AEM
Journals.ASM.org

$WblA_{ch}$, a Pivotal Activator of Natamycin Biosynthesis and Morphological Differentiation in *Streptomyces chattanoogensis* L10, Is Positively Regulated by $AdpA_{ch}$

Pin Yu,[a,b] Shui-Ping Liu,[a,b] Qing-Ting Bu,[a,b] Zhen-Xing Zhou,[a,b] Zhen-Hong Zhu,[a,b] Fang-Liang Huang,[a] Yong-Quan Li[a,b]

College of Life Sciences, Zhejiang University, Hangzhou, China[a]; Key Laboratory of Microbial Biochemistry and Metabolism Engineering of Zhejiang Province, Hangzhou, China[b]

Detailed mechanisms of WhiB-like (Wbl) proteins involved in antibiotic biosynthesis and morphological differentiation are poorly understood. Here, we characterize the role of $WblA_{ch}$, a *Streptomyces chattanoogensis* L10 protein belonging to this superfamily. Based on DNA microarray data and verified by real-time quantitative PCR (qRT-PCR), the expression of $wblA_{ch}$ was shown to be positively regulated by $AdpA_{ch}$. Gel retardation assays and DNase I footprinting experiments showed that $AdpA_{ch}$ has specific DNA-binding activity for the promoter region of $wblA_{ch}$. Gene disruption and genetic complementation revealed that $WblA_{ch}$ acts in a positive manner to regulate natamycin production. When $wblA_{ch}$ was overexpressed in the wild-type strain, the natamycin yield was increased by ~30%. This provides a strategy to generate improved strains for natamycin production. Moreover, transcriptional analysis showed that the expression levels of *whi* genes (including *whiA*, *whiB*, *whiH*, and *whiI*) were severely depressed in the $\Delta wblA_{ch}$ mutant, suggesting that $WblA_{ch}$ plays a part in morphological differentiation by influencing the expression of the *whi* genes.

The Gram-positive filamentous soil bacterial genus *Streptomyces* is characterized by its complex life cycle, which involves the formation of a substrate mycelium that goes on to develop aerial hyphae, the tips of which ultimately coil and septate into spores. These bacteria are well known for the ability to produce a variety of commercially valuable antibiotics and other secondary metabolites (1, 2). Previous investigations suggested that the triggering of antibiotic biosynthesis is closely coordinated with the initiation of morphological differentiation in *Streptomyces* species (1, 3, 4), and both processes have been shown to be stringently controlled via a hierarchical regulatory network involving the integration of various physiological and environmental signals (5, 6). Although a significant number of regulatory genes and mechanisms involved in the regulatory networks governing morphological development and antibiotic production in *Streptomyces* have been elucidated, relatively little is known about the correlation of these two physiological processes.

The *whiB*-like (*wbl*) genes, which encode homologues of WhiB, have received attention because of their important roles in diverse aspects of actinobacterial biology, such as morphological differentiation and antibiotic production (7, 8). The gene products are small cytoplasmic proteins that contain four conserved cysteine residues able to coordinate an Fe-S cluster and are found largely in actinomycetes, including some mycobacteria (9). It has been reported that *wbl* genes are induced by various stimuli, especially oxidative stress, suggesting a unique role in maintaining redox homeostasis (10–12). In *Mycobacterium tuberculosis*, WhiB3 acts as a metabolic regulator that binds to the promoters of polyketide biosynthetic genes (13), while WhiB6 acts as an initial phagosomal signal receptor (14). It has also been shown that WhiB7 is a critical protein for generating resistance to antibiotics in *M. tuberculosis* (15). Recently, *whiB3* (16), *whiB4* (17), *whiB5* (18), and *whiB2* (19) were shown to play essential roles in the virulence of *M. tuberculosis* during progressive infection.

In *Streptomyces coelicolor* M145, there are 11 WhiB-like proteins, among which WblA was reported to act as an important transcriptional regulator involved in antibiotic production and morphological differentiation (7, 20, 21). WblA and its orthologues have been described as crucial antibiotic downregulators for the biosynthesis of various antibiotics such as actinorhodin (21), doxorubicin (22), tautomycetin (23), and moenomycin (24). Moreover, it was discovered that *wblA* is negatively regulated by the pleiotropic regulator AdpA in *S. coelicolor* (25). However, in our study, we revealed differing functions of $wblA_{ch}$ in antibiotic biosynthesis and in the AdpA-WblA regulatory relationship in *Streptomyces chattanoogensis* L10, an industrial strain used for natamycin production. We demonstrate that $wblA_{ch}$ is an $AdpA_{ch}$ regulon that is under general direct positive control of $AdpA_{ch}$. We also find that $WblA_{ch}$ acts as a pivotal activator for natamycin biosynthesis and morphological differentiation in *S. chattanoogensis* L10.

MATERIALS AND METHODS

Media, plasmids, strains, and growth conditions. The strains used in the present study are listed in Table 1. General techniques for bacterial growth and isolation and manipulation of nucleic acids were carried out according to standard protocols for *Escherichia coli* and *S. coelicolor*, respectively (26). *S. chattanoogensis* L10 strains were grown at 28°C on YMG agar (1% malt extract, 0.4% yeast extract, 0.4% glucose, 0.2% $CaCO_3$, and 2% agar,

Received 5 June 2014 Accepted 19 August 2014
Published ahead of print 29 August 2014
Editor: M. Kivisaar
Address correspondence to Yong-Quan Li, lyq@zju.edu.cn.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01849-14.

doi:10.1128/AEM.01849-14

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
S. chattanoogensis		
WT	*S. chattanoogensis* L10 wild type	30
Δ*wblA*$_{ch}$	*wblA*$_{ch}$-disrupted mutant	This study
Δ*adpA*$_{ch}$	*adpA*$_{ch}$-disrupted mutant	28
YC1	*wblA*$_{ch}$-disrupted mutant complemented with *wblA*$_{ch}$ and its own promoter	This study
YC2	Wild-type strain carrying *wblA*$_{ch}$ overexpression	This study
YC3	Wild-type strain carrying an empty vector	This study
Saccharomyces cerevisiae BY4741	Indicator organism for natamycin bioassays	Laboratory stock
E. coli		
TG1	General cloning host	Novagen
ET12567/pUZ8002	Methylation-deficient *E. coli* strain for conjugation with the helper plasmid	41
BL21(DE3)	Host for protein expression	Novagen
BW25113/pIJ790	Strain used for PCR-targeted mutagenesis	27
Plasmids		
pMD19-T vector	General cloning vector	TaKaRa
pT-*wblA*$_{ch}$	pUC18 containing the promoter of *wblA*$_{ch}$	This study
pSET152	Integrative shuttle vector	26
pIJ8630	Integrative shuttle vector	42
pIJ773	Source of apramycin resistance cassette; *aac(3)IV oriT*	27
pMRD312	Cosmid containing *wblA*$_{ch}$	This study
pMRD313	*wblA*$_{ch}$ replaced by *aac(3)IV* cassette in pMRD312	This study
pYP1	*wblA*$_{ch}$ replaced by an 81-bp scar in pMRD312	This study
pYP2	pSET152 carrying *wblA*$_{ch}$ and its promoter	This study
pYP3	pIJ8630 carrying *wblA*$_{ch}$ and *ermE** promoter	This study

pH 7.2) for sporulation and at 30°C in YEME medium (0.3% yeast extract, 0.3% malt extract, 0.5% tryptone, 1% glucose) for natamycin production.

In-frame deletion and complementation of *wblA*$_{ch}$. Disruption of *wblA*$_{ch}$ was performed by gene replacement according to a modified PCR targeting system (27). First, the cosmid pMRD312, containing the *wblA*$_{ch}$ open reading frame (ORF) fragment, was introduced into *E. coli* BW25113/pIJ790. Second, the *wblA*$_{ch}$::FRT (FLP recombination target)-*oriT-aac(3)IV*-FRT disruption cassette was amplified from pIJ773 with primer pair wblA$_{ch}$-del-F/wblA$_{ch}$-del-R and electrotransformed into *E. coli* BW25113/pIJ790/cosmid pMRD312 to generate the disruption cosmid pMRD313, which replaced most of the *wblA*$_{ch}$ coding region (amino acids 50 to 112). Third, the targeted cosmid pMRD313 was transformed into *E. coli* DH5α/BT340 in order to excise the *aac(3)IV* cassette. The resulting cosmid, pYP1, was conjugated by *E. coli* ET12567/pUZ8002 into *S. chattanoogensis* L10. The *wblA*$_{ch}$ disruption mutant was selected by replica plating for thiostrepton-sensitive colonies and confirmed by PCR amplification with primer pair wblA$_{ch}$-F/wblA$_{ch}$-R.

For complementation, the integrative vector pSET152 was used. Primer pair wblA$_{ch}$-BamHI-F1/wblA$_{ch}$-EcoRV-R1 was used to amplify a 750-bp DNA fragment containing the *wblA*$_{ch}$ ORF and its own promoter. The amplified PCR products were then inserted into pSET152, which was digested with BamHI and EcoRV, and the resultant plasmid, pYP2, was integrated into the chromosome of the *wblA*$_{ch}$ deletion mutant at the phage ΦC31 *attB* site for complementation.

Overexpression of *wblA*$_{ch}$. For *wblA*$_{ch}$ overexpression, a 342-bp DNA fragment containing the complete *wblA*$_{ch}$ gene was amplified by using primers wblA$_{ch}$-NdeI-F and wblA$_{ch}$-NotI-F. Afterwards, this PCR product was subcloned into the pMD19 vector (TaKaRa) after dA addition and then digested with NdeI and NotI to give a NdeI-NotI DNA fragment containing *wblA*$_{ch}$. This fragment was ligated into the same sites of pIJ8630, which contained the *ermE** promoter inserted into the BamHI site. The resulting plasmid was then introduced into *E. coli* ET12567/pUZ8002 for *E. coli-Streptomyces* conjugation.

Microarray analysis. Mycelia of *S. chattanoogensis* L10 wild-type (WT) and mutant strains grown in YEME medium were collected at 16 h, 24 h, and 36 h. Total RNA was isolated by using the RNA Extract kit (Qiagen, Germany) according to the manufacturer's instructions and checked for an RNA integrity number (RIN) to inspect RNA integration by using an Agilent Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). Microarray assays, including primer design, labeling, hybridization, and washing, and microarray data normalization were performed by Shanghai Biotechnology Corporation (China) according to standard protocols.

Electrophoretic mobility shift assays. Expression of the recombinant AdpA$_{ch}$ protein with a His tag at its C terminus was performed with *E. coli* BL21, and the protein was purified by using Ni-nitrilotriacetic acid (NTA) His Bind resin (Novagen), as previously described (28). For probe preparation, probe A, probe B, and probe C, covering each binding site of the *wblA*$_{ch}$ promoter, were amplified with the primers listed in Table 2. The promoter region (384 bp) of *wblA*$_{ch}$ was amplified with primer pair wblA$_{ch}$-EMSA-F/wblA$_{ch}$-EMSA-R. Afterwards, the PCR products were cloned into the pMD19 vector (TaKaRa). The biotin-labeled probes were then prepared by PCR amplification with 5′-biotin-labeled M13 universal primers. About 1 ng of probe was incubated with purified His$_6$-AdpA$_{ch}$ at 25°C for 30 min in buffer (20 mM Tris [pH 7.5], 0.01% bovine serum albumin [BSA], 5% glycerol, 50 μg ml^{-1} sheared salmon sperm DNA); the protein concentrations used are indicated in Fig. 2. For the competition assay, 100-fold molar excesses of unlabeled probe and nonspecific DNA were each added to 0.2 μg purified His$_6$-AdpA$_{ch}$. Reactions were displayed on 5% acrylamide gels for separation in 0.5× Tris-borate-EDTA (TBE) buffer. Electrophoretic mobility shift assay (EMSA) gels were then electroblotted onto a nylon membrane and UV fixed by using a UV cross-linker. Labeled DNA was detected with streptavidin-horseradish peroxidase (HRP) and BeyoECL Plus (Beyotime, China) according to the manufacturer's instructions.

DNase I footprinting assay. A DNase I footprinting assay was performed as previously described (29). First, the 6-carboxyfluorescein (FAM)-labeled *wblA*$_{ch}$ probe was amplified by PCR using 5′-FAM-labeled M13 universal primers from plasmid pT-*wblA*$_{ch}$, followed by gel recovery. About 50 ng of fluorescently labeled probe was then added to the reaction

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5′–3′)[a]
Construction of *wblA$_{ch}$* mutant	
wblA$_{ch}$-del-F	TGTCCGGTGCGTACGGAGTGCCTGGCCGACGCCCTGGACATTCCGGGGATCCGTCGACC
wblA$_{ch}$-del-R	CGCGGCGTACTCGTCGAAGGCCTCGCCCTCGCAGTCCACTGTAGGCTGGAGCTGCTTC
wblA$_{ch}$-F	TACGCCTCGGTTCGTCAA
wblA$_{ch}$-R	ATCGCACCACGGAAACC
Construction of complemented strain	
wblA$_{ch}$-BamHI-F1	AGGATCCGCGGACAACCCTACGTTC
wblA$_{ch}$-EcoRV-R1	TGATATCATCGCACCACGGAAACC
Overexpression of *wblA$_{ch}$*	
wblA$_{ch}$-NdeI-F	AACATATGCAAGGAGCGGCGCAGA
wblA$_{ch}$-NotI-R	AAGCGGCCGCCTACCCGACCGCGGCGTA
Construction of His$_6$-tagged WblA$_{ch}$	
wblA$_{ch}$-BamHI-F	AGGATCCGTGCAAGGAGCGGCGCAGA
wblA$_{ch}$-XhoI-R	ACTCGAGCTACCCGACCGCGGCGTA
RT-PCR	
hrdB-RT-F	CTGCTTGAGGTAGTCCTTGACC
hrdB-RT-R	AAGAGCGTCGCAGCGAAGAGTC
whiA-RT-F	TGGTCAGCGGACGCATTG
whiA-RT-R	CACCACCCGCACGACATAG
whiB-RT-F	TGCTGGTCGAGGAGGC
whiB-RT-R	CCCCAGATGCCGAAGC
whiD-RT-F	AGTTCGCTCTTCTTCCACCC
whiD-RT-R	GTTCTTCGCGTTCGTCCTC
whiE-RT-F	GCGGCATCACCCTCTTCA
whiE-RT-R	ATGCGCCAGCGGTTCTC
whiG-RT-F	TGGACACGCTGGAGGACA
whiG-RT-R	TCTGACTCACCCGGCTCT
whiH-RT-F	ACCGCAGCGTGACGACCA
whiH-RT-R	CGAGGGACAGCAGGAGGGT
whiI-RT-F	TATGTCGTCACCGGCACCC
whiI-RT-R	CGATGGCCTTGTTGGACTGG
scnRII-RT-F	TGCCGGCTGAGGTAGAG
scnRII-RT-R	GTGCAGCAGCCGTTAGT
scnRI-RT-F	ATGACCTGCCGTTAGGC
scnRI-RT-R	GACCAGACGCTCGAAGC
wblA$_{ch}$-RT-F	AGCCTGCCGCACTACC
wblA$_{ch}$-RT-R	TACGCACCGGACACCC
EMSA	
wblA$_{ch}$-EMSA-F	GCGGACAACCCTACGTTC
wblA$_{ch}$-EMSA-R	GCTGGCGCCGTCCTCTCC
Site-A-F	TCTTGAATGGCCCGAACGGACTATGCGA
Site-A-R	CGCATAGTCCGTTCGGGCCATTCAAGAA
Site-mA-F	TCTTGAAGATATCGAACGGACTATGCGA
Site-mA-R	CGCATAGTCCGTTCGATATCTTCAAGAA
Site-B-F	ACATGGGACTTCCGGCGACACAAGCGA
Site-B-R	CGCTTGTGTCGCCGGAAGTCCCATGTA
Site-mB-F	ACATGGGACTTCGATATCCACAAGCGA
Site-mB-R	CGCTTGTGGATATCGAAGTCCCATGTA
Site-C-F	CCTCTCTCTGTGCGGCCCCTGCCCCA
Site-C-R	GGGGCAGGGGCCGCACAGAGAGAGGA
Site-mC-F	CCTCTCTCTGTGGATATCCTGCCCCA
Site-mC-R	GGGGCAGGATATCCACAGAGAGAGGA

[a] Sequences representing restriction sites and mutagenesis are underlined.

mixture to a final volume of 50 μl together with the AdpA$_{ch}$ protein, which was ultrafiltered with a centrifugal ultrafiltration device (YM-10; Millipore) for a 10-kDa cutoff and eluted in 20 mM Tris buffer (pH 7.5). After binding of the AdpA$_{ch}$ protein to the *wblA$_{ch}$* probe (30°C for 30 min), 0.01 U of DNase I (Promega) was added for 1 min at 30°C. The reactions were then stopped with an equal volume of 100 mM EDTA, and samples were extracted by using phenol-chloroform. After precipitation with a combination of 10 μl 7.5 M ammonium acetate (NH_4Ac), 40 μg

glycogen, and 100 μl 100% ethanol, the digested DNA mixture was loaded into an ABI 3130 DNA sequencer with the Liz-500 DNA marker (MCLAB). The DNA sequencing ladder was prepared according to instructions provided with the Thermo Sequenase dye primer manual cycle sequencing kit (USB).

Real-time quantitative PCR analysis. Mycelium was harvested from strains grown in YEME medium or on YMG agar medium overlaid with cellophane discs for different times. Total RNAs were then isolated by using TRIzol reagent (Bio Basic Inc.) according to the manufacturer's guidelines. After treatment with DNase I (TaKaRa), PCR was carried out to ensure that there was no genomic DNA. The quality and quantity of RNA samples were then determined by UV spectroscopy and checked by agarose gel electrophoresis. A primeScript First Strand cDNA synthesis kit (TaKaRa) was used to generate cDNA from 2 μg of total RNAs according to the manufacturer's protocol. Quantitative real-time PCR of selected genes was performed on a Roche LightCycler 480 instrument (Roche), and ~1 ng of synthesized cDNA was used as a DNA template in a 20-μl final reaction mixture volume for real-time quantitative PCR (qRT-PCR) with 10 μl Power SYBR green PCR master mix. *hrdB* was used as an internal control, and all experiments were done in triplicate.

Natamycin bioassay and HPLC analysis. Natamycin produced by *S. chattanoogensis* on YMG agar was measured by bioassays using *Saccharomyces cerevisiae* as an indicator organism. Agar plugs were cut from the lawns of *S. chattanoogensis* that had been grown on YMG medium for 10 days by using a core borer (about 0.8 cm in diameter) and were placed onto a lawn of freshly plated *Saccharomyces cerevisiae* cells. After incubation at 30°C for 18 h, the inhibition zones of the bioassay plates were recorded. Natamycin production was further confirmed by high-performance liquid chromatography (HPLC) analysis using the Agilent 1100 HPLC system. An Agilent HC-C_{18} column (5 μm, 4.6 by 250 mm) was used, and the UV detector was set at 303 nm. The mobile phase and gradient elution processes were previously described (30).

Scanning electron microscopy. For scanning electron microscopy, agar plugs ~1 cm in diameter were cut from the lawns of *S. chattanoogensis* strains grown on YMG medium for 10 days and then plunged into liquid nitrogen. The cut agar blocks were observed by scanning electron microscopy (S-3000N; Hitachi) after sputter coating with gold. The detailed process was performed as previously described (28).

Accession numbers. The GenBank accession numbers for the sequences of *wblA$_{ch}$*, *whi* genes, and other genes in the microarray analysis are KM264326 to KM264342. Experimental details and data from the microarrays have been deposited in the Gene Expression Omnibus (GEO) under accession no. GSE59806.

RESULTS

Identification of *wblA$_{ch}$* as a member of the AdpA$_{ch}$ regulon by transcriptional analysis. AdpA is an important pleiotropic regulator in the A-factor regulatory cascade in *Streptomyces griseus* (31). Induction of *adpA* requires the dissociation of ArpA from the *adpA* promoter, and the members of the AdpA regulon are then activated to participate in several important cellular functions (32, 33). In our previous study, we showed that AdpA$_{ch}$ plays an important role in natamycin biosynthesis and morphological differentiation in *S. chattanoogensis* L10 (28). For a more detailed analysis of AdpA$_{ch}$, we compared the pattern of gene expression of the wild-type (WT) strain to that of the Δ*adpA$_{ch}$* mutant strain. Mycelium was harvested after 16 h, 24 h, and 36 h of growth, and RNA was isolated from three independent cultures of each strain.

In the DNA microarray analysis, we used the criterion of a fold change of ≥2 and ≤0.5 ($P < 0.05$) to discriminate AdpA$_{ch}$-responsive genes. According to this criterion, approximately 809 and 431 genes were transcriptionally downregulated and upregulated, respectively, in the *adpA$_{ch}$* mutant strain compared to the WT strain at all tested time points. Among these genes, we noticed

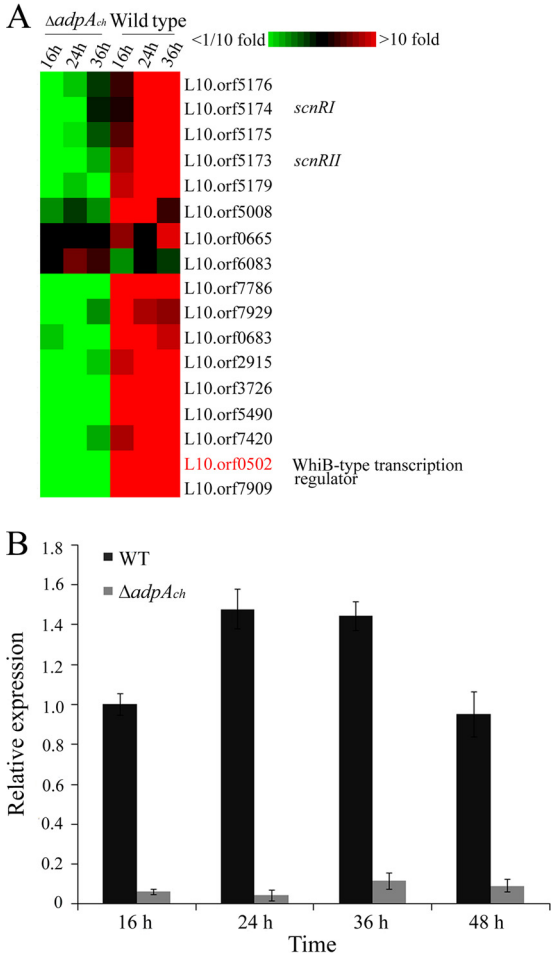

FIG 1 Expression profiles of *wblA$_{ch}$* differentially expressed during growth of the WT strain and the Δ*adpA$_{ch}$* mutant. (A) Microarray analysis of the transcription profiles of selected genes. RNA samples were isolated at 16 h, 24 h, and 36 h during growth on YEME medium. (B) RT-PCR analysis of *wblA$_{ch}$* transcript levels in the WT strain and the Δ*adpA$_{ch}$* mutant. The expression level of *wblA$_{ch}$* is presented relative to the wild-type sample at 16 h, which was arbitrarily assigned a value of 1. The transcription level of *hrdB* was assayed as an internal control. Error bars were calculated by measuring the standard deviations among data from three replicates of each sample.

that L10.orf0502, which encodes a WhiB-type transcription regulator, was downregulated 79-fold at 16 h, 23-fold at 24 h, and 6.4-fold at 36 h in the Δ*adpA$_{ch}$* mutant strain (Fig. 1A). These results suggested that L10.orf0502 is positively regulated by AdpA$_{ch}$. A sequence similarity search showed that L10.orf0502 is an orthologous protein of the WblA protein (GenBank accession no. NP_627776.1) (90% identity) of *Streptomyces coelicolor* A3(2). Thus, it was designated *wblA$_{ch}$*. To further verify the microarray data and clarify the relationship between the *wblA$_{ch}$* and AdpA$_{ch}$ in *S. chattanoogensis* L10, we carried out real-time quantitative PCR (qRT-PCR) to test the expression of the *wblA$_{ch}$* gene in the Δ*adpA$_{ch}$* mutant and in the WT strain. As expected, the transcript levels of *wblA$_{ch}$* in the Δ*adpA$_{ch}$* disruption mutant had decreased significantly in contrast to that of the WT strain at all tested time points (Fig. 1B). These results further confirmed that AdpA$_{ch}$ acts as an activator of *wblA$_{ch}$* transcription in *S. chattanoogensis* L10.

Interaction of AdpA$_{ch}$ with the promoter region of *wblA$_{ch}$*. To determine whether AdpA$_{ch}$ plays a direct role in the regulation

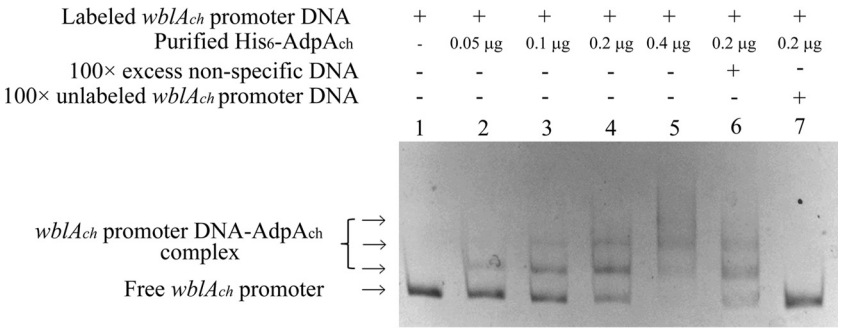

FIG 2 Gel mobility shift assays using a labeled DNA fragment containing the *wblA*$_{ch}$ promoter region and the His$_6$-AdpA$_{ch}$ protein. Lane 1, labeled fragment; lanes 2 to 5, labeled fragment with 0.05 μg, 0.1 μg, 0.2 μg, and 0.4 μg of purified His$_6$-AdpA$_{ch}$ protein, respectively; lane 6, labeled fragment with a 100-fold excess of nonspecific DNA and 0.2 μg purified His$_6$-AdpA$_{ch}$ protein; lane 7, labeled fragment with a 100-fold excess of unlabeled *wblA*$_{ch}$ promoter DNA and 0.2 μg purified His$_6$-AdpA$_{ch}$ protein.

of the *wblA*$_{ch}$ gene, electrophoretic mobility shift assays (EMSAs) were performed. For this purpose, AdpA$_{ch}$ was overexpressed in the *E. coli* BL21 strain as a recombinant His$_6$-tagged protein and purified by Ni-NTA agarose chromatography. A biotin-labeled *wblA*$_{ch}$ probe covering the *wblA*$_{ch}$ promoter region from positions −321 to +63 was prepared. Results from EMSAs showed that purified His$_6$-AdpA$_{ch}$ can bind to the upstream regions of *wblA*$_{ch}$ to form a complex (Fig. 2). There was only one shifted band for the *wblA*$_{ch}$ probe at a low His$_6$-AdpA$_{ch}$ protein concentration. However, when the protein concentration was increased, two other shifted bands were observed, accompanied by the disappearance of the lower band (Fig. 2, lanes 2 to 5). It is therefore likely that there is more than one His$_6$-AdpA$_{ch}$-binding site located on the *wblA*$_{ch}$ promoter region. In order to examine binding specificity, EMSAs with an excess of unlabeled specific and nonspecific competitor DNA were performed. As shown in Fig. 2, the retarded bands (lanes 6 and 7) disappeared in the presence of excess unlabeled *wblA*$_{ch}$ probe but not in the presence of nonspecific DNA. These results suggest that purified AdpA$_{ch}$ regulates the transcription of *wblA*$_{ch}$ by specifically binding to the upstream regions of *wblA*$_{ch}$.

To further define the accurate binding sites of AdpA$_{ch}$ in the upstream region of *wblA*$_{ch}$, DNase I footprinting assays were carried out. The experiments revealed that His$_6$-AdpA$_{ch}$ protected three regions, from nucleotides −83 to −44, −182 to −160, and −226 to −192 relative to the *wblA*$_{ch}$ transcription start point (Fig. 3A and C). After further analysis of the protected DNA regions, we found that there was a highly conserved AdpA$_{ch}$-binding sequence in each binding site, which is identical to that found in *S. griseus* (Fig. 3B) (34). Therefore, series of mutations were constructed to

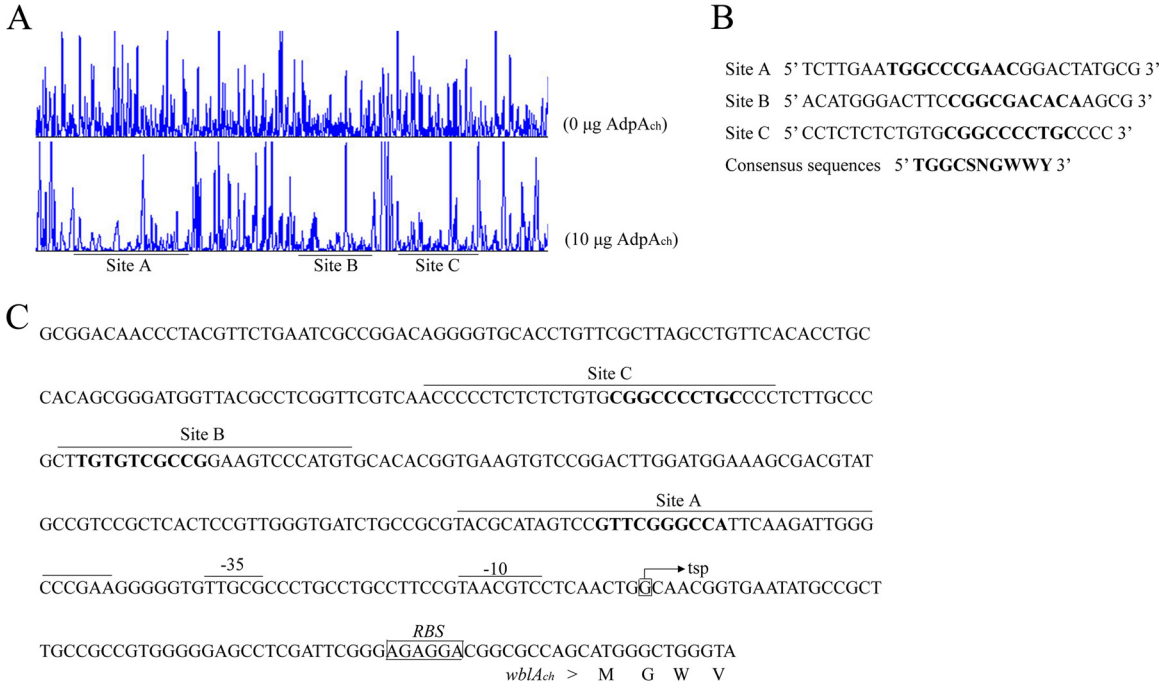

FIG 3 DNase I footprinting assay for determination of the AdpA$_{ch}$-binding site. (A) A 5′-FAM-labeled *wblA*$_{ch}$ probe was used in the DNase I footprinting assay without or with purified AdpA$_{ch}$ (10 μg). The protected regions are underlined. (B) Sequences of the determined AdpA$_{ch}$-binding sites and the consensus AdpA$_{ch}$-binding sequence (in boldface type). (C) Nucleotide sequences of the *wblA*$_{ch}$ promoter region and the predicted AdpA$_{ch}$-binding sites. The transcription start point (tsp) is marked by a bent arrow, the AdpA$_{ch}$-binding sites are overlined, and the ribosome-binding site (RBS) is boxed.

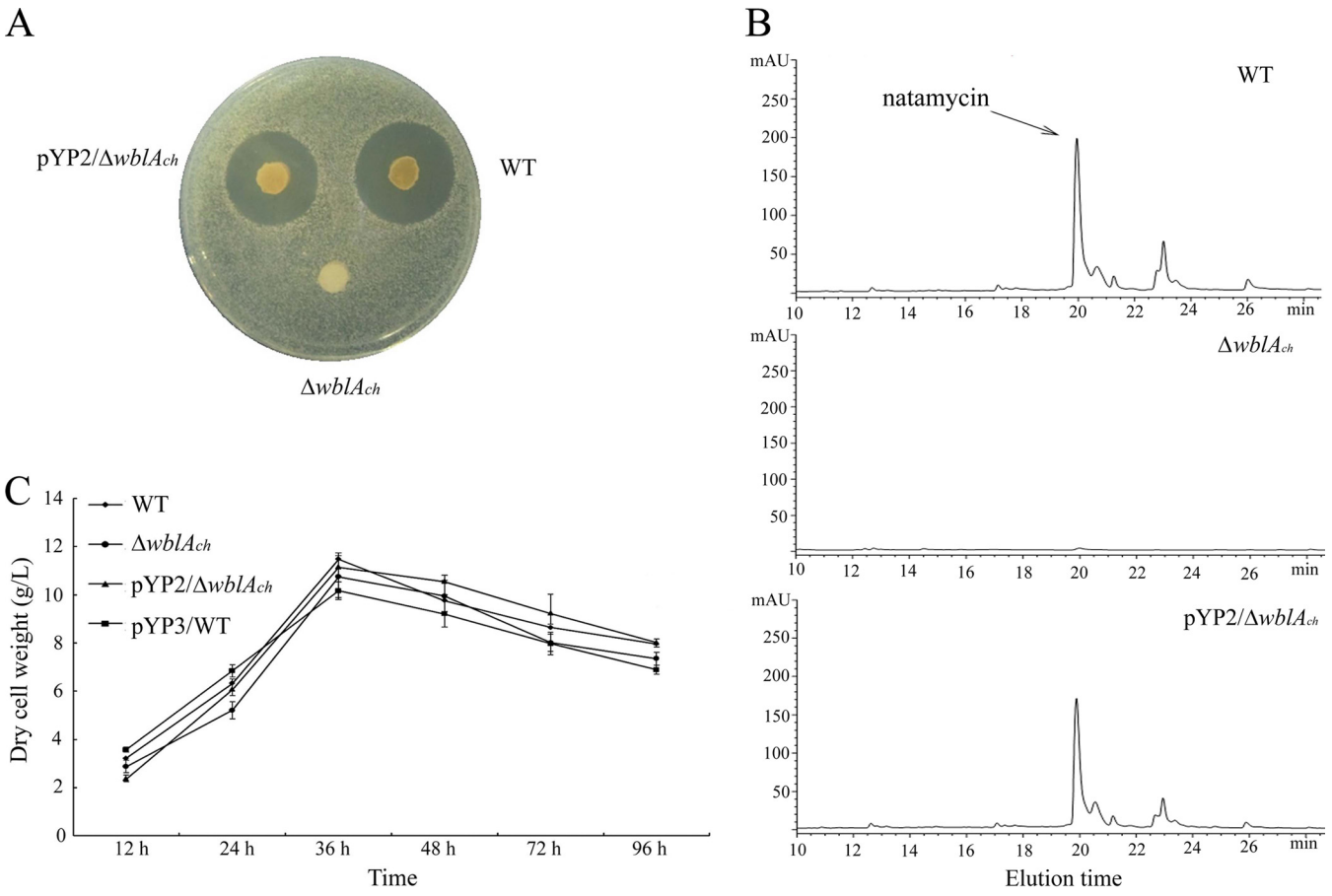

FIG 4 Effect of *wblA*$_{ch}$ disruption on growth and production of natamycin. (A and B) Natamycin bioassay (A) and HPLC analysis of fermentation filtrates (B) from the WT strain, the *wblA*$_{ch}$ deletion mutant, and the *wblA*$_{ch}$-complemented mutant. The peak of natamycin is marked by an arrow. mAU, milli-absorbance units. (C) Growth of the wild-type strain and mutant strains.

determine the actual AdpA$_{ch}$-binding sites. As shown in Fig.S4 in the supplemental material, no binding shift was detected for mutated sites A to C compared with their corresponding wild-type targets. Thus, these consensus sequences are essential for the binding activity of AdpA$_{ch}$.

***wblA*$_{ch}$ is a pivotal activator of natamycin biosynthesis.** Previous reports have shown that WblA and its orthologues act as negative regulators for antibiotic biosynthesis in various *Streptomyces* species and that *wblA* deletion mutants lead to overproduction of the corresponding antibiotics (21–24). In order to identify the function of *wblA*$_{ch}$ in natamycin biosynthesis in *S. chattanoogensis* L10, a *wblA*$_{ch}$ deletion mutant was constructed via homologous recombination, as described Materials and Methods. The disruption mutant was confirmed by PCR analysis and Southern blot analysis (see Fig. S1 in the supplemental material). Meanwhile, the complemented strain was constructed by reintroducing a 750-bp DNA fragment containing *wblA*$_{ch}$ and its own promoter into the mutant strain. To assess natamycin production, *Saccharomyces cerevisiae* was used as an indicator organism for the bioassay. As shown in Fig. 4A, no inhibition was observed with the agar plug from the *wblA*$_{ch}$ deletion mutant, whereas the growth-inhibiting activity against *S. cerevisiae* was restored when plasmid pYP2 was reintroduced into the Δ*wblA*$_{ch}$ mutant. To further confirm this phenotype, high-performance liquid chromatography (HPLC) analysis was carried out. No peak of natamycin was present in the culture filtrates of the *wblA*$_{ch}$ deletion mutant, in contrast to the culture filtrates of the WT strain, and the complemented strain had restored the production of natamycin to a level similar to that of the WT strain (Fig. 4B). Nevertheless, these three strains had comparable growth rates and similar biomasses (Fig. 4C). These results indicate that WblA$_{ch}$ may act as a positive regulator for natamycin biosynthesis.

To test this hypothesis, we carried out qRT-PCR to evaluate the effects of WblA$_{ch}$ on the transcription of *scnRI* and *scnRII*, pathway-specific regulators for natamycin biosynthesis (28). As expected, the transcript levels of *scnRI* and *scnRII* were lower in the *wblA*$_{ch}$ mutant strain than in the WT strain at all tested time points from 16 h to 48 h (Fig. 5). Moreover, WblA$_{ch}$ was expressed as an N-terminally His$_6$-tagged protein in order to test whether WblA$_{ch}$ directly regulates natamycin biosynthesis by its binding activity. Unfortunately, the EMSA result showed that WblA$_{ch}$ cannot directly bind to the promoters of *scnRI*, *scnRII*, or any of the other structural genes in the cluster (data not shown).

Overexpression of *wblA*$_{ch}$ results in increased natamycin production. Previous investigations indicated that overexpression of *wblA* resulted in decreased production of the corresponding antibiotic (21–24). To evaluate the function of *wblA*$_{ch}$ in *S. chattanoogensis* L10, pIJ8630::*wblA*$_{ch}$ was constructed, in which *wblA*$_{ch}$ was under the control of the strong constitutive *ermE** promoter, and this recombinant plasmid was then introduced

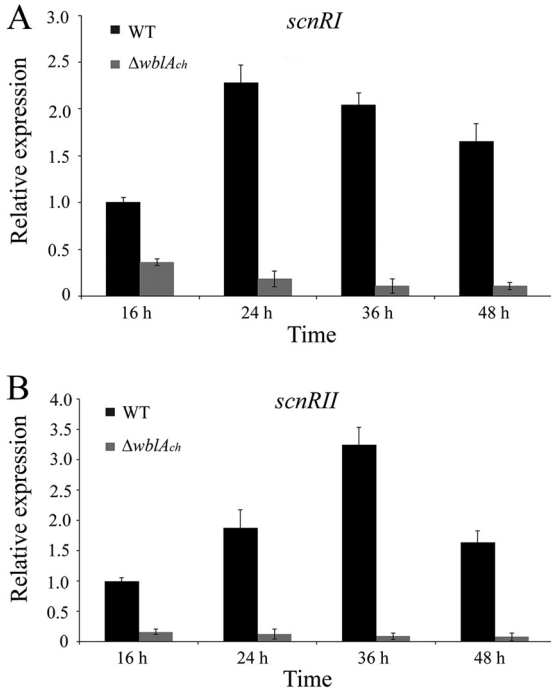

FIG 5 Quantitative RT-PCR analysis of *scnRI* (A) and *scnRII* (B) in the WT strain and the *wblA*$_{ch}$ deletion mutant. The RNA samples were obtained from cultures grown in YEME medium for 16 h, 24 h, 36 h, and 48 h. The expression levels of *scnRI* and *scnRII* are presented relative to the levels in the wild-type sample at 16 h, which was arbitrarily assigned a value of 1. The transcription level of *hrdB* was assayed as an internal control, and error bars were calculated by measuring the standard deviations among data from three replicates of each sample.

into the WT strain. As shown in Fig. 6, the level of natamycin production of the resulting mutant (YC2) was increased ~1.3-fold compared to that of the WT strain after 96 h of incubation, although the biomasses of these strains were similar (Fig. 4C). This result reinforced the evidence that *wblA*$_{ch}$ is a pivotal activator for natamycin production.

Pleiotropic effects of *wblA*$_{ch}$ on morphology differentiation. To investigate the effect of a deletion of *wblA*$_{ch}$ on development, the mutant was compared to the wild-type strain on YMG medium. As shown in Fig.S2A in the supplemental material, disruption of *wblA*$_{ch}$ caused a *whi* phenotype, being able to erect aerial hyphae but defective in sporulation. Scanning electron microscopy of the *wblA*$_{ch}$ mutant grown on YMG medium revealed thin, sparse aerial hyphae, with characteristic aberrant sporulation septation (see Fig. S2B in the supplemental material). The morphological deficiency in the mutant strain was fully complemented by the pSET152 derivative carrying a 750-bp DNA fragment containing *wblA*$_{ch}$ and its own promoter. In addition, we further evaluated the transcription of *whi* genes, which are related to spore formation in *Streptomyces*. Our data showed that the transcription levels of the *whi* genes were severely decreased in the Δ*wblA*$_{ch}$ mutant, with the exception of *whiD*, which exhibited a slight increase (Fig. 7). Other *whi* genes, such as *whiA* and *whiE*, showed low transcriptional activity in the Δ*wblA*$_{ch}$ mutant, ~20% of the wild-type levels, while transcriptional activities of *whiB*, *whiH*, and *whiI* were almost absent in the mutant. These results implied that WblA$_{ch}$ may play a part in morphological differentiation through interacting with other *whi* genes.

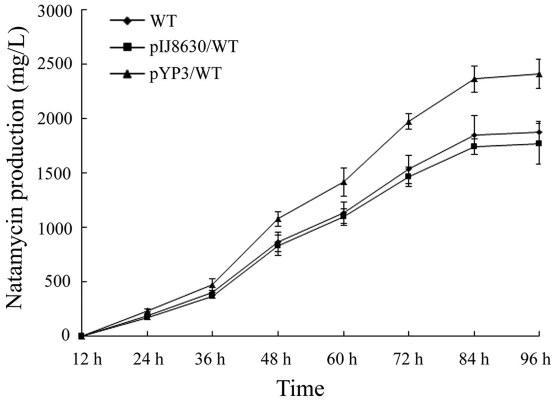

FIG 6 Effect of *wblA*$_{ch}$ overexpression on production of natamycin. Natamycin production of fermentation filtrates in the wild-type strain (WT), the wild-type strain with pIJ8630 (pIJ8630/WT), and the *wblA*$_{ch}$ overexpression strain (pYP3/WT) at different incubation times is shown.

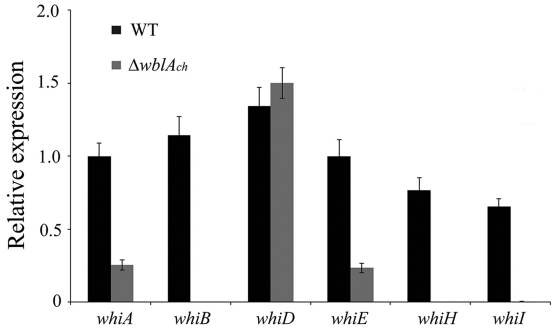

FIG 7 Effect of *wblA*$_{ch}$ disruption on the transcription of *whi* genes. The RNA samples were harvested from strains grown on YMG agar medium overlaid with cellophane discs for 4 days. The expression level of *whiA* is presented relative to the wild-type sample, which was arbitrarily assigned a value of 1. The transcription level of *hrdB* was assayed as an internal control, and error bars were calculated by measuring the standard deviations among data from three replicates of each sample.

DISCUSSION

The biochemical and genetic mechanisms of WblA with regard to secondary metabolite and morphological differentiation have not been determined previously. In this study, we found that AdpA$_{ch}$, a pleiotropic regulator in *S. chattanoogensis* L10, acts as a positive regulator of the expression of *wblA*$_{ch}$. However, *wblA* was previously reported to be negatively regulated by AdpA in *S. coelicolor*, a model organism for studying bacterial differentiation (25). This finding is directly contradictory to our own findings. AdpA$_{ch}$ shares 92% sequence identity with AdpA$_{sc}$ (28), and WblA$_{ch}$ shares 90% sequence identity with WblA$_{sc}$. Although these two types of protein share good amino acid sequence homology, different regulatory patterns are observed in the AdpA-WblA regulatory relationship in these two strains. The host-specific characteristics of the WblA orthologues in different strain backgrounds seem inconceivable because of the important roles of these proteins in controlling antibiotic production and morphological differentiation in *Streptomycetes*. Notably, results of microarray and chromatin immunoprecipitation (ChIP)/chromatin affinity precipitation (ChAP)-seq analysis of *Streptomyces griseus* coincide with our results (35, 36). Moreover, *wblA*$_{ch}$ was shown to encode a

positive regulator participating in natamycin biosynthesis in *S. chattanoogensis* L10, whereas *wblA* and its orthologues were previously determined to be novel antibiotic downregulators in various *Streptomyces* species. Based on these cumulative evidences, $WblA_{ch}$ in *S. chattanoogensis* L10, an industrial strain for natamycin production, is suggested to be involved in a number of functions that differ from those observed in other *Streptomyces* species in previous reports.

The Wbl proteins, which harbor a predicted helix-turn-helix motif, are supposed to be able to bind DNA as transcription factors. A series of Wbl proteins, including WhiB1 (37), WhiB2 (38), WhiB3 (13), and WhiB4 (17), have been experimentally demonstrated to bind with DNA. However, the EMSA results did not support the hypothesis that $WblA_{ch}$ has the binding ability to regulate the expression of genes involved in natamycin biosynthesis directly. Similar to our results, previous genetic studies on WblA orthologues did not reveal the mechanisms by which they regulate antibiotic production in *Streptomyces*. Previous reports showed that Wbl proteins in general may change their regulatory properties in response to dormancy signals, including O_2 and nitric oxide (NO), via their iron-sulfur cluster (39). It was also shown that the Wbl proteins can bind specific target proteins as ligands to modify their activity (40). Thus, it is likely that the binding activity of WblA orthologues may require a redox state or interaction with other cellular proteins.

Natamycin is the major secondary metabolite of *S. chattanoogensis* L10 in YEME medium; simultaneously, a yellow pigment is generated in the same medium. Several lines of evidence imply that these two pathways compete for precursors originating from primary metabolism. In our study, overexpression of $wblA_{ch}$ resulted in increased production of natamycin, whereas the production of yellow pigment had decreased severely (see Fig. S3 and Table S1 in the supplemental material). Notably, the available literature reported that Wbl proteins are involved in the metabolic switchover, such as WhiB3, which was shown to act as an intracellular redox sensor and regulates fatty acid metabolism by binding directly to its promoter sequence in *M. tuberculosis* (13), and WhiB5, which was shown to be involved in metabolic regulation during starvation in *M. tuberculosis* (18). WblA was reported to be able to sense and respond to the intracellular redox environment in a manner considered to be coupled to central metabolism (7, 39). Based on this characteristic of the Wbl protein, we speculated that $WblA_{ch}$ may act as a metabolic regulator where overexpression of $wblA_{ch}$ results in a redirection of the flux toward the biosynthesis of natamycin. Thus, it could provide a strategy for engineering new overproducer strains of natural products where regulation of the enzymes involved in metabolic flux becomes a realistic way to generate improved strains. In sum, our findings in this study should lead to an increased understanding of the biological function of the WhiB-like protein WblA, and further experiments will provide more accurate information on the biological functions of these proteins.

ACKNOWLEDGMENTS

We are grateful to Chris Wood for critical reading and scientific editing.

This work was supported by the Key Program of Zhejiang Provincial Natural Science Foundation of China (grant no. LZ12C01001), the National Basic Research Program of China (973 Program) (grant no. 2012CB721005), the National High-Technology Research and Development Program of China (863 Program) (grant no. 2012AA02A706), the National Key Technology R and D Program (grant no. 2011BAD23B05-2), and the Specialized Research Fund for the Doctoral Program of Higher Education (grant no. 20120101110143).

REFERENCES

1. **Hopwood DA.** 2007. *Streptomyces* in nature and medicine: the antibiotic makers. Oxford University Press, New York, NY.
2. **Demain AL.** 1999. Pharmaceutically active secondary metabolites of microorganisms. Appl. Microbiol. Biotechnol. **52:**455–463. http://dx.doi.org/10.1007/s002530051546.
3. **van Wezel GP, McDowall KJ.** 2011. The regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances. Nat. Prod. Rep. **28:**1311–1333. http://dx.doi.org/10.1039/c1np00003a.
4. **Chater KF.** 1993. Genetics of differentiation in *Streptomyces*. Annu. Rev. Microbiol. **47:**685–713. http://dx.doi.org/10.1146/annurev.mi.47.100193.003345.
5. **Liu G, Chater KF, Chandra G, Niu GQ, Tan HR.** 2013. Molecular regulation of antibiotic biosynthesis in *Streptomyces*. Microbiol. Mol. Biol. Rev. **77:**112–143. http://dx.doi.org/10.1128/MMBR.00054-12.
6. **Takano E.** 2006. γ-Butyrolactones: *Streptomyces* signaling molecules regulating antibiotic production and differentiation. Curr. Opin. Microbiol. **9:**287–294. http://dx.doi.org/10.1016/j.mib.2006.04.003.
7. **Soliveri JA, Gomez J, Bishai WR, Chater KF.** 2000. Multiple paralogous genes related to the *Streptomyces coelicolor* developmental regulatory gene *whiB* are present in *Streptomyces* and other actinomycetes. Microbiology **146:**333–343.
8. **Zheng F, Long QX, Xie JP.** 2012. The function and regulatory network of WhiB and WhiB-like protein from comparative genomics and systems biology perspectives. Cell Biochem. Biophys. **63:**103–108. http://dx.doi.org/10.1007/s12013-012-9348-z.
9. **Geiman DE, Raghunand TR, Agarwal N, Bishai WR.** 2006. Differential gene expression in response to exposure to antimycobacterial agents and other stress conditions among seven *Mycobacterium tuberculosis whiB*-like genes. Antimicrob. Agents Chemother. **50:**2836–2841. http://dx.doi.org/10.1128/AAC.00295-06.
10. **Den Hengst CD, Buttner MJ.** 2008. Redox control in actinobacteria. Biochim. Biophys. Acta **1780:**1201–1216. http://dx.doi.org/10.1016/j.bbagen.2008.01.008.
11. **Rustad TR, Harrell MI, Liao R, Sherman DR.** 2008. The enduring hypoxic response of *Mycobacterium tuberculosis*. PLoS One **3:**e1502. http://dx.doi.org/10.1371/journal.pone.0001502.
12. **Larsson C, Luna B, Ammerman NC, Maiga M, Agarwa N, Bishai WR.** 2012. Gene expression of *Mycobacterium tuberculosis* putative transcription factors *whiB1-7* in redox environments. PLoS One **7:**e37516. http://dx.doi.org/10.1371/journal.pone.0037516.
13. **Singh A, Crossman DK, Mai D, Guidry L, Voskui MI, Renfrow MB, Steyn AJC.** 2009. *Mycobacterium tuberculosis* WhiB3 maintains redox homeostasis by regulating virulence lipid anabolism to modulate macrophage response. PLoS Pathog. **5:**e1000545. http://dx.doi.org/10.1371/journal.ppat.1000545.
14. **Rohde K, Yates RM, Purdy GE, Russell DG.** 2007. *Mycobacterium tuberculosis* and the environment within the phagosome. Immunol. Rev. **219:**37–54. http://dx.doi.org/10.1111/j.1600-065X.2007.00547.x.
15. **Burian J, Yim G, Hsing M, Axerio-Cilies P, Cherkasov A, Spiegelman GB, Thompson CJ.** 2013. The mycobacterial antibiotic resistance determinant WhiB7 acts as a transcriptional activator by binding the primary sigma factor SigA (RpoV). Nucleic Acids Res. **41:**10062–10076. http://dx.doi.org/10.1093/nar/gkt751.
16. **Steyn AJC, Collins DM, Hondalus MK, Jacobs WR, Kawakami RP, Bloom BR.** 2002. *Mycobacterium tuberculosis* WhiB3 interacts with RpoV to affect host survival but is dispensable for in vivo growth. Proc. Natl. Acad. Sci. U. S. A. **99:**3147–3152. http://dx.doi.org/10.1073/pnas.052705399.
17. **Chawla M, Parikh P, Saxena A, Munshi M, Mehta M, Mai D, Srivastava AK, Narasimhulu KV, Redding KE, Vashi N, Kumar D, Steyn AJC, Singh A.** 2012. *Mycobacterium tuberculosis* WhiB4 regulates oxidative stress response to modulate survival and dissemination in vivo. Mol. Microbiol. **85:**1148–1165. http://dx.doi.org/10.1111/j.1365-2958.2012.08165.x.
18. **Casonato S, Sánchez AC, Haruki H, González MR, Provvedi R, Dainese E, Jaouen T, Gola S, Bini E, Vicente M, Johnsson K, Ghisotti D, Palù G, Hernández-Pando R, Manganelli R.** 2012. WhiB5, a transcriptional reg-

ulator that contributes to *Mycobacterium tuberculosis* virulence and reactivation. Infect. Immun. **80:**3132–3144. http://dx.doi.org/10.1128/IAI.06328-11.

19. **Konar M, Alam MS, Arora C, Agrawal P.** 2012. WhiB2/Rv3260c, a cell division-associated protein of *Mycobacterium tuberculosis* H37Rv, has properties of a chaperone. FEBS J. **279:**2781–2792. http://dx.doi.org/10.1111/j.1742-4658.2012.08662.x.
20. **Fowler-Goldsworthy K, Gust B, Mouz S, Chandra G, Findlay KC, Chater KF.** 2011. The actinobacteria-specific gene *wblA* controls major developmental transitions in *Streptomyces coelicolor* A3(2). Microbiology **157:**1312–1328. http://dx.doi.org/10.1099/mic.0.047555-0.
21. **Kang SH, Huang J, Lee HN, Hur YA, Cohen SN, Kim ES.** 2007. Interspecies DNA microarray analysis identifies WblA as a pleiotropic down-regulator of antibiotic biosynthesis in *Streptomyces*. J. Bacteriol. **189:**4315–4319. http://dx.doi.org/10.1128/JB.01789-06.
22. **Noh JH, Kim SH, Lee HN, Lee SY, Kim ES.** 2010. Isolation and genetic manipulation of the antibiotic down-regulatory gene, *wblA* ortholog for doxorubicin-producing *Streptomyces* strain improvement. Appl. Microbiol. Biotechnol. **86:**1145–1153. http://dx.doi.org/10.1007/s00253-009-2391-z.
23. **Nah JH, Park SH, Yoon HM, Choi SS, Lee CH, Kim ES.** 2012. Identification and characterization of *wblA*-dependent *tmcT* regulation during tautomycetin biosynthesis in *Streptomyces* sp. CK4412. Biotechnol. Adv. **30:**202–209. http://dx.doi.org/10.1016/j.biotechadv.2011.05.004.
24. **Rabyk M, Ostash B, Rebets Y, Walker S, Fedorenko V.** 2011. *Streptomyces ghanaensis* pleiotropic regulatory gene *wblA*$_{gh}$ influences morphogenesis and moenomycin production. Biotechnol. Lett. **33:**2481–2486. http://dx.doi.org/10.1007/s10529-011-0728-z.
25. **Lee HN, Kim JS, Kim P, Lee HS, Kim ES.** 2013. Repression of antibiotic downregulator WblA by AdpA in *Streptomyces coelicolor*. Appl. Environ. Microbiol. **79:**4159–4163. http://dx.doi.org/10.1128/AEM.00546-13.
26. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* genetics. John Innes Foundation, Norwich, United Kingdom.
27. **Gust B, Challis GL, Fowler K, Kieser T, Chater KF.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **100:**1541–1546. http://dx.doi.org/10.1073/pnas.0337542100.
28. **Du YL, Li SZ, Zhou Z, Chen SF, Fan WM, Li YQ.** 2011. The pleitropic regulator AdpA$_{ch}$ is required for natamycin biosynthesis and morphological differentiation in *Streptomyces chattanoogensis*. Microbiology **157:**1300–1311. http://dx.doi.org/10.1099/mic.0.046607-0.
29. **Mao XM, Zhou Z, Cheng LY, Hou XP, Guan WJ, Li YQ.** 2009. Involvement of SigT and RstA in the differentiation of *Streptomyces coelicolor*. FEBS Lett. **583:**3145–3150. http://dx.doi.org/10.1016/j.febslet.2009.09.025.
30. **Du YL, Shen XL, Yu P, Bai LQ, Li YQ.** 2011. Gamma-butyrolactone regulatory system of *Streptomyces chattanoogensis* links nutrient utilization, metabolism, and development. Appl. Environ. Microbiol. **77:**8415–8426. http://dx.doi.org/10.1128/AEM.05898-11.
31. **Ohnishi Y, Yamazaki H, Kato JY, Tomono A, Horinouchi S.** 2005. AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. Biosci. Biotechnol. Biochem. **69:**431–439. http://dx.doi.org/10.1271/bbb.69.431.
32. **Onaka H, Horinouchi S.** 1997. DNA-binding activity of the A-factor receptor protein and its recognition DNA sequences. Mol. Microbiol. **24:**991–1000. http://dx.doi.org/10.1046/j.1365-2958.1997.4081772.x.
33. **Kato JY, Miyahisa I, Mashiko M, Ohnishi Y, Horinouchi S.** 2004. A single target is sufficient to account for the biological effects of the A-factor receptor protein of *Streptomyces griseus*. J. Bacteriol. **186:**2206–2211. http://dx.doi.org/10.1128/JB.186.7.2206-2211.2004.
34. **Yamazaki H, Tomono A, Ohnishi Y, Horinouchi S.** 2004. DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. Mol. Microbiol. **53:**555–572. http://dx.doi.org/10.1111/j.1365-2958.2004.04153.x.
35. **Higo A, Hara H, Horinouchi S, Ohnishi Y.** 2012. Genome-wide distribution of AdpA, a global regulator for secondary metabolism and morphological differentiation in *Streptomyces*, revealed the extent and complexity of the AdpA regulatory network. DNA Res. **19:**259–273. http://dx.doi.org/10.1093/dnares/dss010.
36. **Hara H, Ohnishi Y, Horinouchi S.** 2009. DNA microarray analysis of global gene regulation by A-factor in *Streptomyces griseus*. Microbiology **155:**2197–2210. http://dx.doi.org/10.1099/mic.0.027862-0.
37. **Smith LJ, Stapleton MR, Buxton RS, Green J.** 2012. Structure-function relationships of the *Mycobacterium tuberculosis* transcription factor WhiB1. PLoS One **7:**e40407. http://dx.doi.org/10.1371/journal.pone.0040407.
38. **Rybniker J, Nowag A, van Gumpel E, Nissen N, Robinson N, Plum G, Hartmann P.** 2010. Insights into the function of the WhiB-like protein of mycobacteriophage TM4—a transcriptional inhibitor of WhiB2. Mol. Microbiol. **77:**642–657. http://dx.doi.org/10.1111/j.1365-2958.2010.07235.x.
39. **Singh A, Guidry L, Narasimhulu KV, Mai D, Trombley J, Redding KE, Giles GI, Lancaster JR, Steyn AJC.** 2007. *Mycobacterium tuberculosis* WhiB3 responds to O_2 and nitric oxide via its [4Fe-4S] cluster and is essential for nutrient starvation survival. Proc. Natl. Acad. Sci. U. S. A. **104:**11562–11567. http://dx.doi.org/10.1073/pnas.0700490104.
40. **Garg S, Alam MS, Bajpai R, Kishan KR, Agrawal P.** 2009. Redox biology of Mycobacterium tuberculosis H37Rv: protein-protein interaction between GlgB and WhiB1 involves exchange of thiol-disulfide. BMC Biochem. **10:**1. http://dx.doi.org/10.1186/1471-2091-10-1.
41. **MacNeil DJ, Klapko LM.** 1987. Transformation of *Streptomyces avermitilis* by plasmid DNA. J. Ind. Microbiol. **2:**209–218. http://dx.doi.org/10.1007/BF01569542.
42. **Sun J, Kelemen GH, Fernandez-Abalos JM, Bibb MJ.** 1999. Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). Microbiology **145:**2221–2227.