shows S1 mapping analysis of transcripts from these promoters. Some genes produce transcripts initiated primarily from SigR-dependent promoters (1084, 1920, 1936, 1958, 2161, 2194, 2537, 2763, 3091, 3373, 4956, 5705, 6061, 7632, 7784). Others utilize additional SigR-independent promoters (1600, 1758, 2254, 2595, 3296, 4797). Examination of genes with multiple promoters reveals diverse patterns of SigR-dependent modulation. For example, SCO1758 (*engA*) encoding a putative GTPase produces p1 transcript as predicted (250 nt upstream from the start codon; Table S1) and the downstream unpredicted p2 transcripts, whose promoter sequence (GGAT-N16-GTT; ggatcacccggtaaaggggtgtt) resembles the SigR consensus but with a shorter spacing of 16 nucleotides. This implies a flexibility of spacing between −35 and −10

elements of SigR-dependent promoters (Kim et al., 2012).

The p2 promoter of SCO1758 (*engA*) and SCO3442 (*mrxB*) (encoding a paralogue of mycoredoxin and a glutaredoxin-like protein) the SigR-induced transcripts still appear in the Δ*sigR-rsrA* mutant but with delayed response. A similar phenomenon was also observed for SCO3091 (*cfa*) encoding a putative cyclopropane-fatty acyl phospholipid synthase (Fig. III-2.1). The delayed induction kinetics in Δ*sigR-rsrA* mutant suggests that these SigR-dependent promoters may also be recognized by SigR paralogues that recognize similar promoter sequences as SigR. Similar induction pattern has been reported for transcripts encoding Lon protease, which are induced rapidly by SigR but with slower kinetics in the absence of SigR (Kallifidas et al., 2010). These observations indicate that a subset of SigR regulon genes can be recognized by at least one closely related SigR paralogue in addition to or in the absence of SigR (Kim et al., 2012).


Thiol redox homeostasis
WT
ΔsigRrsrA
Diamide
0 20 40 80 0 20 40 80 min
trxA4
mrxB
msrA
msrB
mca1
*
Redox-sensitive cofactors
WT
ΔsigRrsrA
Diamide
0 20 40 80 0 20 40 80 min
sufU
sufA
lipA
Protein degradation
WT
ΔsigRrsrA
Diamide
0 20 40 80 0 20 40 80 min
*
mpa
clpC
Modulating ribosome/translation
WT
ΔsigRrsrA
Diamide
0 20 40 80 0 20 40 80 min
infC
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engA
*
obgE
SCO57

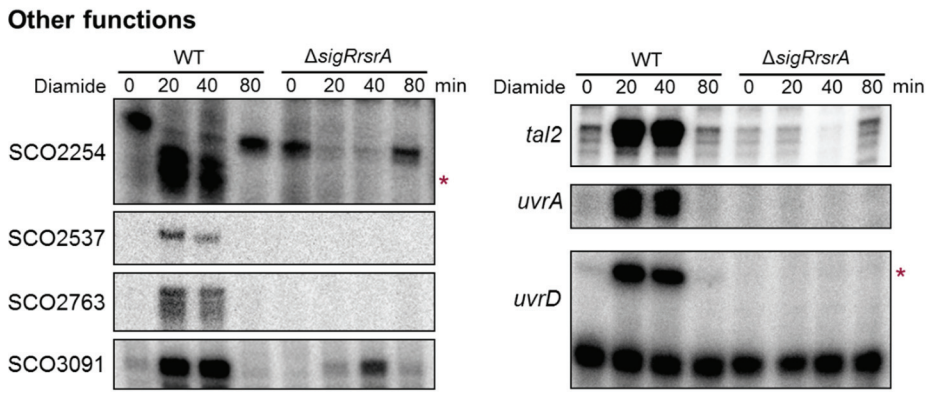


Figure III-2.1. Verification of newly identified targets by S1 mapping analysis

S1 nuclease mapping of selected SigR-dependent promoter regions. RNA samples were prepared from exponentially growing wild-type and Δ*sigRrsrA* cells treated with 0.5 mM diamide for 0, 20, 40 and 80 min. The bands with marked asterisks are from predicted SigR-binding promoters.

III-2.2. SigR maintains the level and activity of the housekeeping sigma factor HrdB during thiol-oxidative stress

The SigR regulon includes several known or predicted transcription factors (SCO1425, 1619, 2331, 2481, 2537, 3207, 3450, 5065, 5552, 6775, 7140), which may significantly increase the number of genes regulated in response to thiol oxidative stress. In addition, I found that the major housekeeping sigma factor HrdB (SCO5820) possesses a previously unrecognized SigR-dependent promoter (GGAAT-N18-GCT) about 50 nt downstream of the known SigR-independent promoter (p1) (Buttner et al., 1990) (Fig. III-2.2A). S1 mapping analysis of *hrdB* transcripts revealed that p1 is indeed the primary promoter in the absence of stress. However, diamide stress rapidly decreased the amount of p1 transcripts and increased the SigR-dependent transcripts from the predicted downstream p2 promoter (Fig. III-2.2B). The decrease in p1 transcripts upon oxidative stress occurred independently of SigR. This indicates that SigR functions to elevate hrdB transcription under oxidative stress conditions. Existence of a second promoter to compensate for loss of transcription from the primary promoter is also observed in SCO2254 encoding a putative efflux membrane protein (Fig. III-2.1), suggesting that this phenomenon may not be rare among SigR-regulated genes. Whereas some primary promoters such as p1 of SCO5820 (*hrdB*) and p1 of SCO2254 are affected dramatically by diamide treatment, the primary promoter p1 of SCO4797 (*uvrD*) is not affected at all. Therefore, the inhibitory effect of oxidative stress on transcription from a major p1 or housekeeping promoter may be a specific response at a subset of genes (Kim et al., 2012).

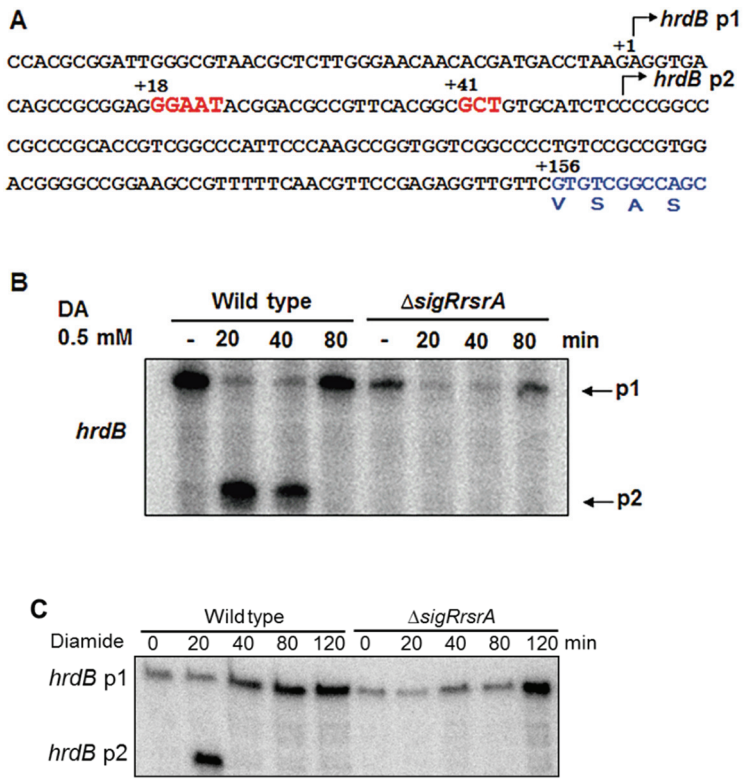


Figure III-2.2. SigR-dependent transcription activation of the *hrdB* gene encoding the major sigma factor in *S. coelicolor*.

A. The position of the SigR-dependent promoter p2 upstream of the *hrdB* coding region predicted from ChIP-chip and sequence pattern. The previously reported promoter p1 resides further upstream.

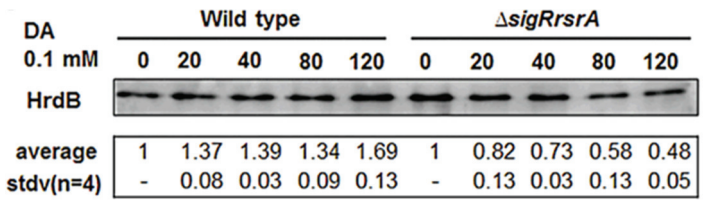
B. S1 nuclease mapping of *hrdB* transcripts from 0.5 mM diamide-treated cells revealing two alternative transcripts.

C. S1 nuclease mapping of *hrdB* transcripts from 0.1 mM diamide-treated cells.

To test whether SigR controls the amount of HrdB protein during oxidative stress, I monitored the amount of HrdB by western blot analysis. To ensure quantitative detection of HrdB, I diluted cell extract with lysis buffer containing BSA as a protein buffer that could minimize uncontrolled loss of proteins in cell extract during liquid handling and electrophoresis. Results in Fig. III-2.3 demonstrate that HrdB is maintained during oxidative stress condition. On the contrary, the level of HrdB decreased significantly in Δ*sigRrsrA* upon oxidative stress condition. Even though it is not clear why the level of HrdB increases in the wild type during the stress, it is obvious that SigR does contribute to maintain the level of HrdB, as predicted from transcript analysis.

I further monitored the activity of HrdB by examining transcripts from HrdB target promoters (*rrnD*p1, p3 and p4) in the ribosomal RNA operon *rrnD*, in the wild type and Δ*sigRrsrA*. All four promoters of the *rrnD* operon share the consensus sequence for HrdB recognition, especially in the −10 region, to varying extent (Baylis and Bibb, 1988; Hahn and Roe, 2007). In vitro transcription from *rrnD* promoters with reconstituted holoenzyme containing HrdB has demonstrated that all four promoters can be transcribed by HrdB, even though transcription from p3 and p4 promoters requires additional cellular factors (Hahn and Roe, 2007). I found that the amounts of the abundant p3 and p4 transcripts were maintained throughout the stress period and even increased up to 50% and 30%, respectively, during 40–80 min diamide treatment. On the other hand, they both decreased continuously in the Δ*sigRrsrA* to about 40% and 60% level, respectively, during the 2 h of stress (Fig. III-2.4). Transcripts from the p1 promoter decreased to 20% level during the stress in the mutant, whereas the level was maintained in the wild type (Fig. III-2.4). These results clearly demonstrate that SigR is necessary to maintain the level and activity of the major housekeeping sigma factor during thiol-oxidative stresses (Kim et al., 2012).

A



B

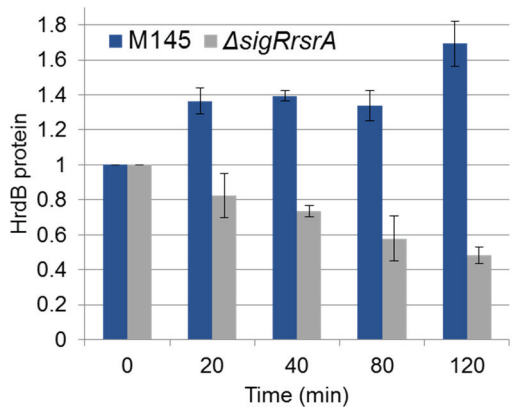
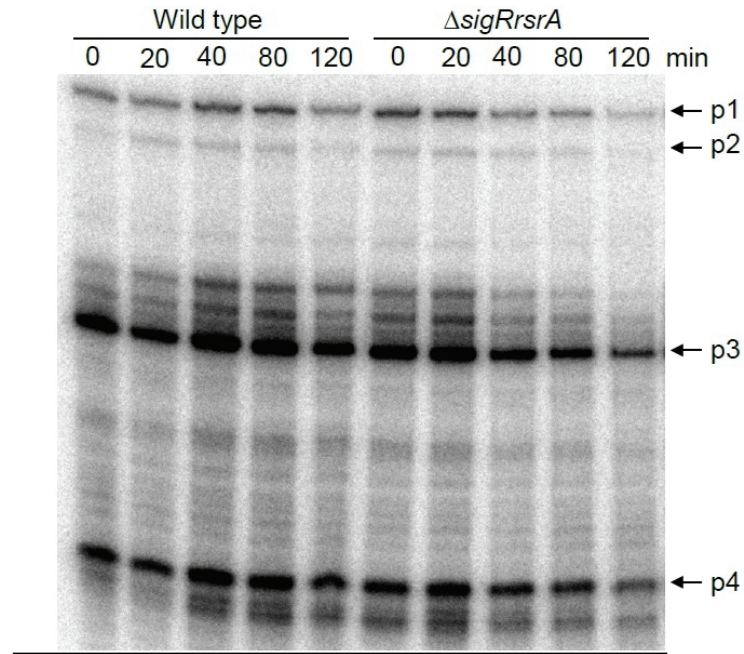


Figure III-2.3. SigR-dependent transcription activation of the *hrdB* gene maintains HrdB under thiol oxidative stress condition.

A. Western blot analysis of HrdB protein. Crude extracts from cells treated with diamide (0.1 mM) for up to 120 min were analysed to quantify the amount of HrdB protein as described in Experimental procedures. Results from four independent experiments were quantified to estimate changes in the level of HrdB protein upon oxidative stress, presented with the average value ± standard deviations (SD).

B. Bar graph of the western blot results in A.



p1	1	1.08 ±0.23	1.40 ±0.48	1.32 ±0.31	1.13 ±0.46	1	0.77 ±0.10	0.41 ±0.02	0.34 ±0.08	0.19 ±0.15
p3	1	1.09 ±0.27	1.42 ±0.47	1.50 ±0.50	1.28 ±0.50	1	1.00 ±0.12	0.59 ±0.01	0.53 ±0.17	0.36 ±0.16
p4	1	1.08 ±0.23	1.32 ±0.45	1.34 ±0.28	1.06 ±0.19	1	1.08 ±0.01	0.82 ±0.14	0.78 ±0.20	0.57 ±0.19

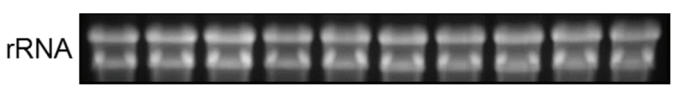


Figure III-2.4. Effect of *sigR* mutation on *rrnD* transcripts.

RNAs were prepared from the wild type and Δ*sigRrsrA* cells treated with 0.1 mM diamide for 0 to 120 min. Transcripts from *rrnD* promoters in each sample (1 μg RNA) were analyzed by S1 mapping. S1 protected bands from p1, p3, and p4 transcripts were quantified. Average values from three independent experiments were presented with standard deviations, relative the amount in non-treated samples.

III-2.3. The UvrA protein is necessary to cope with UV and thiol oxidative stresses

Among the newly discovered SigR targets SCO1958, SCO4797 and SCO5188, which encode putative UvrA, UvrD and a paralogue of UvrD (UvrD2) were predicted to function in the nucleotide excision repair system. The UvrABC endonuclease complex with UvrD (DNA helicase II) has been shown to repair UV-induced DNA damages, the most prominent of which are pyrimidine dimers (Sinha and Hader, 2002). However, whether the UvrABC system is needed under thiol-oxidative stress condition and whether thiol-oxidative stress causes DNA damage have not been known. Consensus SigR promoters (GGCAT-N18-GTT for *uvrA*, GGCAT-N18-GTT for *uvrD*, GGAAA-N19-GTC for *uvrD2*) are located 103, 31 and 7 nt upstream from the start codons of respective genes. The *uvrA* gene is transcribed at a very low level under unstressed conditions. However, upon diamide stress, it is drastically induced from a SigR-dependent promoter (Fig. III-2.1). On the contrary, the *uvrD* gene is transcribed at a relatively high level from a SigR-independent promoter p1 during unstressed condition. Upon induction, the upstream SigR-dependent promoter p2 is induced (Fig. III-2.1). The *uvrD*2 transcripts were not detectable by S1 analysis, probably due to low amount (Kim et al., 2012).

To examine whether UvrA indeed confers UV resistance, and whether SigR and UvrA have related functions in conferring resistance to UV and thiol-reactive compounds in *S. coelicolor*, sensitivity to UV and thiol-reactive compounds was analyzed. Fig. III-2.5 demonstrates that Δ*uvrA* has increased sensitivity to UV irradiation, and Δ*sigRrsrA* is also sensitive to UV, though not as dramatically as Δ*uvrA*. Therefore, SigR is necessary to achieve optimal UV resistance possibly through inducing UvrA system. The SigR regulon genes were not induced by UV

irradiation (Fig. III-2.6), suggesting that UV itself is not capable of activating SigR function. SigR can be activated by thiol-reactive oxidants/electrophiles such as diamide and methyl hydroquinone (MHQ), as predicted from the induction of thiol-specific oxidative stress response by these compounds in *B. subtilis* (Antelmann et al., 2008). Since some electrophiles can react not only with thiols in proteins and small molecules but also with DNA, I examined whether UvrA is needed to cope with chemical stresses caused by thiol-reactive electrophiles such as diamide, methyl hydroquinone (MHQ) and methyl glyoxal (MG). It has been reported that MG induces not only thiol-specific oxidative stress response but also SOS response, suggesting some DNA damage in *B. subtilis* (Nguyen et al., 2009). MG is known to cause DNA damage by reacting with DNA bases to form N-2-(1-carboxylethyl)-2′-deoxyguanosine (CEdG) (Synold et al., 2008). The result in Fig. III-2.5 shows that UvrA is needed to defend cells against these electrophiles. As expected, Δ*sigRrsrA* mutant became very sensitive to thiol-reactive electrophiles. Dramatic resistance of Δ*sigRrsrA* toward MG is unexpected. When the sensitivity of Δ*sigRrsrA* toward MG was monitored in liquid culture during early exponential phase, the mutant was more sensitive to MG than the wild type, suggesting that the resistance phenotype on plate culture could depend on treatment method. Overall, the finding that SigR induces DNA repair proteins expands our understanding of the function of SigR to accommodate response towards UV- and some electrophile-incurred damages (Kim et al., 2012).

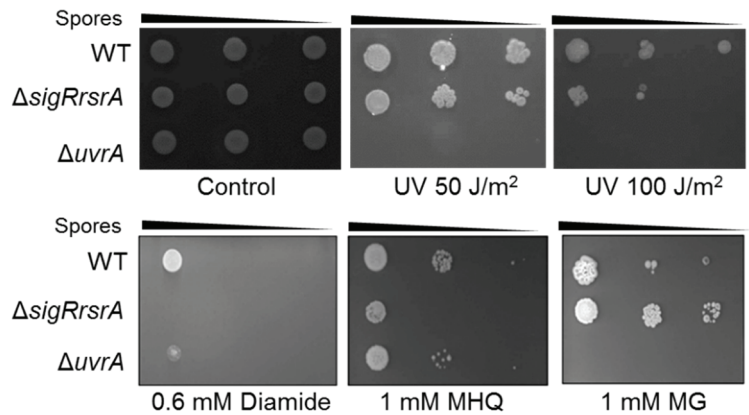


Figure III-2.5. Sensitivity of ΔuvrA and ΔsigRrsrA mutants to UV and thiol-reactive oxidants and electrophiles.

Serially diluted spores of wild-type (WT), Δ*sigRrsrA*, Δ*uvrA* strains were spotted on NA plates with or without added chemicals such as diamide (0.6 mM), methyl hydroquinone (MHQ, 1 mM) and methyl glyoxal (MG, 1 mM). For UV irradiation, the spotted plates were exposed to UV light from UV cross-linker for 30 seconds. Plates were incubated at 30°C for 1 (untreated), 2 (UV, diamide), 5 (MHQ) or 6 days (MG).

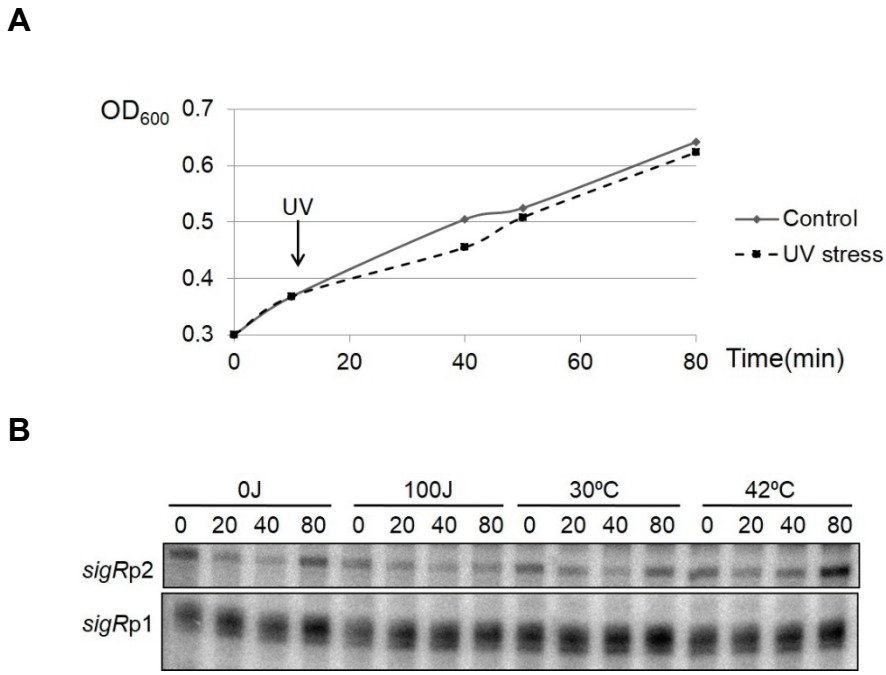


Figure III-2.6. UV 100J does not induce transcription of *sigR*

A. Growth of wild type cells treated with UV.

B. RNAs were prepared from the wild type cells treated with 100 J UV or 42℃ for 0 to 80 min. Transcripts from *sigR* promoters in each sample were analyzed by S1 mapping.

III-3. Role of SigR in antibiotic stress response

III-3.1. Induction of the *sigR* gene expression by translation-inhibiting antibiotics

While performing hygromycin-chase experiment to measure the half-life of σ^R and $\sigma^{R\square}$ proteins, Kim previously observed an increase in transcripts from the *sigR*p1 promoter (Kim et al., 2009). This observation was unexpected since the *sigR*p1 promoter was regarded as constitutive. I examined the effect of other antibiotics and compared it with that of thiol oxidant diamide. Fig. III-3.1B shows the induction profile of *sigR*p1 and *sigR*p2 transcripts after treatment with tetracycline (2 μg/ml) or diamide (0.5 mM) for up to 2 h. Similarly to hygromycin, *sigR*p1 transcripts increased significantly by about 10-fold in response to tetracycline, in a prolonged fashion. This contrasts with the transient induction of *sigR*p2 transcripts by diamide as previously observed (Kang et al., 1999; Paget et al., 1998). The antibiotic induction of *sigR*p1 transcription does not seem to be mediated by SigR itself, unlike *sigR*p2 transcription, since the induction occurred even in the Δ*sigR* mutant (MK1), where all transcriptions from the *sigR*p2 promoter disappeared (Fig. III-3.2) (Yoo et al., 2016).

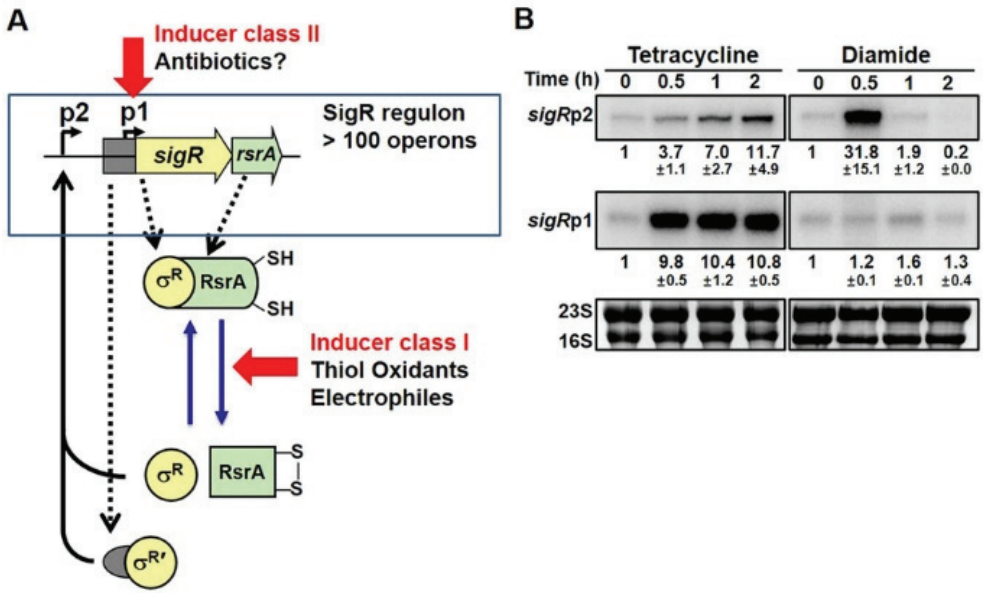

A
Inducer class II
Antibiotics?
p2
p1
sigR
rsrA
SigR regulon
> 100 operons
σR
RsrA
SH
SH
Inducer class I
Thiol Oxidants
Electrophiles
σR
RsrA
S
S
σR'
B
Tetracycline
Diamide
Time (h)
0
0.5
1
2
0
0.5
1
2
sigRp2
1
3.7 ±1.1
7.0 ±2.7
11.7 ±4.9
1
31.8 ±15.1
1.9 ±1.2
0.2 ±0.0
sigRp1
1
9.8 ±0.5
10.4 ±1.2
10.8 ±0.5
1
1.2 ±0.1
1.6 ±0.1
1.3 ±0.4
23S
16S

Figure III-3.1. Induction of *sigR*p1 transcription by antibiotics

A. The regulatory loops in activating SigR regulon. Two isoforms of SigR, σ^R and $\sigma^{R'}$, are produced from the two promoters of *sigR* gene, *sigR*p1 and *sigR*p2, respectively. Under cytoplasmic reducing environment, the reduced RsrA binds SigR (primarily the abundant σ^R), inhibiting SigR-directed transcription. Upon oxidative stress by thiol-oxidants such as diamide, di-sulfide bonds are formed in RsrA, and SigR is released from sequestration. The released SigR directs transcription of more than 100 genes (SigR regulon) that includes its own gene (from the upstream *sigR*p2 promoter). In contrast to σ^R that is very stable, $\sigma^{R'}$ with 55 more N-terminal amino acids is very unstable.

B. Difference between induction by thiol oxidant diamide and antibiotic tetracycline. The wild-type cells were sampled at 0, 30, 60 and 120 min after treatment with tetracycline (2 μg/ml, or 4.16 μM) or diamide (0.5 mM) for S1 nuclease mapping of *sigR*-specific RNAs. The rRNA in each RNA sample were resolved in parallel. Results from three independent experiments were quantified to present values for average fold induction with standard error of the mean (s.e.m.).

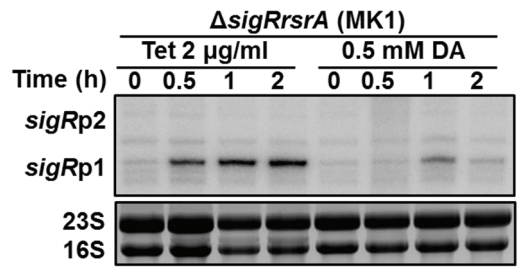


Figure III-3.2. Persistent induction of *sigR*p1 transcripts by tetracycline in the absence of SigR.

The *ΔsigRrsrA* (MK1) cells were sampled at 0, 0.5, 1, and 2 h after treatment with tetracycline (2 μg/ml) or diamide (0.5 mM), followed by S1 nuclease mapping. For each sample, 50 μg RNAs were analyzed for *sigR*-specific transcripts. Transcripts from *sigR*p2 promoter were below detection limit under all conditions. The *sigR*p1 transcripts were induced by tetracycline by about 4 to 6-fold during 0.5 to 2 h treatments. Ribosomal rRNAs in each RNA sample were presented below.

To investigate the induction of *sigR* mRNAs by antibiotics in further detail, I explored diverse antibiotics with different chemical structures and targets. Following 30 min treatments at varying concentrations, the *sigR* transcripts were monitored by S1 mapping. The results demonstrated that translation-inhibiting antibiotics such as chloramphenicol, erythromycin, and lincomycin all induced *sigR*p1 expression significantly (Fig. III-3.3A). Fusidic acid, hygromycin and streptomycin also induced *sigR*p1 transcripts (Fig. III-3.4). On the other hand, ampicillin, norfloxacin, and rifampicin that affects cell wall, DNA replication, and transcription, respectively, failed to increase transcripts from *sigR*p1 (Fig. III-3.3B). Rifampicin induced *sigR*p2 expression at 2 μg/ml, as observed previously in a different *S. coelicolor* strain M600 (Newell et al., 2006). Thus, the *sigR*p1 expression is induced specifically by translation-inhibiting antibiotics. Determination of growth inhibitory concentrations for treated antibiotics (Fig. III-3.5) indicated that the *sigR*p1 induction occurred at sub-inhibitory concentrations (Yoo et al., 2016).

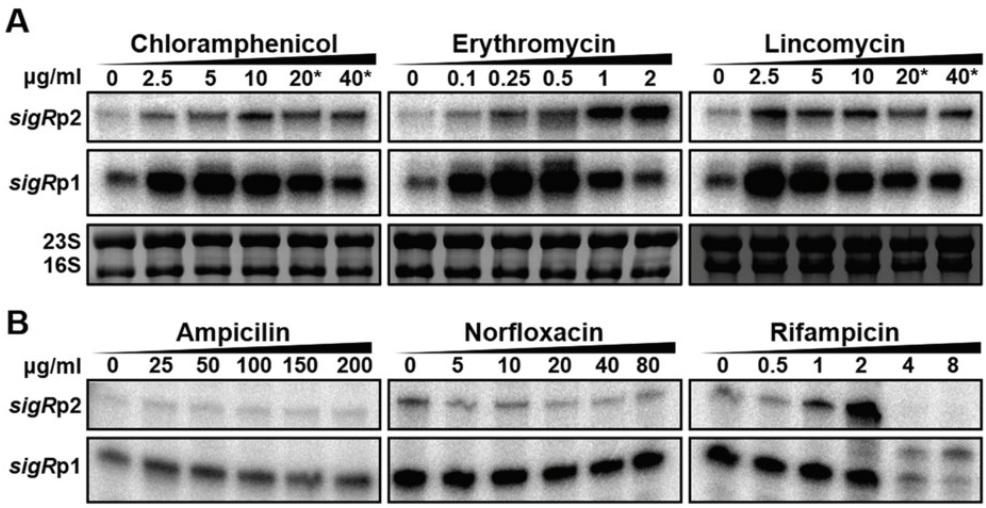


Figure III-3.3. Translation-inhibiting antibiotics induce *sigR*p1 transcription.

A. Effect of antibiotics that target translation. *S. coelicolor* cells were sampled at 30 min after treatments with chloramphenicol (0 to 40 μg/ml), erythromycin (0 to 2 μg/ml), or lincomycin (0 to 40 μg/ml). S1 nuclease protection assay for *sigR*-specific transcripts were done. The rRNA in each sample were presented as a control. The asterisk (*) denotes inhibitory concentration of the antibiotics (see Fig. III-3.5).

B. Effect of antibiotics that target other cellular processes; cell wall synthesis (ampicillin from 0 to 200 μg/ml), DNA replication (norfloxacin from 0 to 80 μg/ml), and transcription (rifampicin from 0 to 8 μg/ml). The *sigR*-specific RNA analysis was done as in panel (A).

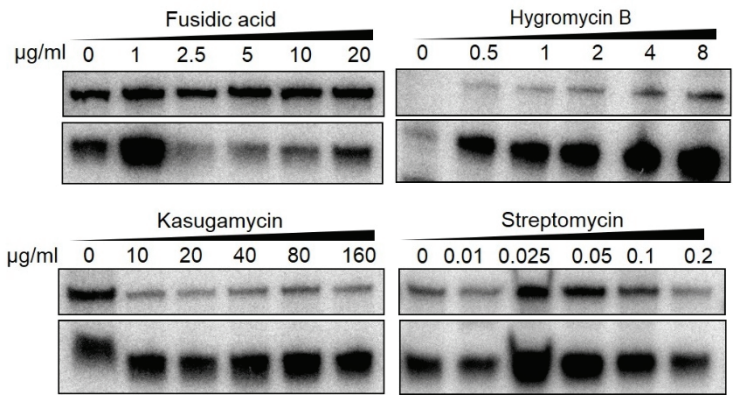


Figure III-3.4. Translation-inhibiting antibiotics induce *sigR*p1 transcription.

S. coelicolor cells were sampled at 30 min or 1 hour after treatments with fusidic acid (0 to 20 μg/ml), hygromycin B (0 to 8 μg/ml), kasugamycin (0 to 160 μg/ml) or streptomycin (0 to 0.2 μg/ml). S1 nuclease protection assay for *sigR*-specific transcripts were done.

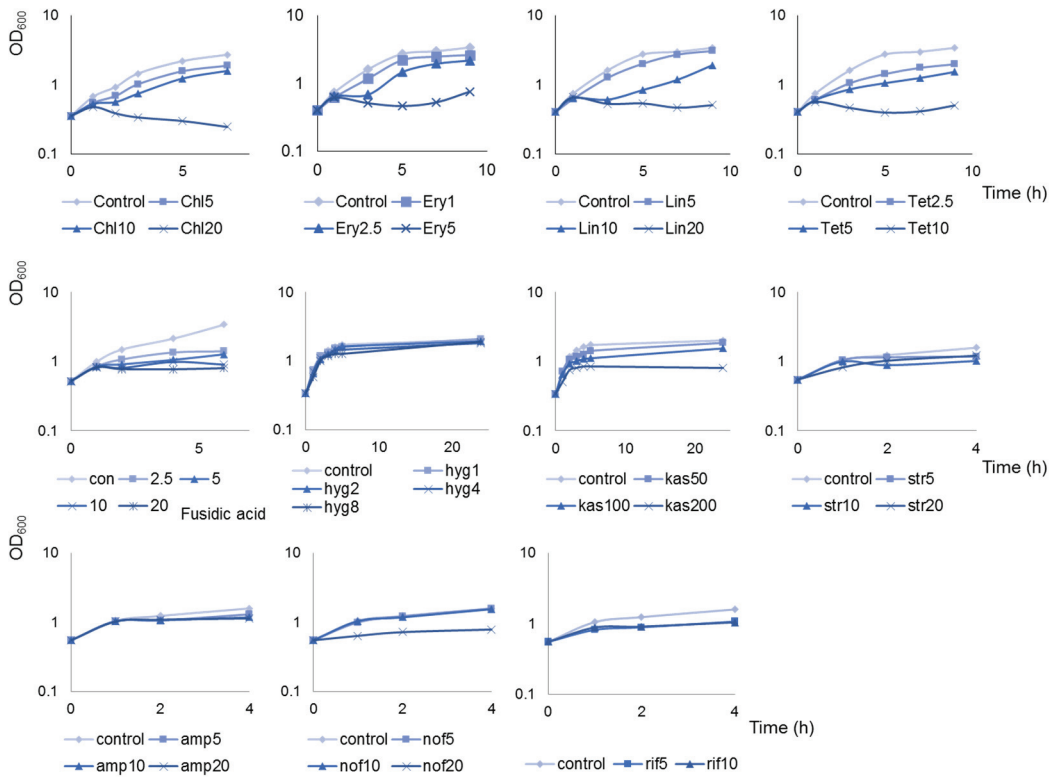


Figure III-3.5. Inhibitory concentration ranges of antibiotics.

Growth of *S. coelicolor* M145 cells in YEME was monitored by OD_{600}. At OD_{600} of ~0.4, varying concentrations of antibiotics were added to the culture medium, and the growth was monitored for up to 10 h. The amounts of antibiotics treated was indicated in μg/ml; Chl for chloramphenicol, Ery for erythromycin, Lin for lincomycin, Tet for tetracycline, Hyg for hygromycin, Kas for kasugamycin, Str for streptomycin, Amp for ampicillin, nof for nofloxacin and rif for rifampicin.

III-3.2. Antibiotic treatment increases SigR protein and steadily induces target gene expression

Whether the increase in *sigR*p1 transcripts leads to increased SigR protein level in the presence of translation-inhibiting antibiotics was then examined. Analytical Western blot analysis with anti-SigR antibody revealed that erythromycin (0.25 μg/ml) increased the level of SigR, but not SigR' protein, continuously for up to 2 h (Fig. III-3.6A). It contrasts with the effect of thiol oxidant diamide which increased the amount of SigR' transiently by about 12-fold, without affecting the level of SigR (Fig. III-3.6A).

Parallel detection of known amounts of SigR protein enabled the estimation that SigR increased steadily by erythromycin to about 3-fold level at 2 h after treatment compared with the untreated level. The basal amounts of σ^R and $\sigma^{R'}$ proteins under non-treated condition were estimated to be about 23 (1.82 μM) and 7 (0.56 μM) fmole/μg proteins in cell extracts, respectively, assuming equal immune-specificity of σ^R and $\sigma^{R'}$ proteins to the antibody used. This corresponds to about 1.8 and 0.6 μM in the cell for σ^R and $\sigma^{R'}$, respectively, assuming that about 43% of dry cell weight is from the protein, and that the wet cell weight is about 5.6 fold of the dry weight, and that cell density is 1 (Shahab et al., 1996).

Following erythromycin treatment, there appeared a non-specific band which is absent in other antibiotic-treated samples (NS in Fig. III-3.6A). Source of this protein band is not certain, except that it is not the product of the *sigR* gene, since it is observed in the *ΔsigR* mutant after erythromycin treatment. Treatments with chloramphenicol, lincomycin, and tetracycline caused similar increase in σ^R without changing the amount of $\sigma^{R'}$ (Fig. III-3.6B). No increase in $\sigma^{R'}$ by antibiotics in spite of some increase in *sigR*p2 transcripts could be due to the unstable nature of $\sigma^{R'}$ (Kim

et al., 2009).

I then examined the expression of a SigR-target gene *trxB* (SCO3890), which encodes thioredoxin reductase. Fig. III-3.6C shows that the SigR-dependent *trxB*p1 transcripts increased significantly by chloramphenicol and tetracycline treatments up to 80 min, consistent with the steady increase in σ^R protein. Therefore, I conclude that the translation-inhibiting antibiotics induce the production of stable σ^R protein, which subsequently induces its target gene expression in a prolonged fashion (Yoo et al., 2016).

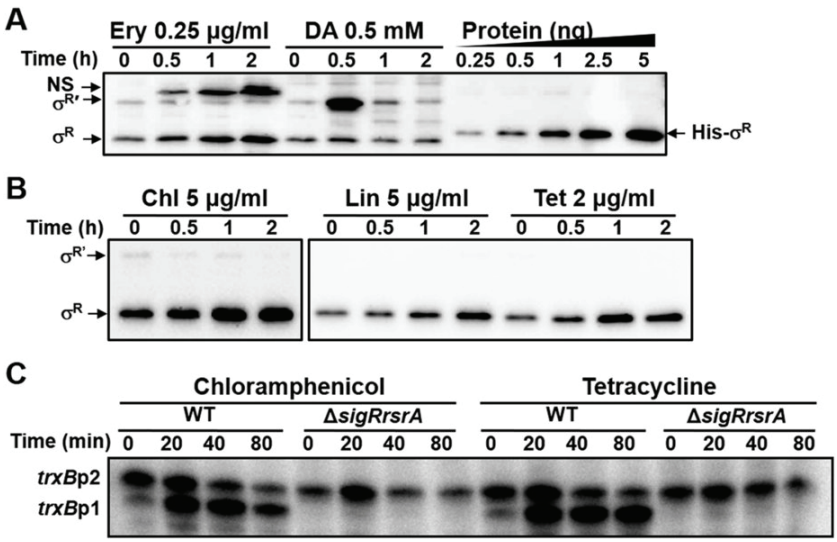


Figure III-3.6. Steady increase in SigR protein by antibiotic treatments and prolonged induction of its target promoter.

A. Steady vs. transient increase in SigR proteins by antibiotic or thiol oxidant. Cells were treated with either erythromycin (0.25 μg/ml) or diamide (0.5 mM) for up to 2 h, followed by western blot analysis with antibody against SigR. The positions of σ^R and $\sigma^{R'}$ were marked by arrows. A non-specific band (NS) produced in erythromycin-treated samples was also indicated. Analytical western blotting of indicated amounts of purified SigR (His-σ^R; from 0.25 to 5 ng) was done in parallel to quantify the amount of SigR-specific protein bands.

B. Western blot analyses of SigR proteins following treatments with chloramphenicol, lincomycin, and tetracycline for 2 h.

C. S1 mapping analysis of *trxB* transcripts. The wild-type and *ΔsigRrsrA* cells were treated with chloramphenicol (17 μg/ml) or tetracycline (5 μg/ml) for up to 80 min, and analyzed for *trxB* transcripts. The *trxB*p1 promoter is under the control of SigR.

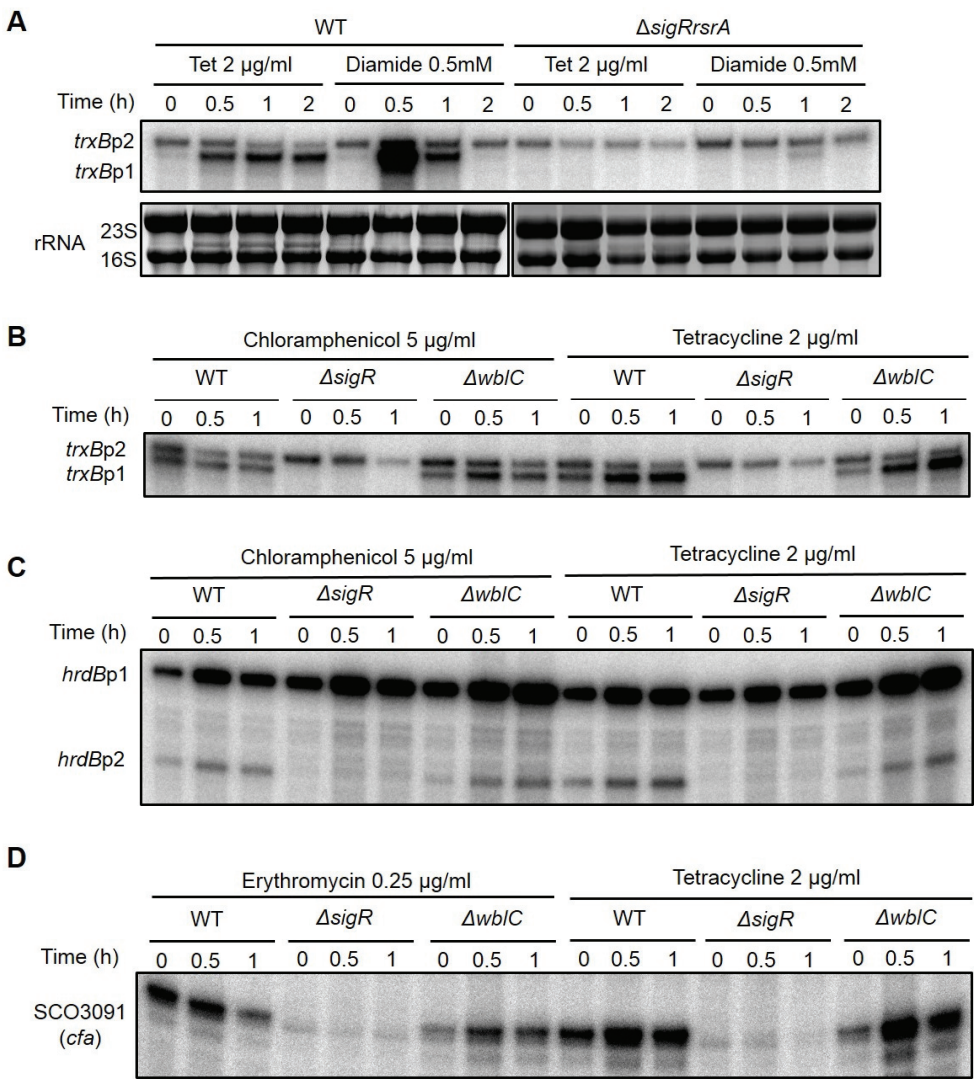


Figure III-3.7. Induction of SigR target promoters by antibiotic treatments.

S1 mapping analysis of SigR target transcripts. The wild-type, Δ*sigRrsrA* and Δ*wblC* cells were treated with tetracycline (2 μg/ml), chroramphenicol (5 μg/ml), erythromycin (0.25 μg/ml) or 0.5 mM diamide for up to 2 hours, and analyzed for *trxB* (A and B), *hrdB* (C) and *cfa* (D) transcripts.

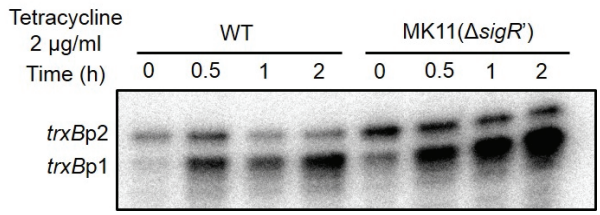


Figure III-3.8. Induction of *trxB*p1 by antibiotic treatments in absence of SigR'.

S1 mapping analysis of *trxB* transcripts. The wild-type and MK11 (*ΔsigR'*) cells were treated with tetracycline (2 μg/ml) for up to 2 hours, and analyzed for *trxB* transcripts. The *trxB*p1 promoter is under the control of SigR.

III-3.3. Antibiotic induction of stable SigR depends on WblC/WhiB7

To find clues to reveal mechanisms behind antibiotic induction of *sigR*p1, I scrutinized its flanking sequences. One prominent feature was a stretch of AT-rich sequence, which is not common in GC-rich actinomycetous genomes, located immediately upstream of the -35 region of the *sigR*p1 promoter (Fig. III-3.9A). The sequence feature is present upstream of the *whiB7* promoter in *Mycobacterium* species, and has been proposed as the binding site of a WhiB-like (Wbl) protein WhiB7 (Burian et al., 2012b; Burian et al., 2013). In *S. coelicolor,* WblC (SCO5190) is the orthologue of WhiB7 of *M. tuberculosis*, and the *wblC* gene also has a putative auto-regulatory WblC-binding signature similarly to the *whiB7* gene of *M. tuberculosis* (Fig. III-3.9A). *wblC* and *whiB7* mutants were reported to be hypersensitive to diverse antibiotics in *S. lividans, S. coelicolor,* and *M. tuberculosis* (Fowler-Goldsworthy et al., 2011; Morris et al., 2005). Inspection of the promoter region of *sigR*-homologous genes (*sigE* and *sigH*) in *M. tuberculosis* H37Rv also revealed the presence of putative WhiB7-binding sites immediately upstream of the promoters (Cortes et al., 2013) (Fig. III-3.9A).

I investigated whether WblC is involved in inducing transcription from the *sigR*p1 promoter upon antibiotic treatment. The wild type and the Δ*wblC* mutant cells (Fowler-Goldsworthy et al., 2011) were treated with tetracycline for up to 3 h, and examined for *sigR*-specific transcripts and their protein products by S1 mapping and Western blot analyses, respectively. Results in Fig. III-3.9B demonstrated that WblC is critically required for the antibiotic induction of *sigR*p1 transcription. The *sigR*p2 transcription, however, was induced by tetracycline regardless of the *wblC* mutation. Immunoblot analysis revealed that the σ^R protein produced from the *sigR*p1 transcripts did not increase in Δ*wblC* mutant, in contrast to the wild type, where σ^R

protein increased about 2.5-fold during the 2 to 3 h treatments with tetracycline (Fig. III-3.9C). It clearly shows that the increase in stable σ^R after antibiotic treatment depends almost entirely on WblC/WhiB7 (Yoo et al., 2016).

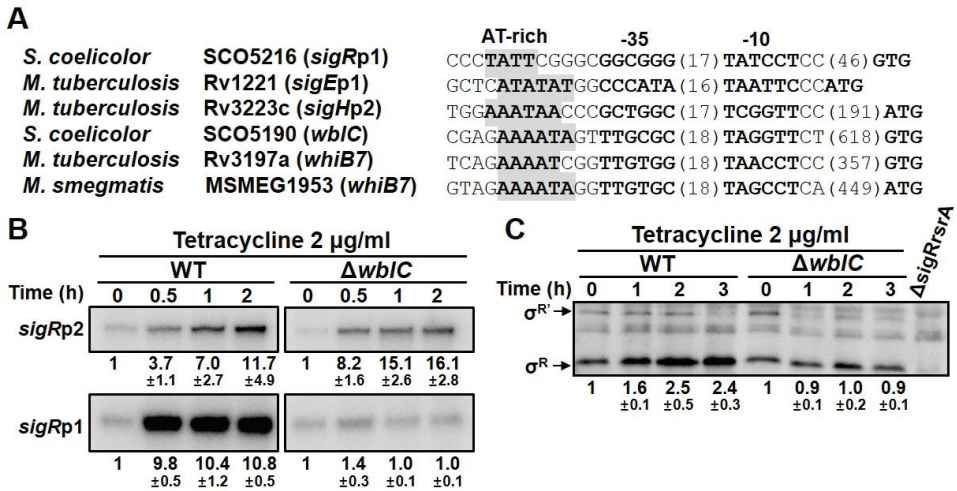


Figure III-3.9. Antibiotic induction of *sigR*p1 transcription and σ^R production depends on WblC/WhiB7.

A. The presence of AT-rich sequence adjacent to the -35 element of promoters for *sigR*p1 in *S. coelicolor* and its homologous genes (*sigE* and *sigH*) in *M. tuberculosis*. The AT-rich sequences upstream of the *wblC* in *S. coelicolor*, and the *whiB7* promoters known to bind WhiB7 protein in mycobacteria, were also presented.

B. Antibiotic induction of *sigR*p1 transcripts depends on WblC/WhiB7. S1 nuclease mapping was done, following treatment of the wild type and Δ*wblC* cells with 2 µg/ml tetracycline for up to 2 h. Results from three independent experiments were quantified to present average values for relative fold change and s.e.m.

C. Western blot analysis of SigR proteins from cells similarly treated as in panel B. Results from three independent experiments were quantified.

III-3.4. Antibiotics increase the amount and the binding of WblC to *sigRp1* promoter *in vivo.*

I then investigated how WblC is involved in antibiotic induction of *sigR*p1 or σ^R. For this purpose, polyclonal antibodies against WblC were raised in rabbits, and used to monitor WblC in cells treated with antibiotics. Fig. III-3.10A shows that the amount of WblC dramatically increased within an hour of erythromycin or tetracycline treatments. The WblC level decreased within 2 h of antibiotic treatment. The decrease at 2 h is more pronounced in erythromycin than tetracycline treated samples. With some slight differences in induction and shut-off kinetics, WblC was induced by other antibiotics such as hygromycin, chloramphenicol, and lincomycin to a maximal level within an hour, and then returned to the basal level within 2 or 3 h (Fig. III-3.10B) (Yoo et al., 2016).

Whether WblC binds directly to the *sigRp1* region *in vivo* was determined by chromatin immunoprecipitation. The wild type and the *ΔwblC* cells were treated with tetracycline (2 μg/ml) for 1 h, and immunoprecipitation with anti-WblC antibody. The amount of *sigR*p1 promoter DNA in the precipitate was estimated by quantitative real-time PCR, along with primer sets for the upstream *sigR*p2 or downstream *rsrA* regions. Fig. III-3.10C demonstrates that tetracycline increased WblC binding to the *sigR*p1 promoter region (from -84 to +7 nucleotide position, relative to the transcription start site of *sigR*p1) by more than 10-fold in the wild type cell, whereas no increased binding was observed in the *ΔwblC* mutant. In comparison, no significant binding of WblC to the *sigR*p2 or *rsrA* regions was observed following tetracycline treatments. Therefore, It is clear that the antibiotic treatments increase the amount of WblC, which specifically binds to the *sigR*p1 promoter region and mediates increased expression of σ^R (Yoo et al., 2016).

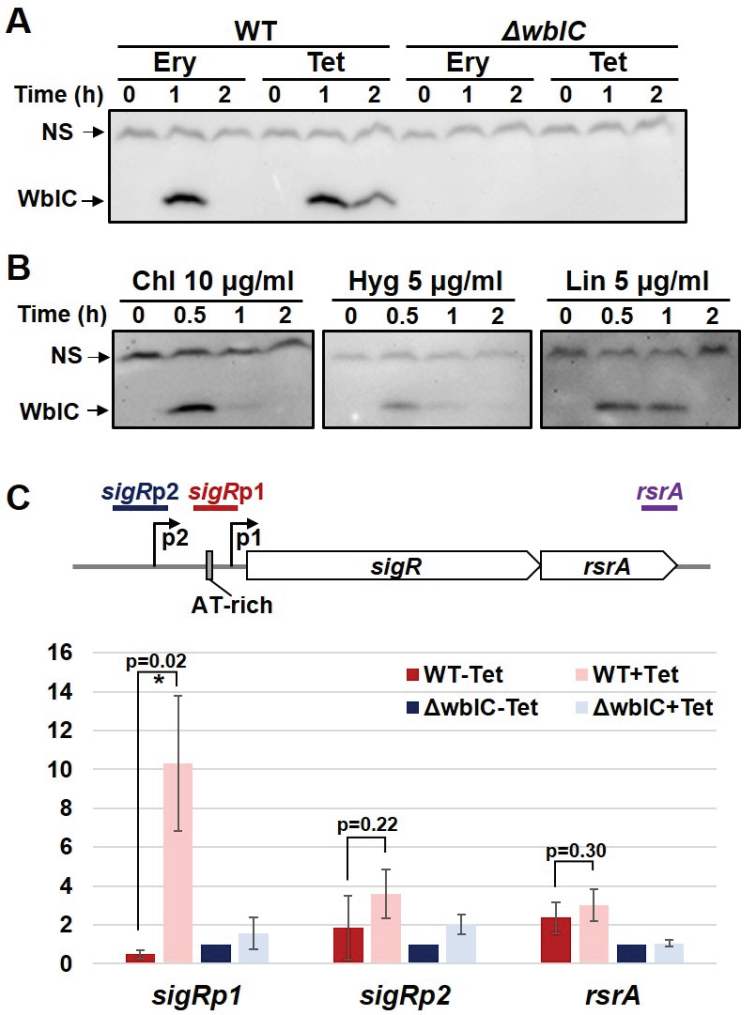

A
WT
ΔwblC
Ery
Tet
Ery
Tet
Time (h)
0 1 2 0 1 2 0 1 2 0 1 2
NS
WblC
B
Chl 10 μg/ml
Hyg 5 μg/ml
Lin 5 μg/ml
Time (h)
0 0.5 1 2
0 0.5 1 2
0 0.5 1 2
NS
WblC
C
sigRp2
sigRp1
rsrA
p2
p1
sigR
rsrA
AT-rich
16
14
12
10
8
6
4
2
0
p=0.02
*
p=0.22
p=0.30
WT-Tet
WT+Tet
ΔwblC-Tet
ΔwblC+Tet
sigRp1
sigRp2
rsrA

Figure III-3.10. Increase in the amount of WblC protein and its binding to the *sigR*p1 promoter *in vivo* upon antibiotic treatments.

A. Western blot analysis of WblC. The wild-type and Δ*wblC* cells were sampled after antibiotic treatments; Ery for 0.25 μg/ml erythromycin, and Tet for 2 μg/ml tetracycline. The WblC-specific band was detected slightly below the 15 kDa marker, coinciding with its predicted size (13.2 kDa). NS denotes non-specific band.

B. Western blot analysis of WblC in wild type cells treated with other translation-inhibiting antibiotics; Chl for 10 μg/ml chloramphenicol, Hyg for 5 μg/ml hygromycin, and Lin for 5 μg/ml lincomycin.

C. Chromatin immunoprecipitation with anti-WblC polyclonal antibody followed by q-PCR with gene-specific primer sets for *sigR*p1, *sigR*p2, and *rsrA* genes. The wild-type and Δ*wblC* cells were processed for immunoprecipitation after 1 h treatment with or without 2 μg/ml tetracycline. The enrichment of each region was estimated by quantitative real-time PCR. The relative average fold with s.e.m. were presented (y-axis), by taking the value for untreated Δ*wblC* sample as 1. The asterisk (*) denotes $p < 0.05$ by Student's t-test (n=3).

III-3.5. SigR confers resistance to translation-inhibiting antibiotics

On the basis of induction by antibiotics, I hypothesized that the *sigR* gene functions in conferring resistance to antibiotics in *S. coelicolor*. So far, the revealed phenotypes of *ΔsigR* mutant are the sensitivity to thiol oxidant diamide (Paget et al., 1998), sensitivity to electrophiles (Park JH, unpublished), and increased protein aggregation in cell extracts that reflects decreased protein quality control (Kallifidas et al., 2010). To assess antibiotic sensitivity, I spotted an equal number of spores from the wild type, *ΔsigR, ΔwblC,* and *ΔsigR* complemented with the chromosomally integrated *sigR* gene, on plates containing various antibiotics. Fig. III-3.11 shows that the *ΔsigR* and *ΔwblC* mutations do not cause sensitivity toward non-inducing antibiotics such as ampicillin, norfloxacin, or rifampicin. However, as predicted, the *ΔsigR* mutant was more susceptible to inducing antibiotics such as chloramphenicol, erythromycin, lincomycin, and tetracycline. The sensitivity was restored to the wild type level by complementation with the wild type *sigR* gene. The *ΔwblC* was more susceptible than the *ΔsigR* mutant to the inducing antibiotics except chloramphenicol. These results demonstrate that the *sigR* gene does play a critical role in ensuring cell viability in the presence of translation-inhibiting antibiotics (Yoo et al., 2016).

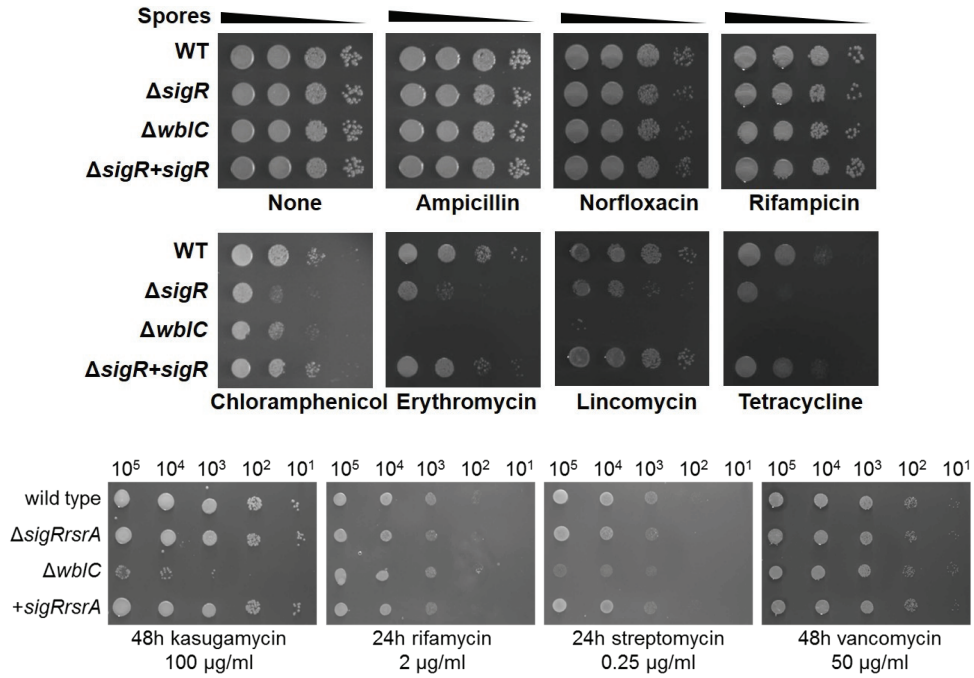


Figure III-3.11. SigR confers resistance to translation-inhibiting antibiotics.

An equal number of spores of the wild-type, *ΔsigR*, *ΔwblC*, and *ΔsigR+sigR* complemented strains were serially diluted by 10-fold and spotted on NA plates with or without antibiotics. Concentrations of antibiotics in the plates were 20 μg/ml ampicillin, 1 μg/ml norfloxacin, 2 μg/ml rifampicin, 10 μg/ml chloramphenicol, 2 μg/ml erythromycin, 10 μg/ml lincomycin, or 2 μg/ml tetracycline. Plates were incubated for 40 to 72 h.

III-3.6. Induction of *sigR*-homologous genes (*sigE* and *sigH*) by antibiotics in *M. tuberculosis*

M. tuberculosis (Mtb) has two close homologs of SigR from *S. coelicolor* (ScoSigR); SigE (Rv1221; MtbSigE) and SigH (Rv3223c; MtbSigH) with 37% and 72% identity, respectively (Fig. III-3.12). SigH is known to regulate the thioredoxin system and heat shock proteins upon oxidative and heat stresses (Raman et al., 2001; Sharp et al., 2016). SigE plays a role in response to oxidative and cell envelop stresses (Manganelli et al., 2001).

The presence of predicted WblC binding sites in the promoter regions of *sigE* and *sigH* (Fig. III-3.9A) led me to examine the expression of these genes in Mtb upon antibiotic treatments. Mtb H37Rv cells were treated with 1 μg/ml each of erythromycin, streptomycin, or tetracycline for up to 3 days and the RNA was examined. Fig. III-3.13 demonstrates the results of S1 nuclease mapping of transcripts from the *sigE* (panel A) and *sigH* (panel B) genes. For Mtb_*sigE* gene, I detected transcripts from the two promoters (transcription start sites) as have been reported (Cortes et al., 2013). The *sigE*p1 promoter contains the WhiB7-binding motif and produces leaderless mRNA (Fig. III-3.9A). Upstream promoter *sigE*p2 does not have WhiB7-binding motif but contains the promoter sequence feature recognizable by MtbSigE or MtbSigH (Song et al., 2008). I found that the *sigE*p1 transcripts increased significantly by all three antibiotics (Fig. III-3.13A). Transcripts from *sigE*p2 increased also by antibiotic treatments, but by less pronounced fold of induction (Yoo et al., 2016).

For Mtb_*sigH* gene, I detected transcripts from two promoters; one from the downstream *sigH*p1 as reported previously (Cortes et al., 2013) and the other from the upstream *sigH*p2 recognizable by MtbSigH (Fernandes et al., 1999; Raman et al., 2001). A WhiB7-binding motif is present in the *sigH*p2 promoter (Fig. III-3.9A).

Results in Fig. III-3.13B show that both *sigH*p1 and *sigH*p2 transcripts increased by antibiotics, even though not as much as the *sigE* transcripts. Based on these observations, I can predict that similar pathways of upregulating SigR-like sigma factors by antibiotics are present in *M. tuberculosis*, and MtbSigE may play a more significant role in orchestrating response against translational blocking antibiotics (Yoo et al., 2016).

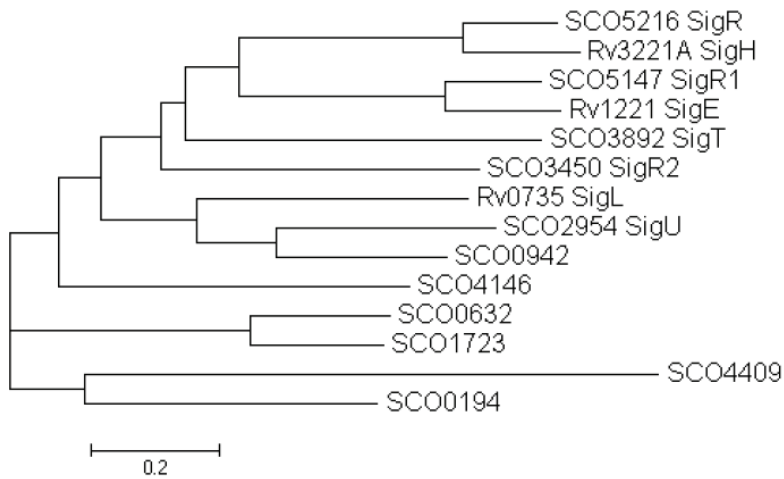


Figure III-3.12. Phylogeny of ZAS-linked ECF Sigma factors from *S. coelicolor* and *M. tuberculosis*

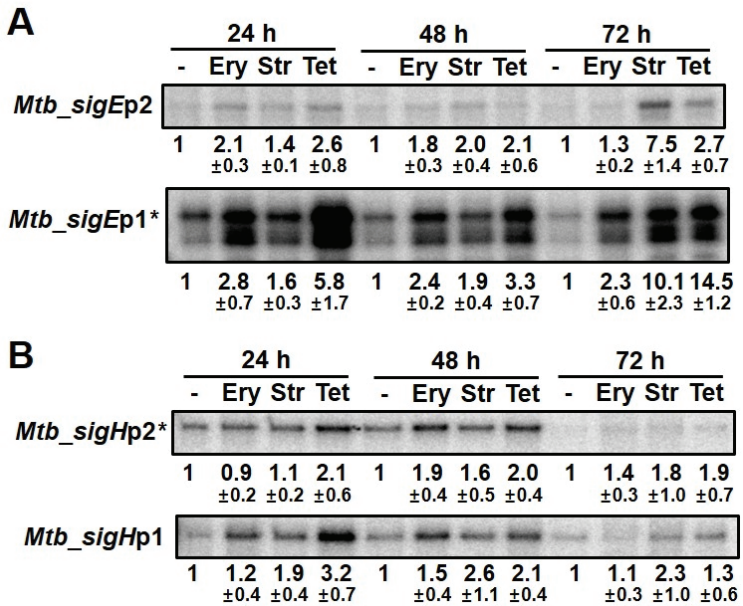


Figure III-3.13. Induction of *sigR*-homologous gene expression by antibiotics.

Transcripts from the *sigE* (panel A) and *sigH* (panel B) genes of *Mtb* H37Rv were analyzed by S1 mapping. RNAs were obtained from cells grown in Middlebrooks 7H9 broth, and either non-treated or treated with 1 μg/ml of antibiotics for 24, 48, and 72 h: Ery, erythromycin; Str, streptomycin; Tet, tetracycline. Results from more than four independent experiments (n=4 for *sigE* and n=6 for *sigH*) were quantified to estimate changes in the level of transcripts, taking the level of untreated sample as 1. The average fold changes with s.e.m. were presented for transcripts from the p1 (downstream) and p2 (upstream) promoters of *sigE* and *sigH* genes. The *sigE*p1 and *sigH*p2 promoters (marked with *) contain putative WhiB7 binding motifs.

III-4. Role of WblC in antibiotic stress response

III-4.1. Induction of *wblC* transcription by antibiotics

In *Mycobacterium*, the transcriptioin of *whiB7* is induced by auto-regulation (Burian et al., 2012a). In *S. coelicolor,* three promoters (transcription start sites) of *wblC* have been reported (Jeong et al., 2016; Romero et al., 2014) (Fig. III-4.1). The upstream promoter sequence of *S. coelicolor wblC* also contains the WblC/WhiB7 consensus for auto-regulation. Downstream promoter *wblC*p2 and *wblC*p3 do not have the WhiB7-binding motif but contain the promoter sequence feature recognizable by SigR (Fig. III-4.1).

The amount of *wblC*-specific mRNA was analyzed by S1 nuclease mapping, using primer sets which encompass the *wblC* promoter region: probe 1 from -840 to -438 nt position (Fig. III-4.2), probe 2 from -691 to -377 nt position (Fig. III-4.2), probe 3 from -750 to -168 nt position (Fig. III-4.3) and probe 4 from -691 to +50 nt position (Fig. III-4.4), relative to the *wblC* start codon. I found that the *wblC*p1p2p3 transcripts increased by antibiotics.

A

TSS	Position	Reference (TSS position ±4)	Promoter sequence
1	5647134	Vockenhuber *et al.*, Romero *et al.* Jeong *et al.*	CGAGAAAATAGTTTGCGCATGCCCGGGGAATCCCCATAGGTTCTTGGTCA
2	5647184	Romero *et al.*	CGGGAACAGCGGCCTTCCCGGAGGCCCTGAATCCGTGTTGTACTTGCATA
3	5647534	Jeong *et al.*	CCGGTGCCGGATTCCGGAAGCAGACGACGCAGCACCACCTGATGTGGGCC

B

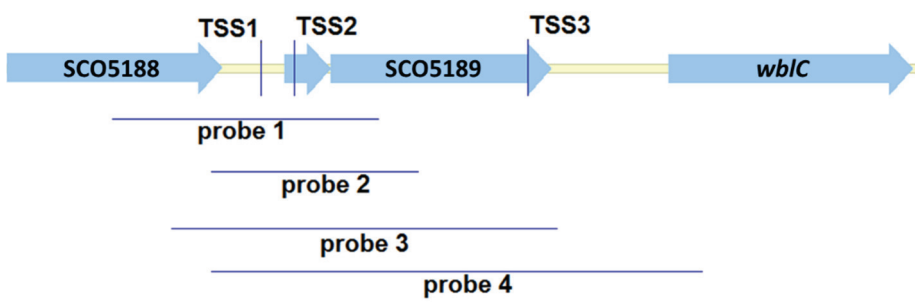
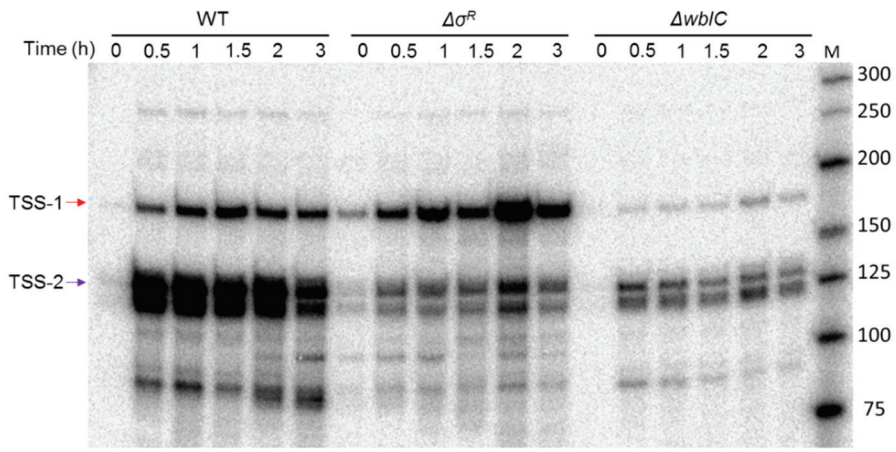


Figure III-4.1. Three transcription start sites of *wblC*.

A. Three transcription start sites of *wblC* and the promoter sequence. Promoter of TSS-1 shows some feature of the WblC consensus.

B. Four probes for detecting transcripts of *wblC*; probe 1 (402 bps, from -840 to -438 nt position relative to the *wblC* start codon), probe 2 (314 bps, from -691 to -377 nt position relative to the *wblC* start codon), probe 3 (582 bps, -750 to -168 nt position relative to the *wblC* start codon) and probe 4 (741 bps, -691 to +50 nt position relative to the *wblC* start codon).

A



B

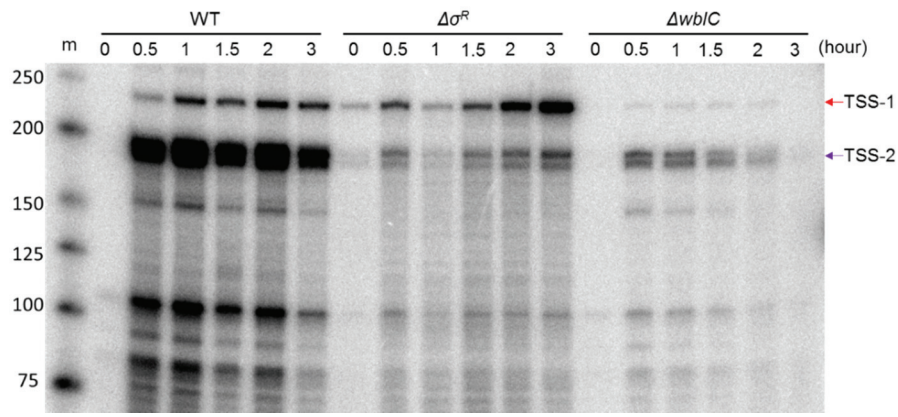


Figure III-4.2. Transcription of *wblC*: probe1 and probe2.

A. S1 mapping analysis using probe 1 (402 bps, from -840 to -438 nt position relative to the *wblC* start codon). Wild type, Δ*sigRrsrA* and Δ*wblC* were treated hygromycin (5 μg/ml) for 3 hours and the RNA samples were used for S1 mapping analysis.

B. S1 mapping analysis using probe 2 (314 bps, from -691 to -377 nt position relative to the *wblC* start codon).

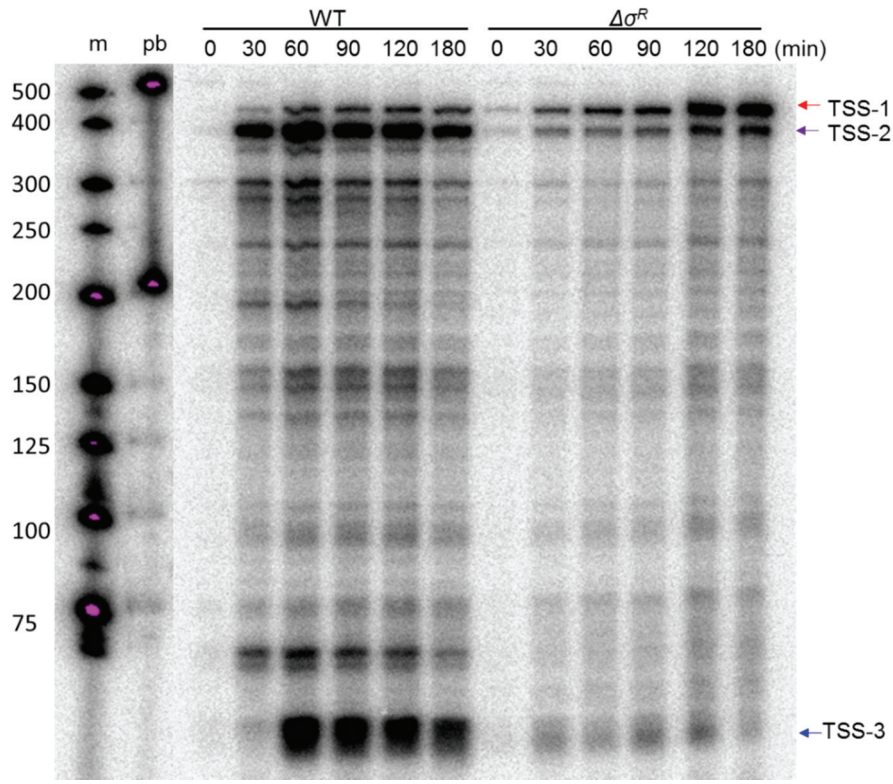


Figure III-4.3. Transcription of *wblC*: probe3.

S1 mapping analysis using probe 3 (582 bps, -750 to -168 nt position relative to the *wblC* start codon). Wild type and Δ*sigRrsrA* were treated hygromycin (5 μg/ml) for 3 hours and the RNA samples were used for S1 mapping analysis.

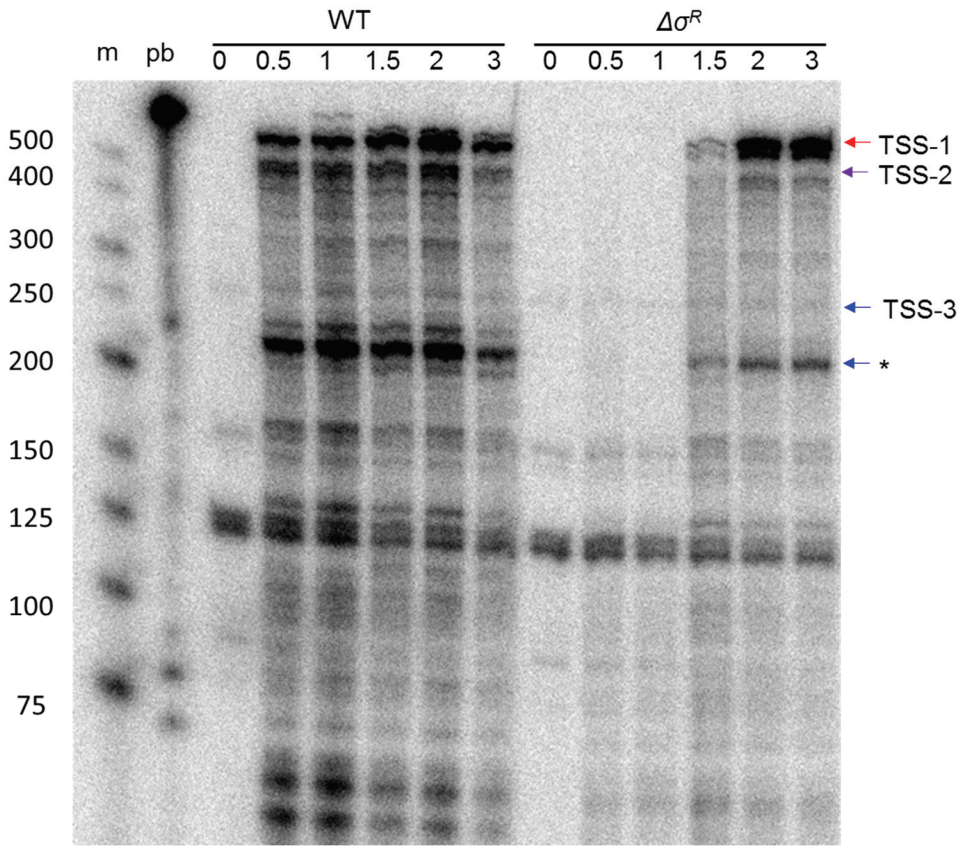


Figure III-4.4. Transcription of *wblC*: probe4.

S1 mapping analysis using probe 4 (741 bps, -691 to +50 nt position relative to the *wblC* start codon). Wild type and Δ*sigRrsrA* were treated hygromycin (5 μg/ml) for 3 hours and the RNA samples were used for S1 mapping analysis.

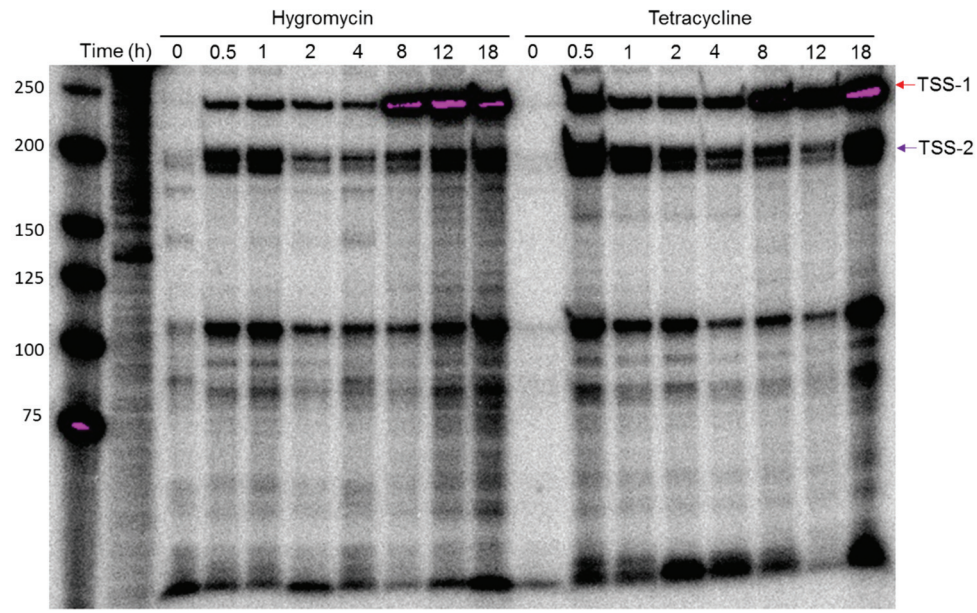


Figure III-4.5. Prolonged induction of *wblC*.

S1 mapping analysis using probe 2 (314 bps, from -691 to -377 nt position relative to the *wblC* start codon). Wild type cells were treated hygromycin (5 μg/ml) or tetracycline (2 μg/ml) for 18 hours and the RNA samples were used for S1 mapping analysis.

III-4.2. The 5′ -phosphate status of *wblC* transcripts

To confirm the three *wblC* promoters are real transcription start sites, I analyzed 5′ -phosphate status of the *wblC* mRNA. For enrichment of 5′ -triphosphate-RNA, the RNA samples were treated with or without Tobacco Acid Pyrophosphatase (TAP). Further digestion with Terminator Exonuclease (TEX) was carried out and the sample was extracted and analyzed by S1 nuclease mapping (Fig. III-4.6).

Fig. III-4.6 shows that *wblC*p1 and *wblC*p3 are real transcription start sites. The result of *wblC*p2 is ambiguous. Two bands from *wblC*p2 were detected, the enrichment pattern of upper band shows the feature of 5′ -monophosphate-RNA. The enrichment pattern of lower bands shows some feature of 5′ -triphosphate-RNA.

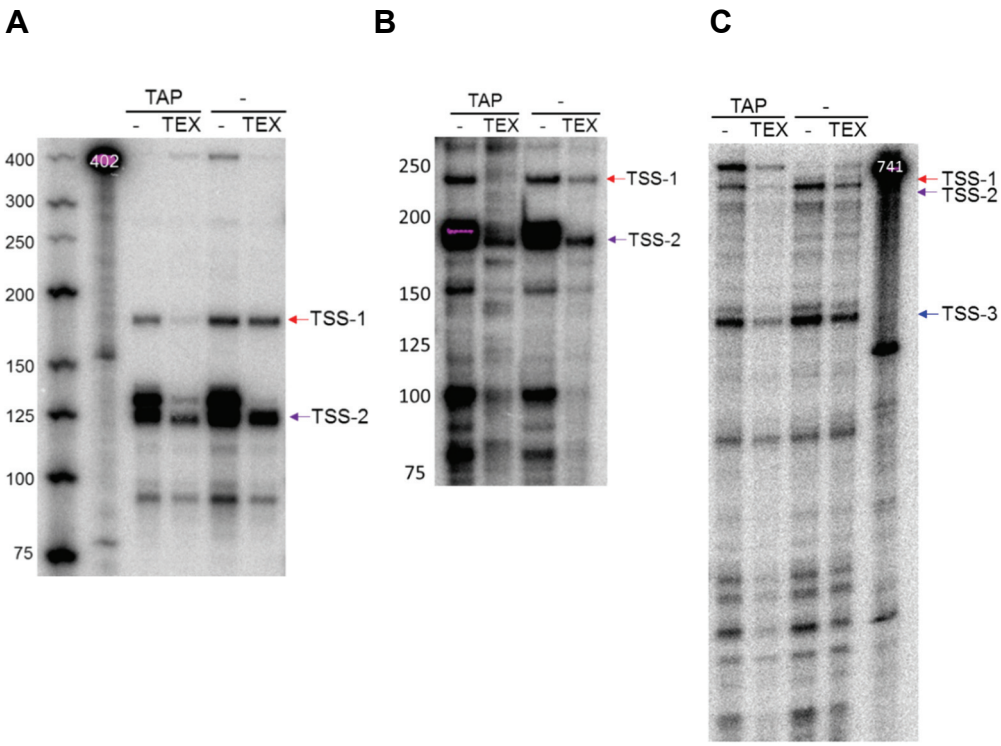


Figure III-4.6. The 5′ -phosphate status of *wblC*.

Wild type cells were treated hygromycin (5 μg/ml) for 30 minutes and the 10 μg RNA samples were treated with or without Tobacco Acid Pyrophosphatase (TAP). Further digestion with Terminator Exonuclease (TEX) was carried out and the sample was extracted and analyzed by S1 nuclease mapping using probe 1 (A), probe 2 (B) and probe 4 (C).

CHAPTER IV. DISCUSSION

IV-1. Role of SigR-RsrA in thiol oxidative stress response

Genome-wide identification of direct SigR binding sites under thiol-oxidative stress conditions, combined with transcript and sequence analyses, revealed many new genes of SigR regulon in *S. coelicolor*. The wide spectrum of target genes that include cascades of regulators indicates that SigR is a global regulator. As expected, SigR induces many genes related with homeostasis of thiols in proteins and small molecules (mycothiols), and proper protein quality control, which are most prominently affected by thiol-reactive/oxidative stressors. Additionally, SigR induces many genes for ribosome-associated components, transcriptional regulators including sigma factors, numerous oxidoreductases (many of which are predicted to contain flavin), DNA damage repair, cofactor metabolism that include several Fe-S containing enzymes/proteins, and lipid synthesis. The full list of SigR target genes enabled prediction and experimental demonstration of new physiological functions of the SigR regulon under thiol-oxidative stress condition (Kim et al., 2012).

I found that SigR directly contributes to maintain the level and activity of the major housekeeping sigma factor HrdB through increasing its transcription during oxidative stress. SigR also confers resistance to UV and thiol-reactive compounds, likely by inducing enzymes of DNA damage repair. Generation of a refined SigR promoter model enabled comparative genomics analysis of the SigR regulon, which revealed the existence of a core SigR regulon conserved across 42 selected Actinomycetes. The most prominent functions of the conserved SigR-orthologue regulons are related with thiol redox homeostasis, including the maintenance of sulphur-containing amino acids, and protein quality control involving chaperones and proteolytic systems. In addition, genes for translational modulation, transcriptional regulatory cascades, oxidoreductases and Fe-S assembly functions

are also included. The bioinformatic analysis also predicts the existence of variable extended regulons specific for each genus. These results complement the experimental characterization of the SigR regulon in *S. coelicolor* to identify genes and functions that are most likely to be critical for the biological response to thiol oxidative stress. Comparison of SigR-regulated gene functions with those of thiol-oxidative stress response system (Nrf2-Keap1) in mammals reveals many shared gene functions, suggesting a robust physiological mechanism to deal with thiol-reactive stresses across distantly related life forms (Kim et al., 2012).

IV-2. Role of SigR in antibiotic stress response

I demonstrated that the *sigR* gene expression is induced by translation-inhibiting antibiotics to produce a stable isoform of SigR, σ^{R}, which elevates its target gene expression for a prolonged period, in contrast to a transient induction of $\sigma^{R'}$ by thiol-oxidative stresses. I also found that the *sigR* gene confers resistance to these inducing antibiotics. Previously, 108 direct target genes of SigR were identified by ChIP-chip analysis (Kim et al., 2012). Since the ChIP experiment was done after diamide treatment for 30 min, when the majority of the *sigR* gene product was $\sigma^{R'}$ (more than 80% of the total SigR; Fig. III-3A), the SigR regulon I determined reflects primarily the promoters preferentially bound by $\sigma^{R'}$. Since $\sigma^{R'}$ differs from $\sigma^{R'}$ only by the N-terminal 55 amino acids, which may not affect promoter recognition, I consider the $\sigma^{R'}$-bound genes may not differ from σ^{R}-binding genes. Quite a number of SigR-target genes encode functions for thiol redox homeostasis, proteolysis, and ribosome modulation (Yoo et al., 2016).

Treatment with translation-inhibiting antibiotics will not only slow down the synthesis of new proteins, but also result in misfolded protein products due to

mistranslation or protein truncation (Kohanski et al., 2008; Ling et al., 2012). Stalled ribosomes uncoupled with transcription can cause mRNA cleavage, resulting in ribosome stuck at non-stop mRNA, which produces non-functional truncated protein upon ribosome rescue (Keiler, 2015; Subramaniam et al., 2014). Therefore, the cellular damages caused by thiol-disturbing oxidative stress can overlap with those by translation-inhibiting antibiotics to quite an extent. In light of this, the functions of predicted ribosome-associated proteins of SigR regulon such as tmRNA (*ssrA*), RelA, HflX, peptide-releasing factor PrfA, EngA, and ObgE need be further investigated (Yoo et al., 2016).

Then, why is prolonged induction of SigR required to cope with antibiotics, whereas transient induction is sufficient to cope with oxidative stress? The results implicate that *S. coelicolor* takes longer time to overcome antibiotic stress than thiol-oxidative stress. Thiol oxidants and electrophiles that elicit thiol-oxidative stress are efficiently removed in *Streptomyces* by mycothiol a functional equivalent of glutathione in actinomycetes (Park and Roe, 2008). Increased production and recycle of mycothiol, along with increased thiol-reducing systems, after thiol-oxidative stress will efficiently remove chemical stressors and return the thiol redox environment back to normal in a relatively short period of time. On the contrary, the antibiotics that bind to the ribosome is harder to be cleared from the cell, affecting cell physiology for longer period of time (Yonath, 2005). This may necessitate the utilization of stable regulator, such as stable σ^R, that can carry out the response for prolonged period of time (Yoo et al., 2016).

I observed that the antibiotics that induced *sigR*p1 transcription also induced *sigR*p2 transcription, even though to a lesser extent (Figs 1B, 2A, and 4B). The antibiotic induction of *sigR*p2 almost entirely depends on SigR, since no *sigR*p2 transcripts were observed in Δ*sigR* mutant (Fig. III-3.). Part of the reason that *sigR*p2 is induced by antibiotics is due to the secondary effect of increased σ^R that recognizes *sigR*p2.

The results in Fig. III-3.4B, which show that the *sigR*p2 is still induced by tetracycline in the Δ*wblC* mutant is not easy to explain. In the absence of WblC, no increase in σ^R is observed, and therefore, the *sigR*p2 induction is likely to occur via the pathway of inactivating RsrA (Fig. III-3.1A). It can be speculated that somehow the intracellular environment of Δ*wblC* is more oxidized than the wild type following antibiotic treatment (Yoo et al., 2016).

IV-3. Induction of WblC in antibiotic stress response

The more interesting question is how the production WblC protein is drastically elevated in the presence of translation-inhibiting antibiotics. The *wblC/whiB7* mRNA contains unusually long 5' UTR with possible ORF for a small protein. This feature appears conserved across actinomycetes (Dinan et al., 2014), and may play some role in elevating WblC expression upon slowing down translation. There is also a possibility that the *wblC* gene expression partly depends on SigR, as predicted from the presence of SigR-dependent promoter sequence upstream of the *wblC* gene. The finding that the extent of antibiotic induction of the *sigR*p1 transcription in the *ΔsigR* mutant reduced to about half of the wild type level supports this idea. Further studies are in need to unravel the underlying mechanism.

In this study, I demonstrate that multiple antibiotics induce the SigR system via yet another pathway of signal transduction, different from what conveys the thiol-perturbing signals. I show that the antibiotic induction of the SigR system proceeds via increasing the production of stable σ^R, and this induction is mediated by WblC/WhiB7 (Yoo et al., 2016).

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국문초록

세포는 환경 변화에 적응하기 위해 유전자 발현을 조절한다. 환경 변화에 대응하는 조절인자들 중, 박테리아에서 전사를 조절하는 시그마 팩터에 속하는 SigR은 *Streptomyces coelicolor*에서 산화 스트레스에 대응하는 데 중요한 역할을 한다. SigR의 항 시그마 인자인 RsrA는 아연과 결합하는 히스티딘과 시스테인 잔기를 통해 산화 환원 상태를 감지하여 SigR의 활성을 조절한다.

SigR의 생리적 기능에 대해 보다 자세히 이해하기 위해서 염색질 결합 면역침전 실험과 함께 염기 서열 분석이 이루어졌고, 이를 통해 SigR이 조절하는 프로모터 108개가 밝혀졌다. 이미 보고된 산화환원 항상성, 단백질 분해 및 리보솜 조절에 기능하는 SigR 표적 유전자들 외에도, 64개의 추가 표적 유전자를 새로이 발견하여 SigR이 글로벌 조절 인자로써 기능하는 것을 규명하였다. 그리고 새롭게 찾은 표적 유전자를 분석함으로써 SigR이 산화환원 스트레스 상황에서 시그마 인자 HrdB의 수준과 활성을 유지하는데 필요하며, UvrA을 조절하여 자외선 및 thiol-산화환원 손상에 대해 세포를 방어함을 알아냈다.

다음으로 SigR이 산화 스트레스뿐만 아니라 항생제 스트레스에도 관여하는 것을 발견하였다. 항생제는 산화 스트레스와는 다른 경로를 통해 SigR과 표적 유전자를 유도하여 항생제 내성을 일으킨다. 산화 스트레스에 대한 반응은 SigR'이라는 불안정한 isoform을 생성함으로써 일어나며, SigR'은 금방 분해되어 이 스트레스 반응이 짧은 시간 동안

일어나게 한다. 이와 달리, 항생제는 안정한 SigR 단백질을 생성하여 오래 지속되는 반응을 유도한다. 항생제 중에서도 특히 최소 생장 억제 농도 이하의 번역을 억제하는 항생제들에 의해 *sigR*p1 프로모터 전사가 유도되어 안정한 SigR 단백질의 합성이 증가했다. 그리고 항생제에 의한 SigR의 발현 증가가 WhiB와 유사한 DNA 결합 단백질인 WblC에 의해 매개되는 것을 새롭게 규명하였다. WblC는 세 개의 전사 시작점을 가지며 번역 저해 항생제를 처리하면 세 프로모터 모두에서 전사가 증가한다. WblC 단백질 역시 번역 저해 항생제를 처리하면 그 양이 증가하며 증가한 WblC는 *sigR*p1 프로모터와 결합하여 안정한 SigR을 암호화하는 *sigR*p1 전사체를 유도한다. 돌연변이체와 야생형 균주의 항생제 감수성을 비교하여 WblC와 SigR가 번역 억제 항생제에 내성을 부여하고 있음을 확인하였다. 추가적으로 결핵균 (*Mycobacterium tuberculosis*) 내의 *sigR* 상동성 유전자들도 항생제에 의해 유도가 일어나는 것을 보았으며, 이러한 현상이 다른 방선균에도 보존되어 있을 가능성을 확인하였다.

이러한 발견은 방선균 내에 보존되어 있는 항생제에 의해 유도되는 항생제 저항 기작을 새로이 밝혔으며, 산화 스트레스와 번역 저해 스트레스가 연관되어 있음을 시사한다.

주요어: 박테리아 유전학, 전사 조절자, 산화 스트레스, 항생제,

방선균, *Streptomyces coelicolor*, SigR, WblC/WhiB7

학 번: 2009-20345