Coordinative Modulation of Chlorothricin Biosynthesis by Binding of the Glycosylated Intermediates and End Product to a Responsive Regulator ChlF1*§

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Chlorothricin, isolated from Streptomyces antibioticus, is a parent member of spirotetronate family of antibiotics that have long been appreciated for their remarkable biological activities. ChlF1 plays bifunctional roles in chlorothricin biosynthesis by binding to its target genes (chlF, chlF1, chlG, and chlK). The dissociation constants of ChlF1 to these genes are ∼102–140 nM. A consensus sequence, 5′-GTAANNATTAC-3′, was found in these binding sites. ChlF1 represses the transcription of chlF1, chlG, and chlK but activates chlF, which encodes a key enzyme acyl-CoA carboxyl transferase involved in the chlorothricin biosynthesis. We demonstrate that the end product chlorothricin and likewise its biosynthetic intermediates (demethylsalicyloyl chlorothricin and deschloro-chlorothricin) can act as signaling molecules to modulate the binding of ChlF1 to its target genes. Intriguingly, a correlation between the antibacterial activity and binding ability of signaling molecules to the regulator ChlF1 is clearly observed. These features of the signaling molecules are associated with the glycosylation of spirotetronate macrolide aglycone. The findings provide new insights into the TetR family regulators responding to special structure of the signaling molecules and reveal the regulatory mini-network mediated by ChlF1 in chlorothricin biosynthesis for the first time.

Streptomyces produce abundant secondary metabolites and provide more than half of medically important antimicrobial and antitumor agents (1). The genes relevant to secondary metabolite biosynthesis in Streptomyces are often clustered together, which is beneficial for exerting their function to control complex antibiotic biosynthesis efficiently (1–3). Expression and regulation of some gene clusters are associated with signal transduction systems mediated by the regulators and their ligands, which are generally divided into two categories (one component and two component systems). Tet family regulators (TFRs),3 a large family in one-component system, widely exist in prokaryotes acting as transcriptional repressors, activators, or both. They usually contain two functional domains: helix-turn-helix DNA-binding domain and ligand-binding domain. The helix-turn-helix DNA-binding domain plays primary roles in regulation of gene expression through binding to DNA sequence, and the ligand-binding domain interacts with small molecules to modulate the binding activity of the regulators (4, 5). AvaR1, an autoregulator receptor of Streptomyces avermitilis, negatively regulates avermectins production, whereas the signaling molecule avenolide can dissociate AvaR1 from the target genes to relieve the inhibition effect (6). In Pseudomonas putidae, the expression of genes required for the degradation of nicotinic acid is regulated by NicS, which can respond to the nicotinic acid and hydroxynicotinic acid of its ligands (7). Understanding the roles of regulators and their ligands in secondary metabolite biosynthesis would be valuable for improving the yield of important antibiotics.

Chlorothricin (CHL), produced by Streptomyces antibioticus, belongs to a large family of spirotetronate/spirotetramate natural products that possess a characteristic pentacyclic aglycone comprising a trans-decalin system and a tetronate or tetramate spiro-conjugate (8, 9). The members in this family exhibit a wide variety of remarkable biological activities (10–12) and thus have attracted considerable attention in drug discovery and development to inspire the investigation into their modes of action, such as chlorothricin, kijanimicin, pyrrolosporin, tetrocarcin, lobophorin, versipelostatin, nomimicin, and so on (9, 13, 14), but the regulatory mechanism on the biosynthesis has not been elucidated for this family of antibiotics as we know. Over the past several years, we have cloned and sequenced CHL biosynthetic gene (chl) cluster, and it encompasses 35 biosynthetic genes, which span a 101.8-kb DNA region on the chromosome of S. antibioticus (8). There are more than 30 structural genes in chl cluster, which encode a

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§ This article contains supplemental Table S1.

3 The abbreviations used are: TFR, TetR family regulator; CHL, chlorothricin; MST, microscale thermophoresis assay; DM-CHL, demethylsalicyloyl chlorothricin; qRT-PCR, quantitative real time PCR; ARE, autoregulator element.
modular type I polyketide synthase system to template the main carbon skeleton assembly and other proteins responsible for three-carbon unit incorporation, cross-bridging to form the pentacyclic core, and successive oxidation to afford aglycone, as well as deoxysugar formation, appending and functionalization with an iterative type I polyketide synthase-programmed 2-methoxy-5-chloro-6-methylsalicyloyl-CHL, which plays an “editor” role and can hydrolyze aberrantly loaded substrates in chlorothricin biosynthesis by repressing the transcription of chlF1, chlG, and chlK and activating chlL. The glycosylation of chlorothricin and its biosynthetic intermediates is significant for both their bioactivity and binding to ChlF1. Our results will provide the basis for more detailed research on the fine-tuned regulation and spirotetronate family of antibiotics in response to different signaling molecules.

**Experimental Procedures**

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used in this study are summarized in Table 1. Molecular biological reagents and restriction enzymes were purchased from standard commercial sources. *S. antibioticus* DSM 40725 and its derivatives were grown on MS agar or in liquid YEME medium (0.3% yeast extract, 0.5% tryptone, 34% sucrose, 5mM MgCl2, 1% glucose, 0.5% glycine) at 28 °C (17). *Escherichia coli* DH5α was used as a general host for propagating plasmids at 37 °C. *E. coli* C41 (DE3) was used as a host for expression of ChlF1. *E. coli* ET12567/pUZ8002 was used for *E. coli*-Streptomyces conjugal transfer. When necessary, antibiotics were used at the following final concentrations: 100 μg ml⁻¹ ampicillin, 100 μg ml⁻¹ kanamycin, and 50 μg ml⁻¹ chloramphenicol in LB for *E. coli*; 25 μg ml⁻¹ nalidixic acid, 50 μg ml⁻¹ ampicillin, and 50 μg ml⁻¹ kanamycin in MS and YEME for Streptomyces.

DNA Manipulation and Sequence Analysis—DNA isolation and manipulation in *E. coli* and Streptomyces were performed.
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according to standard methods (17, 18). PCR amplifications were carried out on a Bio-Rad DNA Engine Peltier Thermal Cycler using either Taq DNA polymerase (Puemx) or KOD plus DNA polymerase (Toyobo). Primer synthesis and DNA sequencing were performed in Shanghai Invitrogen Biotech. Primers used in this study are listed in supplemental Table S1 in the supplemental data. Analyses of the binding sites were performed with MEME.

Gene Disruption, Complementation, and High Level Expression—To construct *S. antibioticus* mutants, gene disruption via homologous recombination was performed. The selected gene was first cloned into the plasmid pKC1139, which was then introduced into *S. antibioticus* DSM 40725 by conjugal transfer from *E. coli* ET12567/pUC8002 (17). Exconjugants conferring apramycin resistance were obtained, and then the colonies that had lost the apramycin resistance via double cross-over were selected and further confirmed by PCR analysis. For complementation or high level expression of genes in the relevant strains, a DNA fragment containing the complete target gene coding region and its promoter region was inserted into the integrative vector pSET152, and the resulting plasmid was introduced into the *S. antibioticus* mutant or the wild-type strain to generate the corresponding complementary strain or high level expression strain, respectively. Colonies conferring apramycin resistance were further confirmed by PCR analysis.

To inactivate chlF1, two fragments corresponding to 1.8 kb of the upstream and downstream sequences of *chlF1* were generated by PCR using the primer pairs F1DML-F/R and F1DMR-F/R. The two fragments were digested with HindIII/BgIII and BgIII/XbaI, respectively, and then ligated with a 1.0-kb BamHI/BgIII-cleaved *kan* resistance cassette, which was amplified from pUC119::neo with primers Neo-F/R. The resulting 4.6-kb HindIII/XbaI fragment was inserted into the corresponding sites of pKC1139 to generate pLY101 for chlF1 disruption. Primers (P1-F/R) were used to confirm the chlF1 disruption mutant (ΔchlF1). For complementation of ΔchlF1 or high level expression of chlF1, a fragment covering the coding region of chlF1 and its own promoter region was amplified by PCR using primers F1-Zsp-F/R and then digested with XbaI/EcoRI and subsequently was inserted into the same sites of pSET152 to generate pLY105. The pLY105 was then introduced into ΔchlF1 or WT by conjugation to get complementary strain ΔchlF1/pLY105 or high level expression strain WT/pLY105, respectively. Primers (P2-F/R) were used to confirm the complementary strain ΔchlF1/pLY105 and high level expression strain WT/pLY105.

To inactivate chlJ, two 2.6-kb HindIII/Ndel and Ndel/XbaI fragments located in the upstream and downstream of *chlF1* were amplified from wild-type genomic DNA by PCR and then inserted into the HindIII/XbaI-digested pKC1139 to generate plasmid pLY102. Primers (P3-F/R) were used to confirm the mutant AchlJ. To complement chlF1, a 2.0-kb XbaI/EcoRI fragment containing the intact chlF1 and its own promoter region was inserted into the same sites of pSET152 to yield pLY106. Primers (P4-F/R) were used to confirm the complementary strain ΔchlJ/pLY106 and high level expression strain WT/pLY106.

To inactivate chlG, two 3.0-kb HindIII/BamHI and BamHI/XbaI fragments located in the upstream and downstream of *chlG* were amplified from wild-type genomic DNA by PCR, and subsequently inserted into the HindIII/XbaI-digested pKC1139 to generate plasmid pLY103. Primers (P5-F/R) were used to confirm the mutant AchlG. To complement chlF1, a 1.8-kb XbaI/EcoRI fragment containing the intact chlG and its own promoter region was inserted into the same sites of pSET152 to generate pLY107. Primers (P6-F/R) were used to confirm the complementary strain ΔchlG/pLY107 and high level expression strain WT/pLY107.

Site-directed Mutagenesis of ChlF1 Binding Sequences—To assess the specificity of the conserved ChlF1 binding sequence, site-directed mutagenesis was performed. Two fragments containing wild-type ChlF1 binding sequences (ARE-JF1 and ARE-G) corresponding to the probes P7-JF1 and P7-G were ligated into the EcoRV-digested plasmid M13 (Stratagene), and the resulting plasmids were named pLY109 and pLY110, respectively. To mutate sequences in AREs, pLY109 and pLY110 were further amplified by PCR using primer pair muJF1-F/R or muG-F/R, and the generated plasmids were named pLY111 or pLY112, respectively. The mutated sequence 5′-TGGCNNAT-GCCA-3′ at the binding sites was further confirmed by sequencing. Then pLY111 and pLY112 were digested with HindIII/XbaI to generate probes P7-JF1-m and P7-G-m with mutated sequences Mu-JF1 and Mu-G, respectively. The binding activity of ChlF1–His<sub>6</sub> to these probes was subsequently determined by EMSAs.

Expression and Purification of ChlF1–His<sub>6</sub>—The ChlF1 coding region was amplified by PCR using primers F1EX-F/R. The amplified fragment was digested with NdeI/XhoI and subsequently inserted into the same sites of pET23b (Novagen) to generate expression plasmid pET23b::chlF1, which was subsequently introduced into *E. coli* C41 (DE3) for protein expression. The detailed procedures for expression and purification of ChlF1–His<sub>6</sub> were performed as described previously (19).

EMSAs and DNase I Footprinting—The EMSAs were performed as described previously (20, 21). The intergenic region of chlF1-chlF1 and the upstream regions of *chlG/chlK* were amplified by PCR using the genomic DNA of *S. antibioticus* with primer pairs JF1-F/R, G-F/R, and K-F/R to generate probes P7-JF1 (264 bp), P7-G (259 bp), and P7-K (259 bp), respectively. The DNA probes were incubated individually with various concentrations of ChlF1 at 25 °C for 30 min in 20 μl of reaction mixture. Then the samples were loaded on 4% (w/v) nondenaturing polyacrylamide gels. After electrophoresis, DNA in the gel was stained with SYBR Gold nucleic acid gel stain for 30 min and
photographed under ultraviolet transillumination using Quant-ity One.

DNase I footprinting assays were performed according to the fluorescent labeling procedure (22). Briefly, DNA fragments were prepared by PCR using fluorescently labeled primers FAM-JF1-F/HEX-JF1-R, FAM-K-F/HEX-K-R, and FAM-G-F/HEX-G-R, and the purified PCR products were used as probes. The probes (200 ng) and proteins with different concentrations were added to a final reaction volume of 50 μl and incubated at 25 °C for 30 min. DNase I (Promega) digestions were carried out for 50 s at 25 °C and stopped with EGTA. The purified samples were added to 9.5 μl of HiDi formamide and 0.5 μl of GeneScan-LIZ500 size standard, and the mixture was then analyzed with 3730XL DNA analyzer. The results were then processed with GeneMarker v2.2.

Microscale Thermophoresis Assay (MST)—The MST assay was performed as previously described (23). Purified ChlF1 was labeled with the NT-495-NHS fluorescent dye using the BLUE-NHS labeling kit (NanoTemper Technologies) for 30 min at room temperature. The probes F_{1F}, F_{Gr}, and F_{K} were prepared by denaturing for 10 min at 98 °C and annealing for 30 min at 50 °C using 10 μM 35-bp primer pairs MJF1-F/R, MG-F/R, and MK-F/R, respectively. Measurements were performed in MST buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM MgCl₂) by using 40% LED power and 80% MST power. 200 nl labeled protein was used for each analysis including a range of probe concentrations from 10,000 nM to 0.3052 nM by 2-fold serial dilution, and the samples were loaded into silica capillaries for determining the K_{D} values using Monolith NT.115 (Nanotemper Technologies). The assays were performed in three independent experiments, and data analyses were processed with NTAffinity Analysis MST v2.0.2 software.

RNA Isolation and Quantitative Real Time PCR (qRT-PCR)—Total RNA was isolated from cultures of S. antibioticus grown at several time points (48, 72, and 96 h). At each time point, the mycelia of S. antibioticus were triturated with liquid nitrogen and then suspended in 1 ml of TRIzol (Cwbio). RNA was isolated with RNeasy Mini Kit, and genomic DNA was removed with RQ1 RNase-free DNase I (Promega). 500 ng of RNA was used to generate cDNA as described previously (19). The synthesized cDNA was used as template for qRT-PCR using primer pairs YGJ-F/R, YGF1-F/R, YGG-F/R, and YGK-F/R. Transcription of 16S rRNA coding gene using primers YG16S-F/R was used as internal control. qRT-PCR was performed in a Rotor-Gene Q with FastFire qPCR PreMix (SYBR Green) kit according to the manufacturer’s instructions.

Effect of des-CHL and CHL Addition on Transcription of ChlF1 Target Genes in Vivo—10 μM purified CHL and des-CHL was added to the cultures after incubation for 30 h, respectively. Meanwhile, addition of DMSO was used as control. Total RNA at three time points (36, 48, and 72 h) were prepared, and the transcript levels of target genes chlJ, chlF1, chlG, chlK, and non-target gene chlB6 were determined by quantitative real time PCR.

Liquid Chromatography-Mass Spectrometry Analysis—LC-MS analysis was performed on Triple Quadrupole LC/MS system (Agilent 1260/6460) in negative mode with an Agilent ZORBAX SB-C18 column (3.5 μm, 2.1 × 100 mm). LC conditions were as follows: gradient elution with buffer A (5 mM HCOONH₄ in double distilled H₂O) and buffer B (acetonitrile), flow rate at 0.25 ml/min, ultraviolet detection at wavelength of 222 nm. The elution profile was a hold at 15% buffer B over 3 min, a linear gradient of 15–95% buffer B over 7 min, a hold at 95% buffer B over 3 min, a linear gradient of 95–100% buffer B over 0.5 min followed by a hold at 100% buffer B over 3.5 min, and then a linear gradient of 100–15% for 1 min and a final hold at 15% buffer B over 12 min. The fragmentor voltage was 130 V, and scanning range was 150–1600 m/z.

Bioassay of Chlorothricolide, DM-CHL, Deschloro-CHL, and CHL.—To detect the biological activities of the compounds against Bacillus subtilis, the purified compounds (dissolved in 30 μl methanol) were added into the holes with 0.8-cm diameter in LB agar medium containing 1% (v/v) B. subtilis culture. The plate was incubated at 37 °C for 8 h, and the antibacterial activity was estimated by measuring the diameter of the inhibition zones.

Results

Effect of chlF1 Disruption on Chlorothricin Production.—In the chl cluster, chlF1 is adjacent to chlJ and chlG (Fig. 1A). ChlF1 shares 49% sequence identity with SCE4974 from Sorangium celulosum So ce56 and 46% identity with KUTG_04524 from Kutzneria sp. 744. ChlF1 contains a helix-turn-helix DNA-binding domain at its N terminus. To investigate the role of ChlF1 in CHL biosynthesis, a chlF1 disruption mutant (∆chlF1) was constructed via homologous recombination with a kanamycin-resistant gene insertion. HPLC analysis showed that the peak corresponding to CHL was present in the culture extract of wild-type strain, but not in that of ∆chlF1 (Fig. 1B), whereas the growth rate and biomass accumulation in both strains were similar (data not shown). Then bioactivity assays were performed against B. subtilis with the fractions corresponding to CHL from the wild type and ∆chlF1 on HPLC chromatograms. An inhibition zone was observed with the fractions from wild type but not with that from ∆chlF1 (Fig. 1B), which was consistent with the HPLC data. CHL production was almost restored in the complementary strain ∆chlF1/pLY105 and increased in the high level expression strain WT/pLY105 compared with that in WT strain (Fig. 1B). The incomplete restoration of CHL production in ∆chlF1 was probably due to the integration of vector pLY105 at _C31 attB_ site on the chromosome, as previously suggested (24). Both HPLC and bioassay
analyses indicated that ChlF1 plays a positive regulatory role in CHL biosynthesis.

Identification of the Target Genes of ChlF1 and Its Binding Sequence—To find the direct targets of ChlF1 in chl cluster, recombinant ChlF1 with a His6 tag (ChlF1-His6) was expressed in E. coli C41 (DE3) and purified by chromatography on nickel-nitrilotriacetic acid resin and detected by EMSAs with 17 possible ChlF1-binding regions (probes) in the gene cluster. The results showed that ChlF1 can bind to the upstream sequences of chlJ-chlF1, chlG, and chlK in a concentration-dependent manner (Fig. 2, A–D), but no complex was found for the other probes (such as $P_{A1}$, $P_{B1}$, $P_{C3}$, and so on; see supplemental Table S1), and a nonspecific probe $P_{16S}$ rRNA was used as negative control (data not shown). The results indicated that ChlF1 regulates CHL biosynthesis through modulating the transcription of chlJ, chlF1, chlG, and chlK. The binding of ChlF1 to probes $P_{F1}$, $P_{G}$, and $P_{K}$ was measured by MST (Fig. 2, E–G). The $K_d$ values of ChlF1 to these probes were 139 ± 2.62, 40 ± 0.08, and 140 ± 3.76 nM, respectively. To further dissect the binding sequences of ChlF1 to chlJ-chlF1, chlG, and chlK, DNase I footprinting sequencing with labeled primers was performed to analyze the protected regions in the presence or absence of ChlF1 (Fig. 3, A–C). ChlF1 protected three binding sites (sites I, II, and III), which are 30–32 bp close to or spanning over the translation start codons (Fig. 3, D–F).

Analysis of the three binding sites with MEME program (25) revealed a highly conserved inverted repeat autoregulator element (ARE) sequence with a 4-bp space (5′-GTAANNATT-3′) (Fig. 4A), and the specific sequence for each binding site was named ARE-F1, ARE-G, or ARE-K, respectively. Competitive EMSAs performed with stepwise dilution of DNA showed that these sequences exhibited similar binding ability to ChlF1 (Fig. 2, B–D). It is probably due to the highly conserved inverted repeat sequence in the binding sites.

To assess whether the inverted repeat sequence of the binding region is specific for ChlF1, site-directed mutagenesis of probes $P_{F1}$ and $P_{G}$ was performed on the ARE sequence 5′-GTAANNATT-3′ to generate 5′-GTCGNNATG-3′ (Fig. 4B). The resulting mutated probes were designated as $P_{F1-m}$ and $P_{G-m}$. EMSAs with the mutated probes showed that ChlF1 completely lost binding ability to $P_{F1-m}$ and $P_{G-m}$ in comparison with wild-type probes $P_{F1}$ and $P_{G}$ (Fig. 4C). It was confirmed that the consensus sequence of ARE is essential for the binding of ChlF1 to its target genes.

ChlF1 Represses chlF1, chlG, and chlK but Activates chlJ—To find out how chlF1 disruption affects its target genes, total RNA was prepared from cultures of S. antibioticus DSM 40725 and ΔchlF1 grown at three time points (48, 72, and 96 h). The transcriptional profiles of chlJ, chlF1, chlG, and chlK were analyzed by qRT-PCR. Interestingly, the transcription of chlJ in ΔchlF1 was significantly declined 25-fold compared with that in WT grown for 48 h (Fig. 5A), whereas the transcription of chlF1, chlG, and chlK in ΔchlF1 was increased (Fig. 5, B–D). These results demonstrated that ChlF1 plays a dual role in the transcription of CHL biosynthetic genes.

Roles of chlJ, chlG, and chlK in Chlorothricin Biosynthesis—To reveal the roles of chlJ, chlG, and chlK in CHL biosynthesis, individual gene disruption mutant (ΔchlJ, ΔchlG, and ΔchlK) and the corresponding complementary and high level expression strains were constructed. The production of CHL in those strains was determined by HPLC along with bioassays against indicator strain B. subtilis.

Because ChlJ shares 93% identity of amino acids sequence with acetyl-CoA carboxylase from Streptomyces livi-
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**FIGURE 2. EMSAs of ChlF1 binding to its target genes.** A, ChlF1-binding regions and corresponding probes (P_{JF1}, P_G, and P_K) are indicated by black lines in the partial chl cluster. B–D, EMSAs of ChlF1 binding to the upstream regions of chlJ-chlF1 (B), chlG (C), and chlK (D). Each lane contains 10 ng of DNA probes. E–G, K_d values of ChlF1 binding to P_{JF1} (E), P_G (F), P_K (G) were determined by MST. The results are presented as means ± S.D. from three independent experiments.

dans 1326 and methylmalonyl-CoA carboxyl transferase from *Streptomyces nodosus*, ChlJ may be decisive for initiating the CHL biosynthesis. As expected, disruption of chlJ caused significant decrease in CHL production compared with that in WT strain according to HPLC and bioassay analyses, whereas the CHL production was restored in the complementary strain (H9004 ΔchlJ/pLY106) and increased in the high level expression strain (WT/pLY106), consistent with the bioassays (Fig. 6A). The results further confirmed that chlJ is indispensable for CHL biosynthesis.

chlG, adjacent to chlF1, encodes a major facilitator superfamily transporter. To identify the role of ChlG in transporting CHL to the extracellular space, we examined the intracellular and extracellular distribution of CHL by HPLC and antibacterial bioassays. The majority of CHL was detected intracellularly in the WT strain. Upon chlG disruption, intracellular CHL was increased slightly in ΔchlG and returned to the same level as in WT in the complemented strain (ΔchlG/pLY106), whereas a slightly lower intracellular CHL was detected in WT/pLY106 (Fig. 6B). These results indicated that CHL is largely accumulated in intracellular system. Thus, unlike the homologous protein ActA in *Streptomyces coelicolor* coordinating with another export pump ActB to transport actinorhodin into extracellular space (26, 27), ChlG might not be significant for CHL export.

BLAST analysis indicated that ChlK is a type II thioesterase, which probably removes aberrantly loaded substrates from the
polyketide chain to promote the specific loading efficiency (28, 29). Both HPLC analysis and bioassays showed that the CHL production in ΔchlK was distinctly lower than that in wild type and was almost fully restored in the complementary strain (H9004/ΔchlK/pLY108). Introduction of one extra copy of chlK into WT (WT/pLY108) resulted in increased CHL production (Fig. 6C). Thus, ChlK also serves as another key enzyme in CHL biosynthesis.

Chlorothricin and Its Pathway Intermediates Modulate the DNA Binding Activity of ChlF1—It has been evidenced that antibiotic itself can act as a signaling molecule to regulate antibiotic production (26, 30). To test whether the activity of ChlF1 is modulated by such small molecules, EMSAs were first carried out with the crude extract from S. antibioticus DSM 40725. ChlF1 could dissociate from PJF1, PG, and PK with the addition of culture extract from DSM 40725 in contrast to the solvent control (methanol). To determine the relationship between ChlF1 and its ligands, we purified the end product CHL, chlorothricolide (the spirotetronate macrolide skeleton of CHL) and other two intermediates with different moieties attached on chlorothricolide, DM-CHL, and deschloro-CHL (Fig. 7, A and B). The characterization of these compounds was determined by HPLC and UV spectra in comparison with those of standards and further confirmed by mass spectrometry. Probe PJF1 was randomly chosen to carry out the dissociation experiments with various concentrations of the above compounds. As shown in Fig. 7C, DM-CHL, deschloro-CHL, and CHL caused ChlF1 dissociation from PJF1 in a concentration-dependent manner from 2 to 128 μM, although the dissociation was not changed distinctly with the increasing of signaling molecules, whereas ChlF1 could not dissociate from PJF1 with the addition of chlorothricolide. Meanwhile, bioassays against B. subtilis...
were performed with the above compounds. It was shown that 
CHL, deschloro-CHL, and DM-CHL containing deoxysugar 
oieties displayed antibacterial activity, whereas no inhibition 
zone was observed with chlorothricolide lacking these moieties (Fig. 7D). These results indicated that ChlF1 can respond to 
CHL and its analogues containing deoxysugar moieties, which 
are essential for antibacterial activity. Glycosylation of spirote-
tronate macrolide skeleton is crucial not only for the antibac-
terial activity of the signaling molecules but also for their bind-
ing to ChlF1.

Effect of des-CHL and CHL Addition on the Transcription of 
ChlF1 Target Genes in Vivo—To confirm whether CHL and its 
biosynthetic intermediates can modulate the regulatory activity 
of ChlF1 in vivo, we measured the transcript levels of ChlF1 target and nontarget genes in response to exogenously added 
the end product CHL and its intermediate des-CHL as signaling 
molecules. The transcription of chlF1 was dramatically 
increased 30-fold at 48 h and 25-fold at 72 h after the addition of 
des-CHL and CHL, respectively (Fig. 8A), demonstrating that 
chlF1 was effectively relieved from ChlF1 repression by the 
modulation of these signal molecules, and it resulted in the 
transcription alteration. Transcription of chlG showed a similar 
profile to that of chlF1 (Fig. 8B). chlJ transcription was 
increased 4-fold at 48 h with the addition of des-CHL and then 
returned to the level as in WT at 72 h (Fig. 8C). Surprisingly, no 
change of chlK transcription was observed at any time points 
(Fig. 8D), implying that the transcription of chlK is probably 
insensitive to des-CHL and CHL in vivo. A nontarget gene 
chlb6 was used as a negative control, and its transcription was 
not altered by the addition of these ligands (Fig. 8E). In sum-
mary, the in vivo study further confirmed that signal molecules 
can effectively dissociate ChlF1 from target genes to modulate 
its regulatory activity.

Discussion

Biosynthesis of secondary metabolites in Streptomyces is reg-
ulated stringently, including one or two component system 
associating with regulators and their signaling molecules. TFRs 
belong to one component system that can recognize diverse 
ligands, such as autoregulators and antibiotics as signals (4). In 
this study, we demonstrated that ChlF1 acts as a positive regu-
lator for CHL biosynthesis and plays dual role in regulating the 
transcription of genes situated in chl cluster. It represses the 
transcription of chlF1, chlG, and chlK, whereas activates chlJ. 
Disruption of chlJ led to massive decrease of CHL production, 
and a trace amount of chlorothricin was detected by HPLC, 
implying that chlJ function may be partially replaced by other 
homologous genes usually involved in primary metabolism. As 
mentioned above, chlJ encodes acyl-CoA carboxyl transferase. 
In general, carboxyl transferase along with biotin carboxyl car-
rier protein and biotin carboxylase is responsible for acyl-CoA 
carboxylation in fatty acid metabolism and TCA cycle, and their 
coding genes are usually situated out of the secondary metabo-
lite biosynthetic gene cluster. The carboxylated products serve
as communal substrates for both primary and secondary metabolite biosynthesis in *Streptomyces* (31). However, acyl-CoA carboxyl transferase coding gene *chlJ* is stringently controlled by the pathway-specific regulator ChlF1. Similarly, *jadJ* is a cluster-situated gene in jadomycin biosynthetic gene cluster, and its encoding protein is involved in acetyl-CoA carboxylation for jadomycin biosynthesis in *Streptomyces venezuelae* (32). This phenomenon could be representative in *Streptomyces* with appealing functions, and it can be assumed that the existence of the enzyme coding genes for acyl-CoA carboxylation in antibiotic biosynthetic gene cluster of *Streptomyces* is probably beneficial for antibiotics biosynthesis. In the regulation of CHL biosynthesis, we suggest that *chlJ* might be the most important target gene of ChlF1 in CHL biosynthesis.

Because of the vast diversity of TFR ligands and their important effects on antibiotic biosynthesis in *Streptomyces* (31). However, acyl-CoA carboxyl transferase coding gene *chlJ* is stringently controlled by the pathway-specific regulator ChlF1. Similarly, *jadJ* is a cluster-situated gene in jadomycin biosynthetic gene cluster, and its encoding protein is involved in acetyl-CoA carboxylation for jadomycin biosynthesis in *Streptomyces venezuelae* (32). This phenomenon could be representative in *Streptomyces* with appealing functions, and it can be assumed that the existence of the enzyme coding genes for acyl-CoA carboxylation in antibiotic biosynthetic gene cluster of *Streptomyces* is probably beneficial for antibiotics biosynthesis. In the regulation of CHL biosynthesis, we suggest that *chlJ* might be the most important target gene of ChlF1 in CHL biosynthesis.

Because of the vast diversity of TFR ligands and their important effects on antibiotic biosynthesis, increasing interests have been elicited on the interactions of TFRs with small ligands. Using BLAST program, no ligand-binding domain in ChlF1 can be predicted, it is probably due to the high diversity in the ligand-binding domain sequence of different regulators to accommodate various structures of signal molecules. Therefore, the ligand-binding domain of ChlF1 is atypical. The binding of ChlF1 to its target genes was affected by addition of CHL and biosynthetic intermediates DM-CHL, as well as deschloro-CHL in vitro EMSA assays, but not affected by chlorothricolide lacking the deoxysugar moieties (Fig. 7C). Glycosylation of chlorothricolide confers bioactivity of DM-CHL, deschloro-CHL, and CHL against *B. subtilis* (33), which is further enhanced by the 2-methoxy-6-methylsalicylic acid and chloro group. The correlation between the antibacterial activity of antibiotics as signaling molecules and their binding to ChlF1 is associated with glycosylation of the signaling molecules at a certain stage of biosynthetic process. Coincidently, kijanimicin, another spirotetronate family of antibiotic, was shown to interact with TFR SCO7719 to induce the expression of deglycosylase SCO7720, whereas the deglycosylated derivative of kijanimicin catalyzed by SCO7720 lost bioactivity, as well as the ability inducing SCO7720 expression (34). Thus, this kind of correlation and the regulation mechanism may be widespread in the biosynthesis of spirotetronate family of antibiotics. The regulator sensing various active metabolites could be beneficial for cells to respond to their surroundings.

The influence of signal molecules on ChlF1 regulatory activity was further confirmed by *in vivo* experiments. Upon the addition of des-CHL and CHL, *chlF1* transcription was significantly increased, demonstrating that these ligands could dissociate ChlF1 from *chlF1* to activate its transcription. However, the transcriptions of other ChlF1 target genes (*chlJ*, *chlG*, and *chlK*) could be more complicated, because they are influenced not only by the relief of ChlF1 but also by the elevated ChlF1 itself, which can in turn bind to its targets. Thus, the transcrip-
FIGURE 8. Effect of exogenous des-CHL and CHL addition on the transcription of ChlF1 target genes in vivo. Purified des-CHL and CHL were dissolved in DMSO and added at 10 μM in the cultures. The transcription levels of ChlF1 target genes chlJ (A), chlF1 (B), chlG (C), chlK (D), and nontarget gene chlB6 (E) were measured by qRT-PCR. The data are presented as the average results from three independent experiments. Relative values were obtained using the transcription of 16S rRNA coding gene as reference. The error bars were calculated from three independent experiments. cDNA used as template in this experiment was diluted 3-fold for the five genes but diluted 1000-fold for the 16S rRNA coding gene.

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