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Repression of Antibiotic Downregulator WblA by AdpA in *Streptomyces coelicolor*

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The upstream region of antibiotic downregulatory *wblA* in *Streptomyces coelicolor* was found to contain AdpA binding motifs. A key morphological regulator, AdpA was shown to specifically bind these motifs by electrophoretic mobility shift assay. An *adpA* disruption mutant exhibited increased *wblA* transcription, suggesting that AdpA negatively regulates *wblA* transcription in *S. coelicolor*.

Streptomyces is a Gram-positive, filamentous bacterial genus known for its ability to produce valuable antibiotics (and other secondary metabolites) and its complex life cycle, which involves significant changes in morphological differentiation (1, 2). An important member of this genus, *Streptomyces coelicolor* A3 (2), is a model species widely used in studies of morphological development and antibiotic regulation, and its genome was the first to be completely sequenced among the streptomycetes (3). The regulation of morphological differentiation in *Streptomyces* species is usually accompanied by the production of secondary metabolites and involves multiple regulatory networks (4–6). Among the complex *Streptomyces* regulatory networks, the A factor regulatory cascade is one of the best-characterized systems, working at both the onset of secondary metabolism and morphological differentiation (7, 8). A factor (2-isocapryloyl-3-R-hydroxymethyl-γ-butyrolactone), initially found in the streptomycin producer *S. griseus* (9, 10), derepresses the transcription of *adpA* (A-factor dependent protein A) by binding to ArpA (A-factor receptor protein A), which is bound to the *adpA* promoter (11). The *S. griseus* AdpA (designated $AdpA_{SG}$) plays a key role as a transcriptional regulator by binding to the promoter regions of various genes involved in the regulation of morphological differentiation and secondary metabolite biosynthesis (7, 12–14). Recently, chromatin immunoprecipitation studies found that $AdpA_{SG}$ directly controls more than 500 genes in cooperation with other regulatory proteins, acting as a transcriptional repressor or activator in *S. griseus* (14). In *S. coelicolor*, however, an *adpA* ortholog gene (*bldH*) was found not to be under the control of an A factor-like γ-butyrolactone (3, 15, 16, 17), indicating that regulatory cascades are different between these two species. The *S. coelicolor adpA* mutation, however, causes a bald phenotype and a reduction of actinorhodin (15), suggesting that *S. coelicolor* AdpA (designated $AdpA_{SC}$) also has an important role in the regulation of both morphological differentiation and secondary metabolism (15, 16), and its functions are not identical to those of $AdpA_{SG}$ (12, 17). Recently, $AdpA_{SG}$ was identified as a transcriptional activator for its ability to influence the expression of STI (a protease inhibitor), RamR (an atypical response regulator required for aerial growth), and ClpP1 (an ATP-dependent protease) (18, 19). $AdpA_{SG}$ contained a dual helix-turn-helix (HTH) motif in its C-terminal DNA binding domain that belongs to the AraC/XylS family of transcription regulators, and its consensus binding sequence is 5′-TGGCS NGWWY-3′ (S, G or C; W, A or T; Y, T or C; N, any nucleotide) (20, 21).

Another transcriptional regulatory gene, *wblA* (*whiB*-like gene A), identified in *S. coelicolor* as well as other *Streptomyces* species, encodes a pleiotropic downregulator of the biosynthesis of various antibiotics, including actinorhodin (22), doxorubicin (23), moenomycin (24), and tautomycetin (25). In addition, the *S. coelicolor* Δ*wblA* mutant showed sporulation-deficient aerial mycelium, thin hyphae, and nonsporulation pigments, suggesting that WblA has an important role in the formation of aerial hyphae (26). Recently, WblA was found to function in the response to oxidative stress among actinobacteria such as *Corynebacteria glutamicum* and *S. coelicolor* (27). Interestingly, we found six putative AdpA consensus binding sequences in the promoter region of the *wblA* gene in *S. coelicolor*. It has been demonstrated that the consensus binding sites for $AdpA_{SC}$ are identical to those of $AdpA_{SG}$, and that the consensus binding sites for $AdpA_{SC}$ exist in 157 intergenic regions in the genome of *S. coelicolor* (18). Thus, to test whether $AdpA_{SC}$ directly binds to the *wblA* promoter, the EMSA (electrophoretic mobility shift assay) was performed to examine the ability of $AdpA_{SC}$ binding to a promoter fragment of *wblA*. $adpA_{SC}$ was amplified from the chromosome of *S. coelicolor* with primer pairs (SCO2792 exp-F, 5′-ACTATGAGCCACGACTCCA CCG-3′; SCO2792 exp-R, 5′-CTCGAGTCACGGCGCGCTGC-3′), sequence verified, and cloned into pET21b (+) for expression as a C-terminal His-tagged protein ($AdpA_{SC}$-His) in the *E. coli* BL21 strain. Previously, it was confirmed that a C-terminal His-tagged $AdpA_{SG}$ from *S. griseus* was functionally expressed and successfully bound to the AdpA binding motifs (14). $AdpA_{SC}$-His (predicted size, 45 kDa) was purified using nickel-nitrilotriacetic acid (Ni-NTA) columns (Qiagen) and analyzed by Western blotting as well as matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (Genomine Inc., South Korea). Biotin-labeled PCR fragments of the *wblA* upstream region were prepared by PCR using the following primer pairs: inter1-F, 5′-GGCAGC

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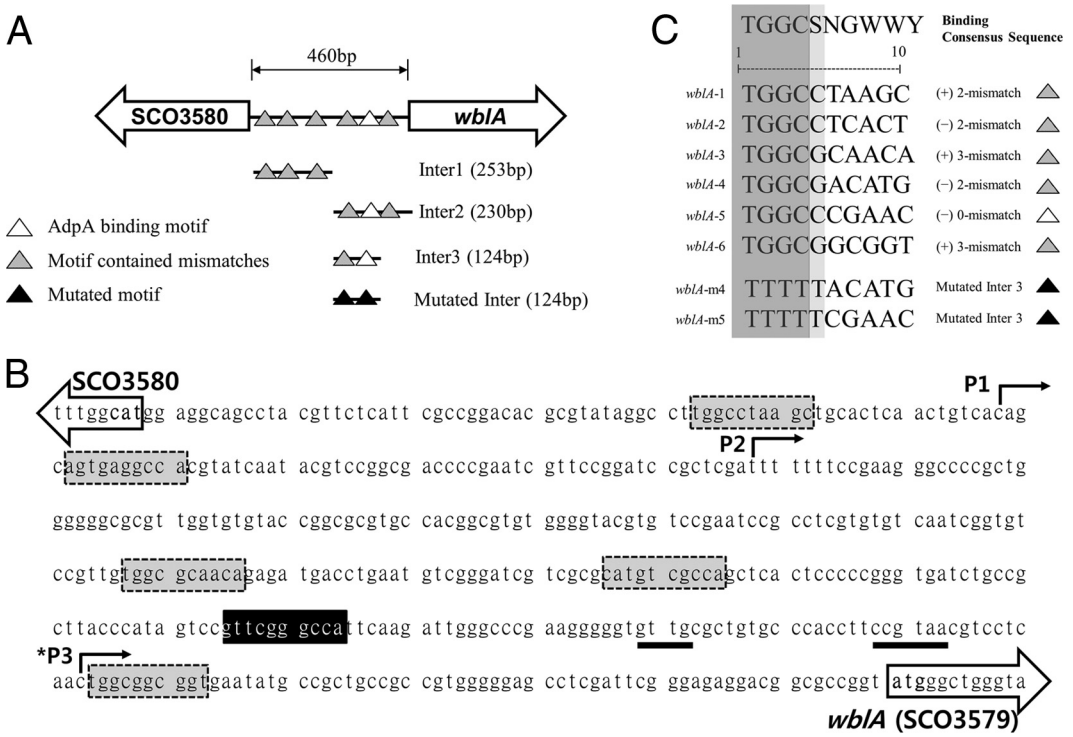

FIG 1 Putative AdpA binding motifs (A) and intergenic sequences (SCO3579 and SCO3580) in the *wblA* gene region of the *S. coelicolor* chromosome (B). Bent arrows, putative promoter designations (P1, P2, and *P3) presented in reference 26; *P3, major transcription start point; underlined sequences, close matches to the consensus sequence for promoters recognized by σ^F in *M. tuberculosis* (34); boxes, putative AdpA binding motifs (gray box, contains 2 to 3 mismatches; black box, contains a perfect match). (C) Alignment of AdpA binding consensus sequences in the upstream region of *wblA* represented with positions and directions.

CTACGTTCTCATTC-3′; inter1-R, 5′-GTCGGTTACCCAGCC CATAC-3′; inter2-R, 5′-TGTTGCGCCACAACGGACAC-3′; inter 3-F, 5′-GAGATGACCTGAATGTCGGG-3′; inter3-R, 5′-GC ACAGCGCAACACCCCCTT-3′; mutated inter3-F, 5′-TCGCGCATGTAAAAAGCTCACTCCCCCGGGTGATCTGCC GCTTACCCATAGTCCGTTCGAAAAATTCAAG-3′; mutated inter3-R, 5′-CTTGAATTTTTTCGAACGGACTATGGGTAA GCGGCAGATCCACCCGGGGGAGTGAGCTTTTTACATG CGCGA-3′ (mutated sequences are underlined); 2792 EMSA-F, 5′-GACAGGGTCTCGGGCCCCC-3′; 2792 EMSA-R, 5′-ACTG CTAAGCCCCCCTCGGT-3′; negative control-F, 5′-AGCGTGG AGGAGCGGAGCTCGT-3′; and negative control-R, 5′-CCAAC CGCCGTGACTCCCAGGGGTACT-3′. As a negative control, a 244-bp DNA probe containing no AdpA binding motif was amplified from the chromosome of the intergenic region between SCO3581, encoding conserved hypothetical protein, and SCO3582, encoding putative secreted protein in *S. coelicolor*. Amplified probes containing the consensus binding sites were incubated with increasing amounts of purified $AdpA_{SC}$ protein in the presence of a nonspecific competitor [poly(dI-dC)] with 2.5% glycerol, 0.05% NP-40, 1 mM EDTA, and 5 mg/ml bovine serum albumin (BSA) for 1 h at room temperature according to instructions of the LightShift chemiluminescent EMSA kit (Thermo Scientific). There are six binding motifs containing 0 to 3 mismatches (in the forward or reverse direction) in the upstream region of the *wblA* gene, and the consensus binding motifs are illustrated in Fig. 1. As a result, both the inter 1 fragment (253 bp) and inter 2 fragment (230 bp) were retarded by increasing the $AdpA_{SC}$ concentration, although fragments possess mismatches, suggesting that $AdpA_{SC}$ directly binds to the promoter of the *wblA* gene and controls the expression of *wblA* at the transcriptional level. The shifts caused by the binding of $AdpA_{SC}$ to the short inter 3 fragment (124 bp), containing both perfect-match sequences and 2-mismatch sequences, were also detected. Perfect-match sequences for AdpA binding are commonly found in the company of mismatch sequences in both *S. griseus* and *S. coelicolor* strains, and these contiguous motifs are related to the function of AdpA (21, 28). Also, the TGGCS sequence is an important recognized motif for the N-terminal domain of $AdpA_{SG}$, with the C in the fourth position being essential for AdpA binding (12, 18). We also constructed a mutated inter fragment (Fig. 1 and 2) containing the TTTTT sequence in the consensus binding motifs of the inter 3 fragment by PCR and then performed EMSA with that fragment. No binding shift was detected (Fig. 2), indicating that the conserved TGGCS sequence was also essential as a binding motif for $AdpA_{SC}$. In an additional experiment, a clear shift in DNA-protein complexes was observed in EMSA performed with the *adpA* promoter fragment (Fig. 2), while shifts of similar strength were observed in EMSA performed with each inter fragment of the *wblA* upstream region. The binding strength of $AdpA_{SC}$ to the *wblA* promoter was not as strong as the binding of $AdpA_{SC}$ to the $adpA_{SC}$ promoter (18). According to the reports (12, 14), however, the strength of the AdpA-DNA interaction is not related to the biological significance of AdpA binding sites. Promoter mapping of the *wblA* upstream region revealed that there are three putative transcriptional start points (26) (Fig. 1B). Among these three positions, P3 is the main transcriptional start point, and transcription begins before the formation of aerial mycelium at

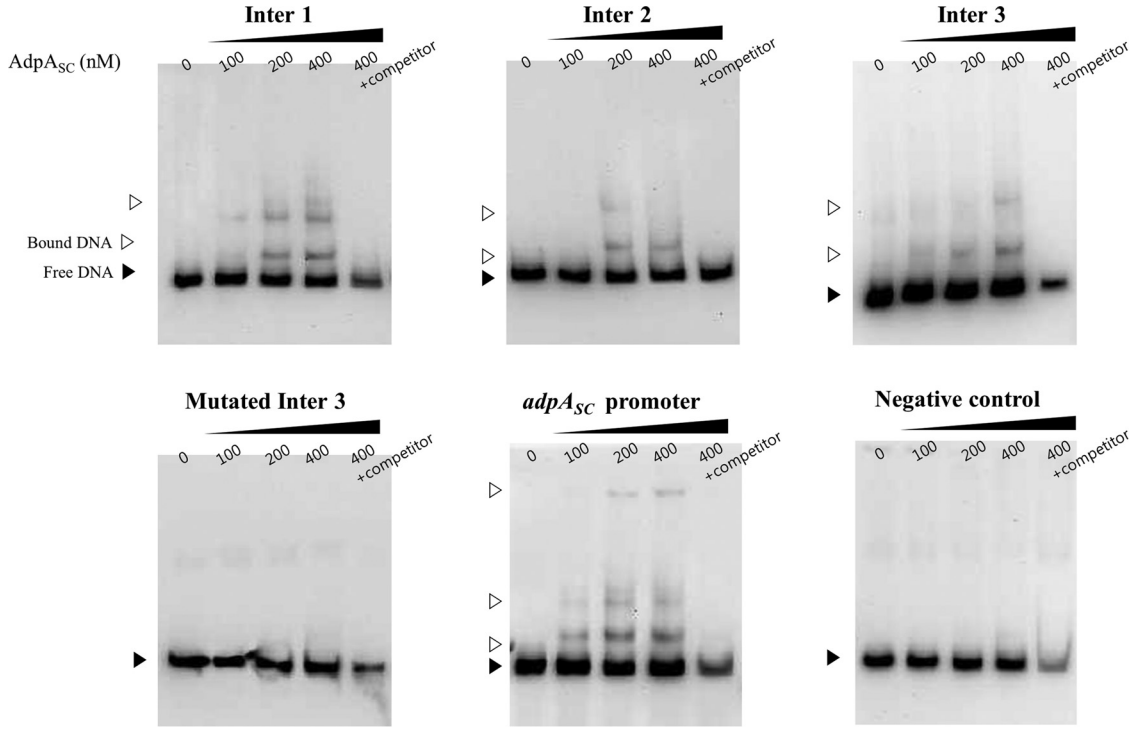

FIG 2 EMSA results. Increasing amounts of AdpA$_{SC}$ protein were incubated with various regions of the *wblA* upstream fragment. PCR-amplified *adpA*$_{SC}$ promoter was used as a positive control, and PCR-amplified intergenic sequence without a binding motif was used as a negative control.

this position. Since putative AdpA binding sites are located throughout the three *wblA* promoter regions, it is not clear which promoter is the major target for the AdpA$_{SC}$ binding in *S. coelicolor*. Thus, we suggest again that AdpA$_{SC}$ directly binds to the upstream region of the *wblA* gene, regulating transcription of *wblA* in *S. coelicolor*. Based on these results, we wondered whether AdpA$_{SC}$ regulates *wblA* gene expression as an activator or repressor. To test this, transcription analysis was conducted with strains of *S. coelicolor* M145, the *S. coelicolor* Δ*wblA* mutant (26, 29), and the *S. coelicolor* Δ*adpA* mutant (19). It was previously reported that the *S. coelicolor* Δ*wblA* mutant produces a much higher level of actinorhodin than *S. coelicolor* M145 (29, 30), whereas the *adpA*-deleted *S. coelicolor* strain does not produce actinorhodin at all in either liquid or plate cultures (15, 16). Each strain was grown in 100 ml of modified R5 (R5 without KH_2PO_4, $CaCl_2 \cdot 2H_2O$, L-proline, and growth factor) liquid medium, and total RNAs were isolated (RNeasy miniprep kit; Qiagen) at 8 h (before any antibiotic production from all strains), 25 h (when there is a slight production of actinorhodin from just the Δ*wblA* mutant), and 35 h (when there is the production of actinorhodin from *S. coelicolor* M145 or the Δ*wblA* mutant and no production of actinorhodin from the Δ*adpA* mutant). cDNA synthesized from RNA isolated at each time point was analyzed for expression of the actinorhodin-specific activator gene *actII-orf4* by normalizing results to the housekeeping gene, *hrdB*. To ensure that contaminating DNA was not present, we also confirmed that there was no real-time PCR amplification of *adpA* and *wblA* from the *S. coelicolor* Δ*adpA* and Δ*wblA* mutant strains, respectively (data not shown). As shown in Fig. 3, real-time PCR analyses demonstrated that the levels of *actII-orf4* transcripts were higher in the Δ*wblA* mutant during the time course than in *S. coelicolor* M145 and the Δ*adpA* mutant. It is consistent that the Δ*wblA* mutant produced the highest level of actinorhodin and the Δ*adpA* mutant did not produce actinorhodin at all (15, 29, 30). The levels of *adpA* transcripts from *S. coelicolor* M145 were similar to those in the Δ*wblA* mutant, suggesting that the expression of *adpA* was not influenced by the *wblA* gene, and suggesting that the transcription of *adpA* occurs upstream of *wblA* transcription. The transcription of *adpA* was maintained at relatively high levels compared to those for *wblA* until 35 h. This result is similar to the facts that *adpA* promoter activity reached the peak during substrate mycelium growth in *S. coelicolor* (from 30 to 45 h) (15) and the maximal production of AdpA$_{SC}$ protein was reached during the early stage of aerial mycelium formation in *S. coelicolor* (from 36 to 60 h) (18). In contrast to *adpA*, the level of *wblA* transcripts in the Δ*adpA* mutant was about 2-fold higher than those in the *S. coelicolor* M145 strain at 25 h, implying that *adpA* negatively controls *wblA* gene transcription. Interestingly, however, the regulation of *wblA* gene transcription by AdpA$_{SC}$ seems to be transient (with apparently no significant differences at both 8 and 35 h), and its mechanism needs to be further characterized. This report shows that AdpA$_{SC}$ regulates the transcription of *wblA* negatively in *S. coelicolor*, despite the fact that several genes related to the formation of aerial mycelium are regulated positively by AdpA$_{SC}$ in *S. coelicolor* (6, 18, 31, 32). In addition, since the transcription of an actinorhodin-specific regulatory gene was reduced in the *adpA* disruption mutant, actinorhodin downregulation by WblA could be dependent on the AdpA regulatory pathway in *S. coelicolor*. By analyzing DNA microarray data from *S. griseus*, it was reported that the promoter region of *wblA* (SGR3340) also has an AdpA binding motif and that AdpA$_{SG}$ binds to the promoter region of *wblA* (33), acting as a transcriptional activator for the *wblA* gene (14). Interestingly,

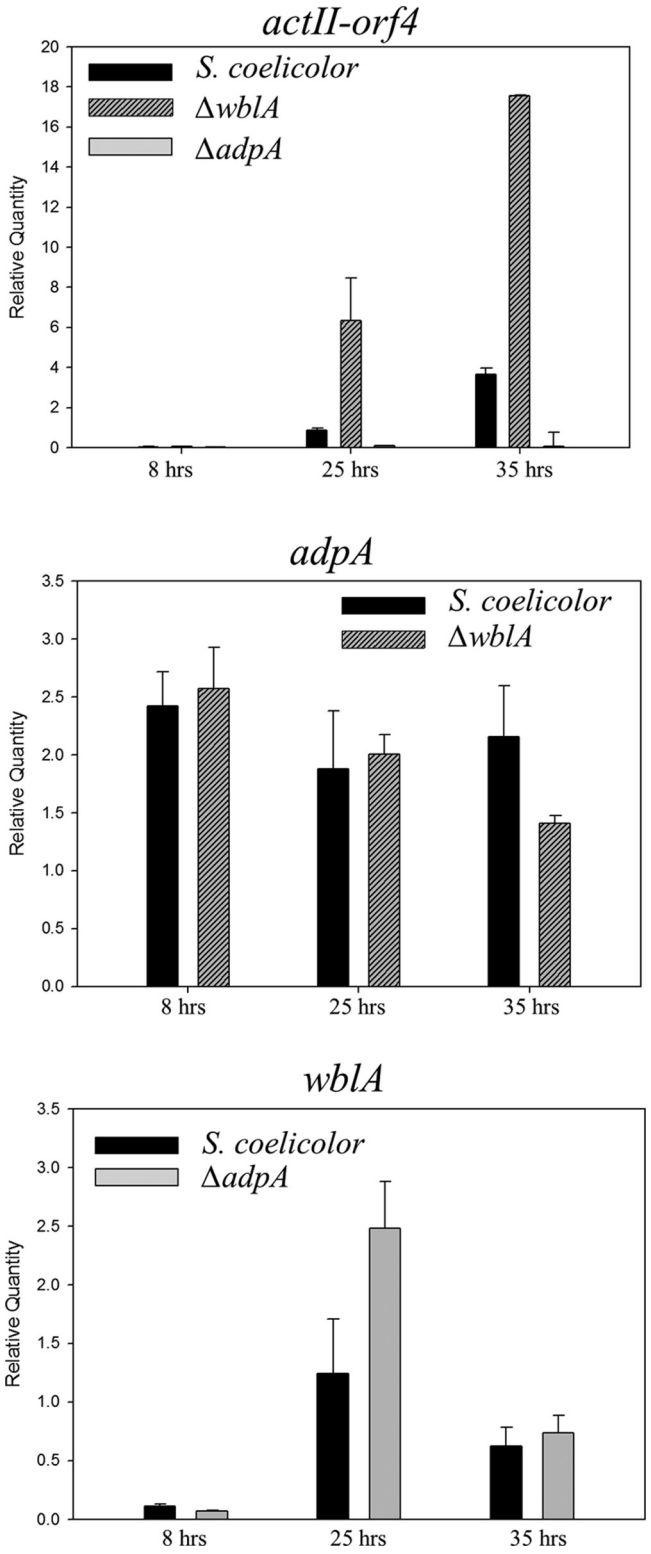

FIG 3 Transcriptional analysis of target genes from *S. coelicolor* M145, the *S. coelicolor* Δ*adpA*$_{SC}$ mutant, and the *S. coelicolor* Δ*wblA* mutant at different time points (8, 25, and 35 h). All cultures were performed in triplicate, and the averages are shown. The *y* axis scale represents the expression value relative to that of *hrdB*, a housekeeping sigma factor (which was set to 1).

this *S. griseus* result is the opposite of our current *S. coelicolor* result, indicating that the AdpA-WblA regulatory cascade mechanism for secondary metabolism and morphological differentiation is different between these two species. In conclusion, we report for the first time the direct influence of AdpA$_{SC}$, a key regulator of morphological differentiation and secondary metabolism in *Streptomyces*, on the *wblA* gene in *S. coelicolor* via EMSA analysis. We also show that *wblA* gene transcription was regulated by *adpA*$_{SC}$, suggesting that AdpA$_{SC}$ not only acts as a central transcriptional regulator of several genes but also regulates *wblA*, which is known as a negative regulator in the antibiotic production and the formation of aerial mycelium.

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