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A WblA-Binding Protein, SpiA, Involved in *Streptomyces* Oxidative Stress Response

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The *Streptomyces coelicolor wblA* gene is known to play a negative role in both antibiotic biosynthesis and the expression of genes responding to oxidative stress. Recently, WhcA, a WblA ortholog protein, was confirmed to interact with dioxygenase-encoding SpiA (stress protein interacting with WhcA) in *Corynebacterium glutamicum*. We describe here the identification of a SpiA ortholog SCO2553 protein ($SpiA_{sc}$) that interacts with WblA in *S. coelicolor*. Using heterologous expression in *E. coli* and *in vitro* pull-down assays, we show that WblA specifically binds $SpiA_{sc}$, and is influenced by oxidants such as diamide. These data indicate that the interaction between WblA and $SpiA_{sc}$ is not only specific but also modulated by the redox status of the cell. Moreover, a $spiA_{sc}$-disruption mutant exhibited a less sensitive response to the oxidative stress induced by diamide present in solid plate culture. Real-time RT-PCR analysis also showed that transcription levels of oxidative stress response genes (*sodF*, *sodF2*, and *trxB*) were higher in the $spiA_{sc}$-deletion mutant than in wild-type *S. coelicolor*. These results show that $SpiA_{sc}$ negatively regulates WblA during oxidative stress responses in *S. coelicolor*.

Keywords: *Streptomyces coelicolor*, oxidative stress, *wblA*, *spiA*

Introduction

Streptomyces coelicolor is a gram-positive filamentous bacterium and the best studied representative of the *Streptomyces* genus [2, 10]. We previously discovered and characterized a novel global antibiotic down-regulator in *S. coelicolor*, named *whiB*-like gene A (*wblA*, SCO3579), as well as *wblA* orthologs in several other *Streptomyces* species [11, 16, 17]. The *whiB* gene was originally proved or defined as one of the developmental regulatory genes essential for the sporulation of aerial hyphae in *S. coelicolor* [4]. Interestingly, the *whiB* homologs are only found in the order Actinomycetales including the genera of *Mycobacterium* and *Corynebacterium* [25], and 11 *whiB*-like genes are present in *S. coelicolor* [1, 2]. Seven *whiB*-like genes were identified in the *Mycobacterium tuberculosis* genome, whereas only four were found in *Corynebacterium glutamicum* [14]. The *whiB*-like genes control diverse cellular processes, such as cell division, differentiation, pathogenesis, starvation survival, and stress response [3, 7, 8, 12, 22–24]. Extensive genetic studies of *wblA* in *S. coelicolor* were also reported [6]. All WhiB-like proteins contain four conserved cysteine residues that presumably bind to a redox-sensitive Fe-S cluster, implying the role for WhiB-like proteins in redox regulation in Actinobacteria [5]. The cluster loss reaction, followed by oxidation of the coordinating cysteine thiols and formation of disulfide bridges, is believed to be important for its activity. Oxidative stress is a process characterized by elevated levels of intracellular reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·). These ROS are capable of damaging cellular macromolecules, including DNA, proteins, and lipids. To protect cells against ROS, all aerobic bacteria have oxidative defense and repair systems, such as the thioredoxin system [5].

Recently, WhcA, a WblA ortholog protein, was confirmed

to play a negative role in the oxidative stress response of *C. glutamicum* [3]. Genes responsive to oxidative stress were expressed higher in the *whcA*-disruption mutant compared with wild type. Subsequently, WhcA was confirmed to interact with the dioxygenase-encoding protein named SpiA (Stress protein interacting with WhcA) in *C. glutamicum*, an interaction modulated by cellular redox conditions [20]. Our previous study also confirmed that the *S. coelicolor whcA*-ortholog, *wblA*, downregulates the expression of genes responding to oxidative stress [12]. Based on the high homology between WblA in *S. coelicolor* and WhcA in *C. glutamicum*, and their similar role in responses to oxidative stress, we identified a SpiA ortholog SCO2553 protein (named $SpiA_{sc}$). We report here a *S. coelicolor* WblA-$SpiA_{sc}$ interaction *in vitro* and confirm the role of $spiA_{sc}$ responses to oxidative stress in *S. coelicolor*.

Materials and Methods

Construction of Plasmids Containing *wblA* and $spiA_{sc}$

The plasmid for the His_6-WblA fusion protein, expressing the His_6 at the C-terminal, was constructed *via* amplification of the *wblA* gene using the primers 5′-CATATGGGCTGGGTAACCGAC-3′ and 5′-AAGCTTGCCCACGGCCGC-3′, and ligating the product DNA into pET21b. The plasmid for the GST-$SpiA_{sc}$ fusion protein, expressing the GST protein at the N-terminal, was constructed by ligating the $spiA_{sc}$ gene, which was amplified using the primers 5′-GGATCC ATGTCCTCCGCACT-3′ and 5′-CTCGAGTCATCGGGCGCC-3′, into pGEX-4T3. The following restriction enzymes were used for cloning of the fusion protein constructs: His_6-WblA fusion protein, *Nde*I and *Hind*III; GST-$SpiA_{sc}$ fusion protein, *Bam*HI and *Xho*I.

Strains, Media, and Growth Conditions

Escherichia coli DH5α was utilized for the construction and propagation of plasmids. The *wblA* (His_6-WblA) and $spiA_{sc}$ (GST-$SpiA_{sc}$) plasmids were expressed in *E. coli* BL21 DE3 cells. A chaperone plasmid named pTf166 (TaKaRa) was used for expression of the soluble form of GST-$SpiA_{sc}$. *E. coli* cells were cultured at 37°C in LB. *S. coelicolor* A3 (2) M145 and *wblA* mutant were used to construct a Δ*spiA* mutant. *S. coelicolor* strains were cultured at 30°C. TSB (Tryptic Soy Broth) and modified R5 liquid media were used as the seed culture and main culture media, respectively. The sensitivity of *S. coelicolor* strains to diamide was assessed on R2YE plates. Antibiotics were added at the following concentrations when needed: 100 μg/ml ampicillin, 50 μg/ml apramycin, 25 μg/ml chloramphenicol, 50 μg/ml hygromycin.

Protein Purification and Pull-Down Assay

The GST-$SpiA_{sc}$ fusion protein was expressed with chaperone proteins and purified using the GSTrap FF column (GE Healthcare), in accordance with the manufacturer's instructions. Pull-down assays were performed with purified recombinant proteins. His_6-WblA was overexpressed, denatured using 8 M urea, and refolded on a HisTrap FF column (GE Healthcare) as previously described [18]. The purified GST-$SpiA_{sc}$ fusion protein was then applied to a HisTrap FF column. Eluted protein samples were analyzed by SDS-PAGE. To confirm the effect of oxidants on protein-protein interactions, 1 mM diamide (a thiol-specific oxidant) was added to the refolding buffer. Purified maltose-binding protein was applied to a Ni-NTA column with His_6-WblA protein and treated, as described above, to assess nonspecific binding.

Construction of $spiA_{sc}$-Disrupted *S. coelicolor* Mutant Strains

The *S. coelicolor* mutant strain was constructed using the PCR-targeted gene-disruption system, according to the method described by Gust *et al*. [9]. An apramycin resistance gene/oriT cassette for the replacement of $spiA_{sc}$ was amplified using the pIJ773 DNA fragment as a template and the following primers: 5′-TACAAG ACCGCGGACGGGATGTACCAGGAGATCAAACAGATTCCGG GGATCCGTCGACC-3′ and 5′-AGTTCGGCGACCAGCACCTCC ACCAGTTGTCCGGCGGGCTGTAGGCTGGAGCTGCTTC-3′. The resultant PCR product was used to target the StC77 cosmid (kindly provided by the John Innes Centre in the United Kingdom) containing the $spiA_{sc}$ gene in *E. coli* BW25113/pIJ790. The mutated cosmid was transferred to *S. coelicolor* and *S. coelicolor* Δ*wblA* mutant strains by conjugation *via E. coli* ET12567/pUZ8002. The desired mutants, the products of double crossovers, were confirmed by screening for colonies that were apramycin resistant but kanamycin sensitive. The double-crossover exconjugants were selected using standard apr^R/kan^S methods [9], followed by confirmation of both *S. coelicolor* Δ$spiA_{sc}$ and *S. coelicolor* Δ*wblA*Δ$spiA_{sc}$ genomic DNAs by PCR. The following primers were used to confirm the double-crossover recombinants: 5′-ACTGACCGATCT CTTCCCCT-3′ and 5′-ACAACCCCGACAACGCTGT-3′.

Biochemical Analysis and Real-Time RT-PCR

The sensitivity of the *S. coelicolor* wild-type and mutant strains (Δ*wblA*, Δ$spiA_{sc}$, Δ*wblA*Δ$spiA_{sc}$) to diamide were assessed on plate culture. All strains were spread on R2YE plates. A total of 1.72 mg (10 μl of 1 M) of diamide (*N,N,N′,N′*-tetramethylazodicarboxamide) was then applied to a paper disc positioned at the center of the plate. The plate was then incubated at 30°C for 48 h until the formation of a clear zone had completely occurred. The RNA was prepared with the RNeasy Mini Kit (Qiagen) by following the manufacturer's instructions. The cDNA conversion was carried out with a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa). Real-time RT-PCR was performed using the TaKaRa SYBR Premix Ex Taq (perfect Real Time) with a Thermal Cycler Dice Real Time System Single (code TP850) (TaKaRa). Normalization was performed with *hrdB*, a housekeeping gene in *S. coelicolor*, using the primer pair *hrdB*_F (5′-GCGGTGGAGAAGTTCGACTA-3′) and *hrdB*_R (5′-TTGATGACCTCGACCATGTG-3′). The following primers were used for real-time RT-PCR: SCO0999 (*sodF2*)_F, 5′-GCACATTCT GCACTCGATCT-3′; SCO0999 (*sodF2*)_R, 5′-GTCGAAGACGAG GATCGG-3′; SCO2633 (*sodF*)_F, 5′-ACTCGATCTACTGGCACA

ACA-3′; SCO2633 (*sodF*)_R, 5′-GTCGAAGACGAGGATCGG-3′; SCO3890 (*trxB*)_F, 5′-ACCGAGGTGGAGAACTTCC-3′; and SCO3890 (*trxB*)_R, 5′-AAGAAGAAGCCGTCACAGGT-3′.

Results and Discussion

Identification of a *C. glutamicum spiA* Ortholog ($spiA_{sc}$) in *S. coelicolor*

Recently, the *C. glutamicum* WhcA, which represses the expression of oxidative stress response genes, was found to interact with the dioxigenase-encoding SpiA. This WhcA-SpiA protein-protein interaction was labile to oxidants [3]. In our previous report, we also showed that the *S. coelicolor wblA* gene plays a negative role in both antibiotic biosynthesis and the expression of genes responding to oxidative stress [12]. Through a BLAST search tool, we found a SpiA ortholog SCO2553 protein (named $SpiA_{sc}$). $SpiA_{sc}$, encoding a putative oxidoreductase, exhibited a relatively high query coverage (97%) and identity (36%) with SpiA in *C. glutamicum*. Based on high similarities between WblA and WhcA, we hypothesized that WblA might interact with the SpiA ortholog protein in *S. coelicolor*.

Demonstration of Protein Interactions Between WblA and $SpiA_{sc}$

A direct physical interaction between WblA and $SpiA_{sc}$ was tested by a protein-binding "pull-down" *in vitro* experiment with purified recombinant proteins. The GST-$SpiA_{sc}$ fusion protein was expressed in *E. coli* BL21. To obtain soluble protein, a GST-$SpiA_{sc}$ fusion protein was expressed with the pTf16 chaperone and purified using the GSTrap FF column. The His_6-WblA fusion protein overexpressed in *E. coli* BL21 formed insoluble inclusion bodies; we therefore performed an on-column renaturation technique [18], followed by a protein-protein interaction assay using SDS-PAGE (described below). A 16 kDa His_6-WblA and 73 kDa GST-$SpiA_{sc}$ co-eluted (Fig. 1A), indicating a specific binding interaction between these two proteins. Nonspecific binding of the GST-$SpiA_{sc}$ fusion protein to the HisTrap FF column was not observed. In addition, nonspecific binding of the GST-$SpiA_{sc}$ fusion protein with the denatured His_6-WblA fusion protein was not observed (Figs. 1C and 1D). Since the band intensity of the co-eluted His_6-WblA protein was lighter than expected, the His_6-WblA protein complex likely degraded during on-column renaturation. In addition, a possibility of incomplete recovery of an iron-sulfur cluster could affect the WblA-$SpiA_{sc}$ interation. Nevertheless, we confirmed that WblA specifically interacts with $SpiA_{sc}$ through *in vitro* pull-down assay experiments.

To further study the effect of redox conditions on *in vitro* protein-protein interactions, the pull-down assay was performed in the presence of the oxidant diamide. When diamide was present during WblA refolding, co-elution of WblA and $SpiA_{sc}$ was not observed (Fig. 1B). These data indicate that the WblA-$SpiA_{sc}$ protein-protein interaction is modulated by cellular redox conditions. Since the diamide could have interfered with the refolding of WblA, however, the diamide effect on the WblA-$SpiA_{sc}$ interaction should be further confirmed.

Cell Growth of $spiA_{sc}$ Mutants and Response to Oxidative Stress

WblA specifically interacts with $SpiA_{sc}$ and the interaction is influenced by oxidants. To better understand the role of $spiA_{sc}$ in the oxidative stress response pathway, we devised experiments using both genetic and physiological approaches. We constructed Δ$spiA_{sc}$ and Δ*wblA*Δ$spiA_{sc}$ disruption mutants using *S. coelicolor* wild-type strains, and monitored them for their growth properties (Figs. 2A, 2B). Internal disruption of the $spiA_{sc}$ was verified by PCR (data not shown). The growth pattern of the Δ$spiA_{sc}$ mutant and Δ*wblA*Δ$spiA_{sc}$ mutant strains was almost identical with wild-type strains. Overall, the growth properties of the $spiA_{sc}$ disruption mutants were comparable to *wblA* disruption mutant cells [12]. These results suggest that the deletion of $spiA_{sc}$ does not affect normal cell growth under the culture conditions tested in this study. Previously, *C. glutamicum* wild-type and Δ*spiA* mutant strains were shown to exhibit almost identical growth rates, whereas the *spiA*-overexpressing strain exhibited a retarded growth rate [19].

We compared the $spiA_{sc}$ mutants and *wblA* mutant strains for sensitivity to oxidative stress using a thiol-specific oxidant, diamide. When oxidative stress was induced by treating cells with diamide, the growth of Δ$spiA_{sc}$ mutant and Δ*wblA*Δ$spiA_{sc}$ mutant strains exhibited higher resistance (with a smaller clearing zone) than the wild type (Fig. 2C). These results were similar with *S. coelicolor* Δ*wblA* mutants. Collectively, these data show that the $spiA_{sc}$ gene plays a negative role, similar to the *wblA* gene in oxidative stress response.

$spiA_{sc}$ Negatively Regulates *wblA*-Controlled Oxidative Response Genes

In a previous study, we found that *wblA* plays a negative role in response to oxidative stress [12]. The *wblA* gene is

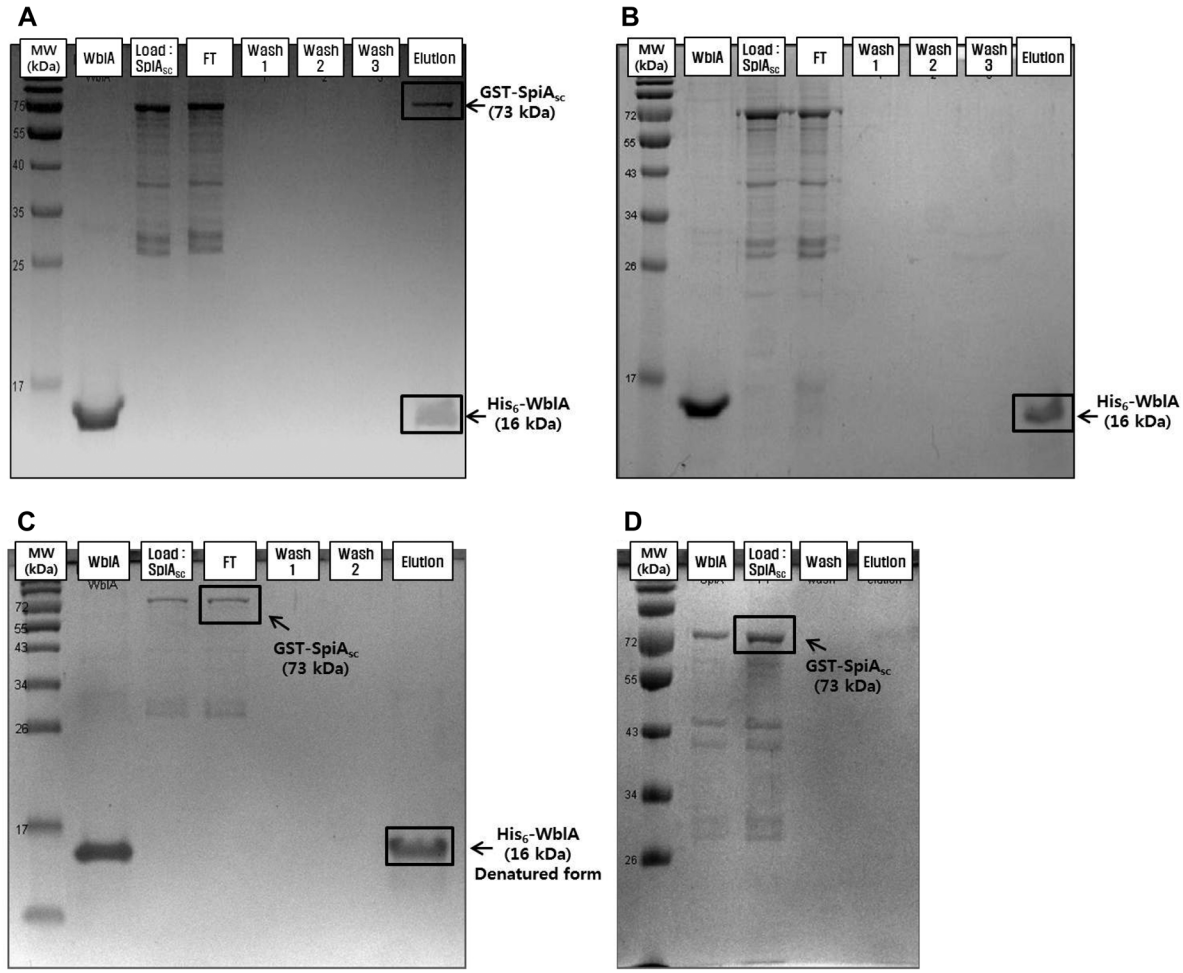

Fig. 1. The physical interaction of WblA with SpiA$_{sc}$ as detected by the *in vitro* pull-down assay.

Purified His$_6$-WblA and GST-SpiA$_{sc}$ proteins were used as bait and prey, respectively. The WblA was refolded in the absence (**A**) and presence (**B**) of the thiol-specific oxidant diamide. SDS-PAGE shows the coelution of the proteins, indicating specific interaction of WblA with SpiA$_{sc}$ protein. Lanes: MW, molecular-weight standards; WblA, denatured WblA; Load, purified SpiA$_{sc}$; FT, flow-through fractions during target load; Wash, column wash fraction after target load. (**C**) Nonspecific binding of the GST-SpiA$_{sc}$ fusion protein with denatured His$_6$-WblA fusion protein was not observed. (**D**) Nonspecific binding of the GST-SpiA$_{sc}$ fusion protein to the HisTrap FF column was not observed.

involved in regulating key members of the oxidative response system, including the superoxide dismutase and thioredoxin reductase genes. As we confirmed that WblA interacts with SpiA$_{sc}$ in *S. coelicolor*, if the *wblA* and *spiA*$_{sc}$ genes function in repressing oxidative stress genes, then genes controlled by *wblA* should also be under the control of *spiA*$_{sc}$. To test this hypothesis, we compared the gene expression of *S. coelicolor* wild-type, Δ*spiA*$_{sc}$, and Δ*wblA* mutant strains. The selected target genes related to oxidative stress response were two superoxide dismutase genes (*sodF*, *sodF2*) and one thioredoxin reductase gene (*trxB*) [12]. The mRNA levels of *trxB* in the Δ*spiA*$_{sc}$ mutant were slightly higher than in the wild type (Fig. 3A). Increases in *trxB* levels were found to a lesser extent in the Δ*wblA* mutant and Δ*spiA*$_{sc}$Δ*wblA* mutant. Unlike the Δ*wblA* mutant, no noticeable differences in *sodF* and *sodF2* expression were detected between the wild type and the Δ*spiA*$_{sc}$ mutant. We also investigated the expression change of the *sod* and *trx* genes under oxidative stress. In the presence of oxidative stress, the mRNA levels of *sod* and *trx* genes in the Δ*spiA*$_{sc}$ mutant were slightly higher than those in the wild type (Fig. 3B). These increases were less prominent in the Δ*wblA* mutant and Δ*spiA*$_{sc}$Δ*wblA* mutant.

We expected transcription levels of target genes in the Δ*spiA*$_{sc}$ mutant to be higher than in the wild type and similar to the Δ*wblA* mutant. Because oxidative stress was

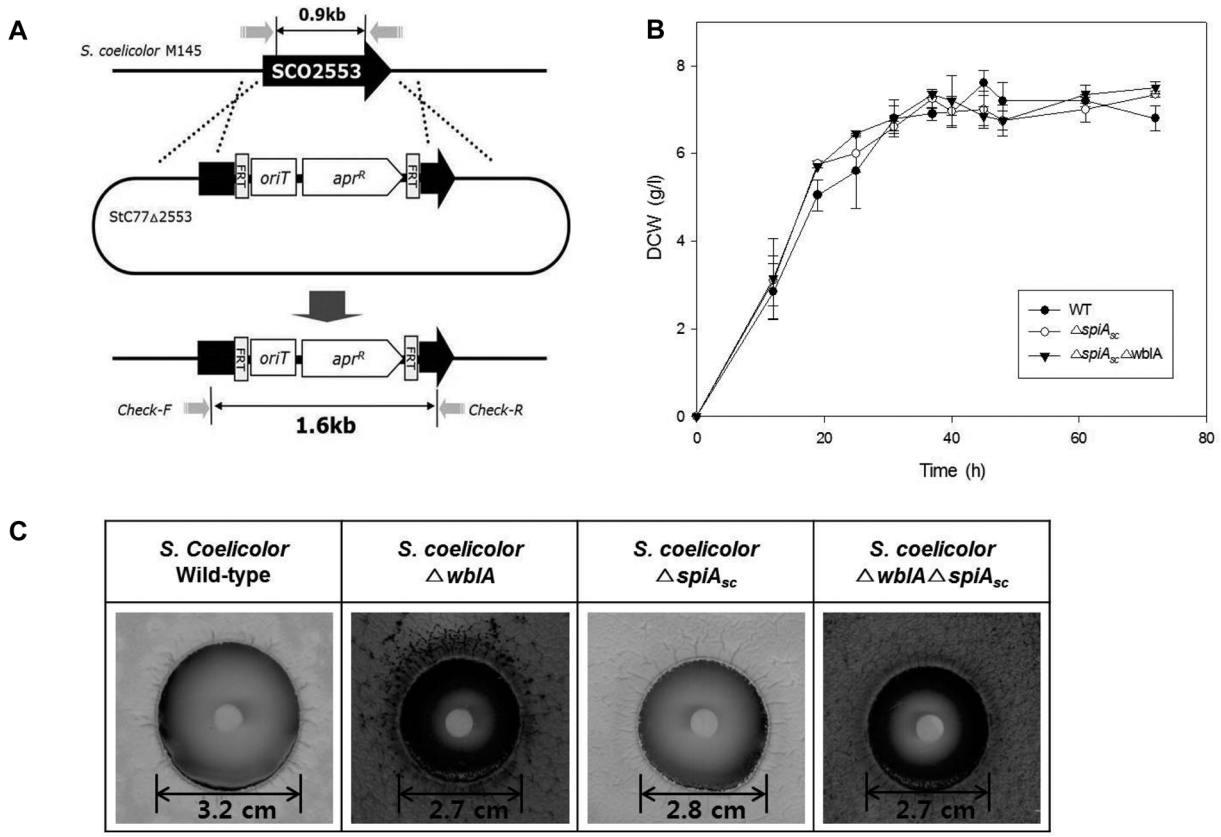

Fig. 2. Growth properties of Δ*spiA*$_{sc}$ and Δ*wblA*Δ*spiA*$_{sc}$ disruption mutants of *S. coelicolor*.
(**A**) PCR-targeted gene-disruption system for *S. coelicolor* Δ*spiA*$_{sc}$ mutants. (**B**) Growth of *S. coelicolor* strains in modified R5 medium for 3 days; wild type (●), Δ*spiA*$_{sc}$ (○), Δ*wblA*Δ*spiA*$_{sc}$ (▼). All cultures were performed in duplicates. (**C**) Sensitivity of the *Streptomyces coelicolor* strains to diamide. A paper disc containing 10 μl of 1 M diamide was placed on each plate containing lawns of either *S. coelicolor* M145 wild type, Δ*wblA* mutant, or Δ*spiA*$_{sc}$ mutant, and followed by incubation at 30°C for 2 days.

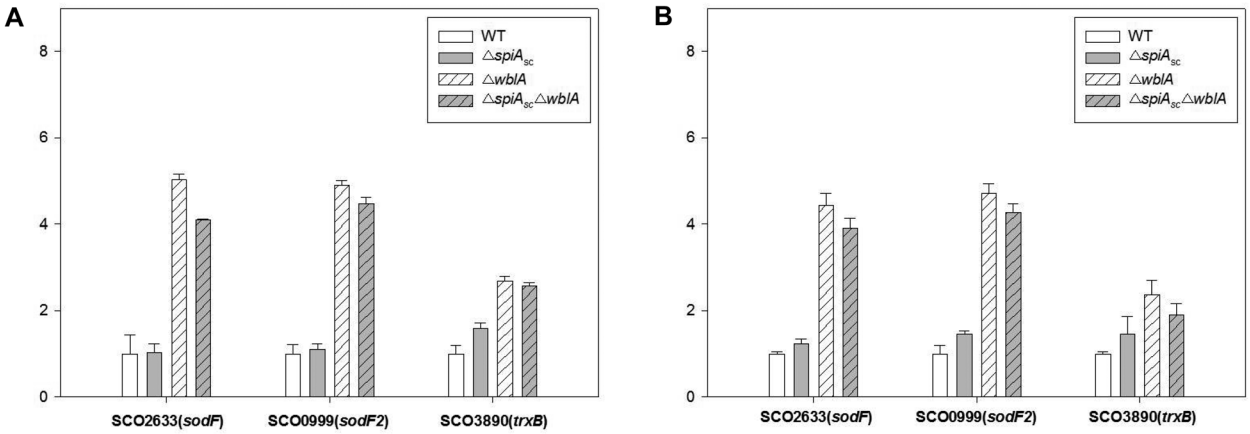

Fig. 3. Real-time RT-PCR analysis of target genes in diamide.
S. coelicolor M145 wild type or Δ*spiA*$_{sc}$ mutant were grown in modified R5 liquid medium to early stationary phase and exposed for 8 h to 1 mM diamide in the absence (**A**) or in the presence of oxidative stress (**B**).

induced, the growth of Δ*spiA*$_{sc}$ mutant and Δ*wblA*Δ*spiA*$_{sc}$ mutant strains exhibited higher resistance (with a smaller clearing zone) than did the wild type. The fact that transcription levels of target genes in the Δ*spiA*$_{sc}$ mutant were lower than those in Δ*wblA* mutants was unexpected and indicates that *spiA*$_{sc}$ plays a role distinct from the *wblA* gene. SpiA$_{sc}$ encodes a putative oxidoreductase – an enzyme that catalyzes the transfer of electrons between molecules. If *spiA*$_{sc}$ encodes a protein with such a function, then deletion of the gene should influence the oxidative stress response mechanism. Therefore, our results suggest that the *spiA*$_{sc}$-dependent oxidative stress response might be controlled by some additional regulatory system in *S. coelicolor*. In *C. glutamicum*, the mRNA levels of *trxB* in the Δ*spiA* mutant were lower than in the wild type [19]. However, the mRNA levels of *trxB* in the Δ*whcA* mutant were higher than in the wild type [3]. SpiA was therefore suggested to encode a dioxygenase that interacts with other cellular proteins in addition to WhcA [19]. In conclusion, a tentative regulatory model showing the action of WblA and SpiA$_{sc}$ has been proposed. In the absence of oxidative stress, WblA may interact with SpiA$_{sc}$ and binds to the regulatory region on the target genes. In the presence of oxidative stress, however, WblA may not interact with SpiA$_{sc}$ and loses its DNA-binding activity, allowing the expression of oxidative stress response genes.

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