Regulation of the biosynthesis of thiopeptide antibiotic cyclothiazomycin by the transcriptional regulator SHJG8833 in Streptomyces hygroscopicus 5008

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Cyclothiazomycin is a member of the thiopeptide antibiotics, which are usually complicated derivatives of ribosomally synthesized peptides. A gene cluster containing 12 ORFs identical to the clt cluster encoding cyclothiazomycin from Streptomyces hygroscopicus 10-22 was revealed by genome sequencing in S. hygroscopicus 5008. Genes SHJG8833 and SHJG8837 of the cluster and flanking gene SHJG8838 were predicted to encode regulatory proteins from different families. In this study, we showed that the newly identified cluster is functional and we investigated the roles of these regulatory genes in the regulation of cyclothiazomycin biosynthesis. We determined that SHJG8833, but not SHJG8837 or SHJG8838, is critical for cyclothiazomycin biosynthesis. The transcriptional start point of SHJG8833 was located to a thymidine 54 nt upstream of the start codon. Inactivation of SHJG8833 abrogated the production of cyclothiazomycin, and synthesis could be restored by reintroducing SHJG8833 into the mutant strain. Gene expression analyses indicated that SHJG8833 regulates a consecutive set of seven genes from SHJG8826 to SHJG8832, whose products are predicted to be involved in different steps in the construction of the main framework of cyclothiazomycin. Transcriptional analysis indicated that these seven genes may form two operons, SHJG8826–27 and SHJG8828–32. Gel-shift analysis demonstrated that the DNA-binding domain of SHJG8833 binds the promoters of SHJG8826 and SHJG8828 and sequences internal to SHJG8826 and SHJG8829, and a conserved binding sequence was deduced. These results indicate that SHJG8833 is a positive regulator that controls cyclothiazomycin biosynthesis by activating structural genes in the clt cluster.

INTRODUCTION

Streptomyces spp. are a large group of Gram-positive, soil-inhabiting filamentous bacteria with high G + C content in their genomes (Bentley et al., 2002). These microbes display a complex process of morphological differentiation and are known mostly for their potential to produce an enormous variety of secondary metabolites, many of which have important biological activities and are clinically useful antibiotics (Hopwood, 2007).

Streptomyces hygroscopicus 5008 (or strain 5008 hereafter), isolated from the Jinggang Mountain area of China in 1974 (Xia & Jiao, 1986), is mostly known as the producer of jinggangmycin, which is a weakly basic water-soluble aminocyclitol antibiotic and was proven to be identical to validamycin (Iwasa et al., 1970). S. hygroscopicus 10-22, a close relative of S. hygroscopicus 5008, can produce jinggangmycin as well, a compound initially named as...
5102-I (Zhou & Liu, 1981), and for which the gene cluster was localized and cloned recently (Jian et al., 2006). Besides jinggangmycin, *S. hygroscopicus* 10-22 also produces another antibiotic, designated 5102-II (Zhang et al., 1982), which was characterized as an acidic sulphur-containing polypeptide antibiotic with biological activities against various fungi (Zhang et al., 1982). The peptide antibiotic 5102-II was recently proven to be the thiopptide antibiotic cyclothiazomycin (Wang et al., 2010). By using a conserved putative cyclodehydratase gene potentially involved in post-translational modification as a probe, the biosynthetic gene cluster for cyclothiazomycin was cloned and sequenced in *S. hygroscopicus* 10-22 (Wang et al., 2010). Heterologous expression in *Streptomyces lividans* 1326 indicated that a minimum set of 15 ORFs is required for the biosynthesis of the final product (Wang et al., 2010).

A chromosomal segment identical to the region of *S. hygroscopicus* 10-22 containing the 12 ORFs from *cltP* to *cltD* required for cyclothiazomycin biosynthesis was identified in *S. hygroscopicus* 5008 after its genome sequence was obtained (Wang et al., 2010; Wu et al., 2012). Interestingly, three putative regulatory genes were identified, with two at the end of, and one flanking, the cluster. Since *S. hygroscopicus* 10-22 is much more difficult to manipulate genetically than *S. hygroscopicus* 5008 and we showed that one of the regulatory genes *cltP* was recently proven to be the thiopeptide antibiotic 5102-II containing the 12 ORFs from *cltP* to *cltD* required for cyclothiazomycin biosynthesis, the final product (Wang et al., 2010).

**METHODS**

**Bacterial strains, plasmids, and culture conditions.** Strains and plasmids used in this work are listed in Table 1. *S. hygroscopicus* 5008 and its derivative strains were grown at 30 °C on SFM agar (Kieser et al., 2000) for spore preparation or in YEME medium (Kieser et al., 2000) for mycelium growth. *Escherichia coli* strains were cultivated in LB liquid medium (Sambrook, 2001).

**DNA manipulation and Southern blot analysis.** Extraction of plasmids from *E. coli* was carried out with columns (BioTeke) according to the manufacturer’s protocol. Genomic DNA was isolated from *S. hygroscopicus* 5008 and its derivatives using the Kirby mix procedure (Kieser et al., 2000). Southern blot analysis was performed on Hybond-N nylon membranes (Amersham Biosciences) with a digoxigenin-labelled probe by using a Dig high prime DNA labelling and detection starter kit (Roche).

**RNA isolation.** *S. hygroscopicus* 5008 and its derivative strains were cultivated in YEME. The mycelium pellet was washed and collected by filtering through layers of filter paper. The collected mycelium was ground in liquid nitrogen before dispensing into Rezol reagent supplied in a RNA extraction kit (SBSBIO). Crude RNA samples were treated with Dnase supplied in Turbo DNA-free reagents (Ambion) to remove chromosomal DNA.

**Reverse transcription (RT)-PCR and real-time PCR.** Reverse transcription was carried out as described previously (Pang et al., 2007). Real-time PCR assays were performed with SYBR Premix Ex Taq (TaKaRa). The recommended thermal cycler conditions compatible with SYBR Premix Ex Taq were used to amplify the cDNA and determine the melting curve of the PCR products and their specificity. Relative quantities of cDNA were normalized for amounts of *hpdB* gene, which encodes the major sigma factor in *Streptomyces*.

**RNA ligase-mediated rapid amplification of cDNA ends (RACE).** A FirstChoice RLM-RACE kit (Ambion) was used to locate the 5’ transcriptional start site of a specific gene following the protocol recommended by the manufacturer and as described previously (Pang et al., 2013). Ten clones were sequenced to determine the transcriptional start point.

**Primer extension analysis.** Primer extension was performed essentially as described by Pang et al. (2007). Briefly, 10 μg of *S. hygroscopicus* 5008 RNA was used to generate cDNA by reverse transcription, using a high-temperature-tolerant Thermoscript reverse transcriptase (Invitrogen) to overcome possible secondary structure due to the high G+C content of *Streptomyces* strains. Reaction products were separated on 8 % denaturing polyacrylamide sequencing gels, alongside four sequencing reactions performed with the same primer using a Sequenase Quick-Denature Plasmid Sequencing kit (USB).

**Deletion of SHJG8833 in *S. hygroscopicus* 5008.** The strategy used for deleting SHJG8833 on the chromosome of *S. hygroscopicus* 5008 involved the following steps:

1. Construction of pSHJG8833-MU. Primers SHJG8833-Left arm-forward (with a SpeI adaptor) and SHJG8833-Left arm-reverse (with a BamHI adaptor) were used to amplify the left arm fragment of 1583 bp. Similarly, primers SHJG8833-Right arm-forward (with a BamHI adaptor) and SHJG8833-Right arm-reverse (with a HindIII adaptor) were used to amplify a 1619 bp fragment, serving as the right arm. The PCR products were ligated into pCR-Blunt (Invitrogen) to generate pLeft-Arm and pRight-Arm, respectively. The right arm was excised and ligated into pLeft-Arm following a BamHI/HindIII digestion, generating pKL-Arm, which is missing a portion of SHJG8833. The fused arms were removed by SpeI and HindIII digestion, and ligated with pTU1278 pretreated with HindIII and SpeI, to yield the deletion construct pSHJG8833-MU. All constructs were verified by sequencing.

2. Conjugation and screening of Δ8833 strain, pSHJG8833-MU was transformed into *E. coli* ET101267 containing pUZ8002 and then was introduced into *S. hygroscopicus* 5008 by intergeneric conjugation as described by Kieser et al. (2000). Single cross-over exconjugants were screened by thiostrepton resistance, and a marker-free double cross-over mutant was selected from a single cross-over exconjugant after rounds of nonselective growth. The Δ8833 mutant strain was confirmed by PCR and Southern analysis.

Other genes were deleted following the same strategy. The primers used in this study are listed in Table S1 (available in the online Supplementary Material).

**Complementation of Δ8833.** To complement SHJG8833, the coding sequence of SHJG8833 (2799 bp) plus 303 bp of its upstream intergenic region was amplified by PCR using primers SHJG8833 com-forward and SHJG8833 com-reverse. The PCR products were gel-purified and inserted into pMD-18T to generate pCom-1. Following sequencing verification, the insert of pCom-1 was released by HindIII digestion and ligated with the pre-cut integrating plasmid pMS82 (Gregory et al., 2003) to generate pSHJG8833-Com, which was introduced into Δ8833 through intergenic conjugation. Exconjugants with resistance to hygromycin were screened and confirmed by PCR analysis.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>S. hygroscopicus</td>
<td>Wild-type cyclothiazomycin-producing strain</td>
<td>Yu et al. (2005)</td>
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<tr>
<td>5008</td>
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<tr>
<td>Δ8824</td>
<td>In-frame deletion 363 bp of SHJG8824</td>
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<td>In-frame deletion 162 bp of SHJG8828</td>
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<td>Δ8833 complemented with p8833-Com</td>
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<td>Δ8833ComCK</td>
<td>Δ8833 transformed with pMS82</td>
<td>This study</td>
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<td>T129</td>
<td>A derivative of S. hygroscopicus 10-22 that does not produce validamycin but still produces cyclothiazomycin</td>
<td>Jian et al. (2006)</td>
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<td>General cloning strain</td>
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<td>Strain used for protein expression</td>
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<td>Kieser et al. (2000)</td>
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<td>He et al. (2010)</td>
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<td>Expression vector</td>
<td>GE Healthcare</td>
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<td>This study</td>
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<td>p8833-Com</td>
<td>pMS82 containing the coding sequence of SHJG8833 plus 303 bp of its upstream intergenic sequence</td>
<td>This study</td>
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<td>pGEX8833DBD</td>
<td>pGEX4T-1 expressing the 90 amino acids of the DNA-binding domain of SHJG8833</td>
<td>This study</td>
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</table>

Construction of an SHJG8833DBD expression plasmid and purification of SHJG8833DBD. To express the DNA-binding domain (DBD) of SHJG8833, the DNA sequence encoding the last section of 90 amino acids was amplified using primers SHJG8833DBD express-Forward/Reverse, and cloned into pGEM-TEasy (Promega). Following sequence verification, the insert was released by EcoRI and XhoI digestion, gel-purified, and ligated into EcoRI/XhoI-cut pGEX4T-1 (Invitrogen), to generate pGEX8833. pGEX8833 was used to transform E. coli Rosetta(DE3)pLysS (Novagen). Expression of SHJG8833DBD was induced by the addition of 0.5 mM IPTG with incubation for 12 h at 16 °C. Bacteria were collected and sonicated in binding buffer [50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 10 mM DTT]. Following sonication, lysates were centrifuged for 10 min at 14,549 g and the tagged SHJG8833DBD was recovered from the supernatant using Proteinlso GST resin (Trans Gen). The resin with tagged SHJG8833DBD was washed with binding buffer before the tagged SHJG8833DBD was eluted off resin with elution buffer [50 mM Tris/HCl (pH 8.0), 10 mM L-glutathione (reduced form)]. The SHJG8833DBD in elution buffer was concentrated using a 10,000 MWCO centrifugal filter (Millipore).

Electrophoresis mobility shift assays (EMSA). DNA fragments were amplified and 3'-end-labelled with biotin-11-UTP using a Biotin 3' End DNA Labelling kit (Thermo Scientific) according to the manufacturer’s instruction. The reaction was performed using a previously described procedure (Pang et al., 2007). For competition assays, an excess of unlabelled competitor DNA was included. Reaction mixtures were analysed on nondenaturing polyacrylamide gels.
Bioassay and HPLC analysis of the cyclothiazomycin production. Cyclothiazomycin produced by S. hygroscopicus 5008 and its mutant strains was extracted and detected as described by Wang et al. (2010). HPLC was performed with a Hypersil ODS (C18) 5 μm column (Thermo Scientific) with UV detection at a flow rate of 1 ml min⁻¹ on a Shimadzu LC-10AT. Solvent A was 0.15 % (v/v) aqueous formic acid and solvent B was acetonitrile. Samples were detected with a gradient of 15% to 90% B in 35 min.

Mass spectrometry analysis. The electrospray ionization mass spectrometry analysis was performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) in the positive ion mode. The Thermo ACCELA HPLC system equipped with an auto-sampler and a PDA detector was used to introduce the samples to the mass spectrometer. The experimental parameters included a spray voltage of 4.5 kV, a capillary voltage of 25 V, a capillary temperature of 275 °C, and a sheath flow rate of 50.

RESULTS

The S. hygroscopicus 5008 genome contains a functional cyclothiazomycin cluster

A total of 29 gene clusters for secondary metabolites were annotated from the genome of S. hygroscopicus 5008 (http://www.ncbi.nlm.nih.gov/nuccore/CP003275.1), including the known Val-A gene cluster (Yu et al., 2005). Among the other 28 clusters, a cluster (Table S2) situated near the right end of the chromosome was found which was identical to that responsible for the biosynthesis of cyclothiazomycin in S. hygroscopicus 10-22 (Wang et al., 2010), suggesting that S. hygroscopicus 5008 has the potential to produce cyclothiazomycin. To investigate whether the newly identified cluster is functional, S. hygroscopicus 5008 was cultured on fermentation medium under conditions that favour the production of cyclothiazomycin by S. hygroscopicus 10-22 (Wang et al., 2010). As the growth of some fungi is sensitive to cyclothiazomycin (Wang et al., 2010; Zhang et al., 1982), we first tested the sensitivity of Cochliobolus heterostrophus to an agar patch of S. hygroscopicus 5008. The results showed that S. hygroscopicus 5008 inhibited the growth of the indicator fungus (Fig. 1a), in a way similar to T129 (Fig. 1b), a S. hygroscopicus 10-22 derivative strain blocked in vali-damycin production (Jian et al., 2006), implying that S. hygroscopicus 5008 may produce cyclothiazomycin. Further, the metabolites of S. hygroscopicus 5008 were extracted and separated on HPLC. A major peak with a retention time of 21.186 min was detected in the S. hygroscopicus 5008 extracts (Fig. 1a), matching closely in retention time to the cyclothiazomycin peak (21.098 min) of T129 (Fig. 1b), suggesting that S. hygroscopicus 5008 produces cyclothiazomycin. To confirm that the peak detected in S. hygroscopicus 5008 is cyclothiazomycin, the elution corresponding to the major peak at 21.186 min was recovered and subjected to mass analysis (c). The photographs in (a) and (b) show the growth inhibition of 5008 and T129 against Cochliobolus heterostrophus. The arrow in (a) indicates the peak corresponding to cyclothiazomycin.

Fig. 1. Analysis of the cyclothiazomycin produced by S. hygroscopicus 5008. The extracts of S. hygroscopicus strains 5008 (a) and T129 (b), a known cyclothiazomycin producer, were tested for their bioactivities and were separated by HPLC. The elution at 21.186 min of S. hygroscopicus 5008 extracts (a) was recovered and subjected to mass analysis (c). The photographs in (a) and (b) show the growth inhibition of 5008 and T129 against Cochliobolus heterostrophus. The arrow in (a) indicates the peak corresponding to cyclothiazomycin.

Sequence analysis of SHJG8833, SHJG8837 and SHJG8838

Three genes predicted to encode regulatory proteins of different families were identified, with two genes (SHJG8833

10-22 (Wang et al., 2010) and the theoretical value for protonized cyclothiazomycin (Aoki et al., 1991). These data confirmed that S. hygroscopicus 5008 produces cyclothiazomycin and indicated that the cyclothiazomycin cluster of S. hygroscopicus 5008 is functional.
and SHJG8837) located at the right end of this cluster, and one (SHJG8838) just flanking the cluster. SHJG8833 was deduced to encode a polypeptide 932 amino acids in length with a predicted molecular mass of 97.49 kDa. The N terminus of SHJG8833 contains an AAA (ATPase associated with various cellular activities) domain (amino acids 11–165) characteristic of AAA proteins (Fig. 2a), which constitute a large family of ATPases associated with diverse cellular activities. The C terminus of SHJG8833 contains a helix–turn–helix (HTH) DNA-binding domain (amino acids 864–918) of LuxR family regulatory proteins (Fig. 2a), which is mainly involved in the binding of the promoter sequence upstream of its target gene (Chen & Xie, 2011; Lazdunski et al., 2004). Apparently, SHJG8833 is a member of the large ATP-binding regulators of the LuxR family (LAL). Sequence BLAST analysis indicated that SHJG8833 is homologous in its full length to several regulatory proteins of the LAL family from several pathways (Fig. 2b, c), e.g. NysRI (32 % identity), a transcriptional regulator for nystatin biosynthesis in Streptomyces noursei ATCC 11455 ( Sekurova et al., 2004); ToyA (31 % identity), which controls the toyocamycin production of Streptomyces rimosus ( McCarty & Bandarian, 2008); AmphRI (31 % identity), one of the regulatory proteins in the amphotericin pathway of Streptomyces nodosus (Caffrey et al., 2001); and FscRIV (30 % identity) of Streptomyces sp. FR-008, which regulates the biosynthesis of a macroline antibiotic FR-008 (Chen et al., 2003). All of these characterized LAL family regulators are critical in the production of the corresponding metabolite, implying that SHJG8833 may also have an important role in the biosynthesis of the thiopeptide antibiotic cyclothiazomycin.

Fig. 2. Amino acid alignment of the two termini of SHJG8833. (a) Predicted domain structure of SHJG8833. (b) Comparisons of the AAA domain and (c) the LuxR domain of SHJG8833 with its homologous protein domains. Numbers indicate the position of amino acid residues from the N terminus of the protein. Identical amino acid residues are highlighted in black, and similar residues in grey. NysRI, the regulator for nystatin biosynthesis of S. noursei ATCC 11455 (AAF71778.1); ToyA, the regulator for toyocamycin biosynthesis in S. rimosus (ACF06633.1); AmphRI, the amphotericin biosynthesis regulator from S. nodosus (AAV37059.1); FscRIV, the heptaene macrolide FR008 regulator from Streptomyces sp. FR-008 (AAQ82554.1).
SHJG8837 is predicted to encode a protein of 297 amino acids. Sequence analysis indicated that it has an N-terminal region (amino acids 12–92) homologous with members of the HTH-XRE family of transcriptional regulators. SHJG8837 is most similar to a putative XRE family transcriptional regulator (48 %) of Streptomyces sp. ExaC1 (Barragan et al., 2005), and a putative XRE family transcriptional regulator (46 %) of Streptomyces auratus. In contrast, SHJG8838, a protein of 635 amino acids, has a C-terminal (residues 536–615) motif characteristic of the HTH-AraC family of transcriptional regulators (Gallegos et al., 1997). SHJG8838 is most similar to an AraC family transcriptional regulator (58 %) of Streptomyces violaceusniger Tu 4113 and NphR (44 %) of Streptomyces aurantiacus.

In-frame deletion of the three regulatory genes

In order to determine the roles of these three genes in the regulation of cyclothiazomycin biosynthesis, we first generated in-frame deletions for SHJG8833, SHJG8837 and SHJG8838. As depicted in Fig. 3, a total of 2307 bp encompassing a portion of the AAA domain and part of the LuxR-like domain of SHJG8833 was removed from the chromosome. The deletion was confirmed by Southern blot analysis using an 807 bp DNA segment flanking the left side of the deleted region as a probe (Fig. 3a, b). A hybridizing band of 3.65 kb was observed for the wild-type, whereas a band of only 1.34 kb was detected in the mutant (Fig. 3b), as expected. The knockout of the 2.3 kb sequence was also verified by PCR analysis using a pair of primers located next to each side of the deleted sequence (Fig. 3c, d). The Southern blot and PCR analysis confirmed that the 2.3 kb sequence within SHJG8833 was completely deleted from the chromosome of S. hygroscopicus 5008, and the resulting SHJG8833 mutant strain was designated Δ8833. Similarly, Δ8837, the deletion mutant for SHJG8837, had a deletion of 510 bp internal to its 873 bp coding sequence, and the SHJG8838 deletion mutant Δ8838 retained only 72 bp of the 1866 bp coding sequence. The deletion of sequences from SHJG8837 and SHJG8838 was confirmed by PCR analysis using primers flanking the deleted regions.

When grown on solid or in liquid medium, these mutant strains showed growth and morphological characteristics identical to those of the wild-type strain, suggesting that the three regulatory genes have no role in bacterial growth or differentiation.

SHJG8833 is required for the biosynthesis of cyclothiazomycin

Production of cyclothiazomycin by mutant strains Δ8833, Δ8837 and Δ8838 was tested, using C. heterostrophus as the indicator strain. A large clear zone of growth inhibition was

![Fig. 3. Schematic representation of the deletion of 2307 bp sequence internal to SHJG8833. The deletion was confirmed by Southern blot analysis (a, b) and PCR analysis (c, d). The black and the grey rectangles represent the AAA domain and the LuxR domain, respectively, of SHJG8833; the dotted line with two arrows indicates the region deleted in the mutant strain. The two bent arrows in (c) denote the primers used in the PCR analysis (d). For, forward primer; Rev, reverse primer; M, size marker.](image)
observed for the wild-type strain 5008 as expected (Fig. 4a). However, no growth inhibition activity against C. heterosporus was detected for Δ8833 (Fig. 4b), suggesting that Δ8833 had abrogated cyclothiazomycin production. The failure of Δ8833 to synthesize cyclothiazomycin was further analysed by HPLC analysis. While a major peak corresponding to cyclothiazomycin was detected in the extracts of S. hygroscopicus 5008 (Fig. 4a), no such peak was present in the extracts of the Δ8833 (Fig. 4b). These results indicated that SHJG8833 is essential for the biosynthesis of cyclothiazomycin. However, deletion of SHJG8837 or SHJG8838 sequence did not affect the production of cyclothiazomycin by Δ8837 or Δ8838 (data not shown), compared to the wild-type strain, suggesting that SHJG8837 and SHJG8838 are not required for cyclothiazomycin biosynthesis.

To confirm that the mutation of SHJG8833 was directly responsible for the defect in cyclothiazomycin production, the whole coding sequence of SHJG8833 plus 303 bp of its upstream intergenic region was cloned into pMS82, an integrative and conjugative plasmid (Gregory et al., 2003), and the resulting plasmid p8833-Com was introduced into Δ8833 to generate Δ8833Com. Δ8833Com-CK, Δ8833 transformed with the vector pMS82 alone, was used as a control in this study. Though Δ8833Com and Δ8833Com-CK showed a wild-type phenotype for growth and morphological differentiation on different media, Δ8833Com was able to synthesize the bioactive compounds as indicated by both bioassay and HPLC analysis (Fig. 4c), while the control strain Δ8833Com-CK1 did not recover the ability (Fig. 4d), confirming that SHJG8833 is directly involved in the regulation of cyclothiazomycin biosynthesis.

Mapping the transcriptional start point (TSP) of SHJG8833

To define the transcription of SHJG8833, we first identified its TSP. Primer-extension analyses with primer SHJG8833 Ext-1 identified a single TSP (Fig. 5a), which is a thymidine residue 54 bases upstream of the predicted SHJG8833 start codon (Fig. 5a, c). The same TSP site was revealed by primer-extension using a second primer SHJG8833 Ext-2 downstream of SHJG8833 Ext-1 (Fig. 5c). To confirm the data obtained from primer-extension analysis, the TSP of SHJG8833 was mapped by 5′-RACE analysis with two gene-specific inner primers SHJG8833 Inner-1 and SHJG8833 Inner-2 (Fig. 5b and data not shown). Two PCR amplicons of different sizes were generated and the TSP of SHJG8833 was localized to the same thymidine residue, consistent with the primer-extension analysis, indicating that SHJG8833 is transcribed from the thymidine 54 bases upstream of the start codon. Analysis of the region upstream of the TSP revealed the presence of a −10 box TAAATT, centred at 10 nt from the TSP, and a −35 box ACATCC, separated by 16 nt from the −10 box (Fig. 5c).

Expression of genes of the cyclothiazomycin biosynthetic pathway in the Δ8833 mutant strain

Because of the loss of cyclothiazomycin production in the SHJG8833 mutant (Fig. 4) and the similarity of the gene to several pathway-specific transcriptional regulators (Fig. 2), it is possible that gene(s) of the cyclothiazomycin pathway might be regulated by SHJG8833. To determine gene(s) regulated by SHJG8833, total RNA was extracted from the wild-type S. hygroscopicus 5008 and Δ8833 strains after growth in YEME for 48 h when cyclothiazomycin was actively expressed. Gene-specific primers for RT-PCR and real-time PCR analysis were designed for each gene (Table S1). Expression of hrdB, encoding the housekeeping sigma factor in S. hygroscopicus 5008, was used as an internal control for both analyses. Beside the three regulatory genes, there are nine structural genes in the pathway (Fig. 6a, Table S2). SHJG8828 was deduced to encode the precursor peptide of cyclothiazomycin. Genes SHJG8826–27 and SHJG8829–32 were expected to be involved in the construction of the thiopeptide framework, while proteins encoded by SHJG8881 and SHJG8882 are likely to be responsible for the formation of
the tertiary thioether (Wang et al., 2010). The deduced product of SHJG8836 is probably a transporter protein, facilitating the export of cyclothiazomycin out of the cell (Wang et al., 2010). Since SHJG8833 regulates cyclothiazomycin production, all these structural genes could be potential targets of SHJG8833, and so their expression was investigated by RT-PCR analysis. SHJG8823, SHJG8824 and SHJG8825, the three genes situated at the 3′ end of the cluster, which have no counterpart in S. hygroscopicus 10-22 (Table S2 and Fig. 6a) and, which display the highest similarity to a chromosome segregation protein (36% identity) of Halosimplex carlsbadense; an RNA-binding protein (30% identity) of Talaromyces stipitatus ATCC 10500, and an epoxide hydrolase, respectively, were also included in the analysis.

The transcripts of SHJG8823, SHJG8824 and SHJG8825 were not altered in the Δ8833 mutant, compared to that in S. hygroscopicus 5008, judged by the band intensity in both samples (Fig. 6b). These data are consistent with the heterologous expression results, in which deletion of genes upstream of cltJ did not affect the biosynthesis of cyclothiazomycin (Wang et al., 2010). However, the transcription of seven consecutive genes, from SHJG8826 to SHJG8832, involved in the construction of the thiopeptide framework, either decreased significantly (SHJG8826) or was not detectable in the mutant Δ8833 (SHJG8827–32), compared to that in the wild-type 5008 (Fig. 6b). Transcription of the other six genes from SHJG8833 to SHJG8838 did not exhibit obvious differences between the wild-type and the mutant strains (Fig. 6b). The transcripts of genes tested in the complemented strain Δ8833Com were similar to that in the wild-type strain. These results indicated that SHJG8833 regulates the expression of the seven genes from SHJG8826 to SHJG8832.

Two sets of primers were used in the RT-PCR analysis of SHJG8833. The pair located in the undeleted region of the coding sequence generated comparable amplicons in strain 5008 and mutant strains (Fig. 6b, middle row), suggesting that SHJG8833 is not autoregulatory. Another pair of primers was located in the deleted region, but no signal was detected in the mutant strain when this pair of primers were used (Fig. 6b, bottom row), showing that SHJG8833 was indeed deleted from its chromosome.

To determine the relative expression levels of these genes in the wild-type and mutant strains, real-time PCR analysis was performed (Fig. 6c), using hrdB encoding the major sigma factor as an internal control. The expression level of each gene in Δ8833 was arbitrarily set to one. Expression of SHJG8823 and SHJG8825 was fairly similar between wild-type 5008 and Δ8833 (Fig. 6c), consistent with the RT-PCR analysis (Fig. 6b). In contrast, SHJG8826 was expressed 135 ± 26-fold higher in the wild-type than in the mutant strain, and a similar level of expression for SHJG8827 (190 ± 34) was detected in strain 5008 (Fig. 6c). SHJG8828 was the most highly upregulated gene tested, and it exhibited a 10498 ± 3800-fold higher expression in the wild-type strain 5008. SHJG8829 was the second most highly upregulated gene in the cluster, showing a 3768 ± 206-fold higher expression in 5008, followed by SHJG8830 (839 ± 281) and SHJG8831 (284.66 ± 26.47), while only a 40 ± 16-fold rise was detected for SHJG8832, the last of the seven genes regulated by SHJG8833. Restoration of SHJG8833 in the complemented strain Δ8833Com resulted in expression levels similar to those found in S. hygroscopicus 5008, confirming that SHJG8833 activates expression of the seven consecutive genes from SHJG8826 to SHJG8832. Expression of SHJG8833 itself was not changed in the mutant strain Δ8833, supporting the conclusion that SHJG8833 is not autoregulatory. A similar level of expression was detected for the three structural genes SHJG8834–36 and the two additional regulatory genes.
SHJG8837 and SHJG8838 (Fig. 6c), confirming that SHJG8833 does not regulate these genes under the conditions tested.

**Genes regulated by SHJG8833 form two operons**

Gene expression analysis indicated that expression of the seven consecutive genes from SHJG8826 to SHJG8832 is positively regulated by SHJG8833. Based on their genetic organization, these seven genes are transcribed in the same direction. As there is only a 34 bp spacer between SHJG8826 and SHJG8827, it is likely that SHJG8826 and SHJG8827 constitute an operon. A 162 bp and a 115 bp intergenic region separates SHJG8828 from its upstream gene SHJG8827 and its downstream gene SHJG8829, respectively, so SHJG8828 might be transcribed alone. Considering the short intergenic regions between SHJG8829 and SHJG8830...
(50 bp), and sequence overlaps between SHJG8830–31–32 (27 bp overlap between SHJG8830 and SHJG8831, and 3 bp overlap between SHJG8831 and SHJG8832), the four genes SHJG8829–30–31–32 could form another operon. We performed RT-PCR to determine whether these genes are co-transcribed, using primers designed to obtain cDNAs corresponding to unaltered transcription between two genes. Transcripts were detected between SHJG8826 and SHJG8827, with products of the same size as that using chromosomal DNA as a template (Fig. 6d). RT-PCR analysis using other primer pairs revealed transcription across the intergenic region between the four genes SHJG8829–30–31–32 (Fig. 6d). Although separated by 115 bp, transcripts were clearly obtained between SHJG8828 and SHJG8829 (Fig. 6d), suggesting that SHJG8828 is co-transcribed with SHJG8829 and genes downstream, rather than transcribed alone. These data suggested that SHJG8826–27 and SHJG8828–32 may form two operons. The co-transcription of SHJG8826 and SHJG8827 was also supported by a similar level of expression detected for these two genes (Fig. 6c). The step-wise lower expression of SHJG8829, SHJG8830, SHJG8831 and SHJG8832, relative to that of SHJG8828, might be caused by the instability of the long mRNA (7.259 kb) of the SHJG8828 operon.

Since SHJG8826 and SHJG8828 are the first genes in the two respective operons, these two genes must have their own promoters, so we performed 5′-RACE analysis to determine the TSPs of SHJG8826 and SHJG8828 (Fig. S1). A single TSP for SHJG8826 was located at the adenine 79 bp upstream of the putative start codon GTG. The TSP of SHJG8828 was identified as an adenine 53 bp upstream of the ATG codon. Though co-transcribed with SHJG8827, a single TSP was also mapped for SHJG8829, which is the adenine 33 bp upstream of the start codon GTG. We also tried to identify TSPs for SHJG8827 and SHJG8830 but were unsuccessful, supporting the idea that they might be co-transcribed with upstream genes.

Genes regulated by SHJG8833 are essential for the biosynthesis of cyclothiazomycin

Of the seven genes downregulated in Δ8833, functions have been deduced as follows: SHJG8826 (cltD), deduced to encode a double-domain protein with its N-terminal domain highly similar to that of a putative M14 peptidase, may have a role in LP cleavage (Wang et al., 2010); SHJG8827 (cltC), expected to encode an NADH oxidase with two McbC-like domains, is likely to participate in the formation of thiazoles (Wang et al., 2010); SHJG8828 (cltA), encodes the 60 aa precursor peptide; SHJG8829 (cltB), encodes a putative cyclodehydratase that may be responsible for the formation of thiazolines (Wang et al., 2010); SHJG8830 (cltE) and SHJG8831 (cltF), both encode proteins homologous to the lantibiotic dehydratases, and may be involved in dehydration of Ser and Thr residues in the peptide modification step (Wang et al., 2010); and SHJG8832 (cltG), which is co-transcribed with SHJG8829/30/31, also encodes a component for the construction of the main framework of cyclothiazomycin (Wang et al., 2010).

According to their deduced functions, each gene regulated by SHJG8833 is required for the formation of the final product. To prove the necessity of these genes in the biosynthesis of cyclothiazomycin, SHJG8828, SHJG8829, SHJG8831 and SHJG8832 were selected and their coding sequence was deleted in-frame to generate mutant strains Δ8828, Δ8829, Δ8831 and Δ8832, respectively. Bioassay analysis demonstrated that these mutants do not produce cyclothiazomycin (Fig. S2), confirming that these genes are required for the biosynthesis of cyclothiazomycin. As a control, the in-frame deletion of SHJG8824, the gene flanking the left ring of the cluster and whose expression is not regulated by SHJG8833, did not affect the production of cyclothiazomycin (data not shown).

SHJG8833DBD binds the promoters of SHJG8826 and SHJG8828

As SHJG8833 activates the transcription of genes from SHJG8826 to SHJG8832, we speculated that SHJG8833 may bind at or near the promoter region of these genes and thereby activate their expression. Owing to its large molecular mass, only the last 90 amino acids encompassing the DNA-binding domain of SHJG8833 were heterologously expressed as a GST fusion protein following cloning into pGEX4T-1. To determine whether SHJG8833 directly regulates the seven genes from SHJG8826 to SHJG8832, DNA fragments covering the upstream sequence of SHJG8826 (Probe 1), SHJG8827 (Probe 2), SHJG8828 (Probe 3), SHJG8829 (Probe 4) and SHJG8830 (Probe 5) were generated by PCR to test for SHJG8833DBD binding (Fig. 7a). Shifting was observed upon incubation of SHJG8833DBD with Probes 1, 2, 3 and 5 (Fig. 7b), indicating that SHJG8833DBD interacts directly with them. Addition of the same unlabelled probe diminished the intensity of the shift band (Fig. 7c and data not shown), suggesting that the binding of SHJG8833DBD to these fragments is specific. The binding of SHJG8833DBD to its own promoter (Probe 6) was also tested. As seen in Fig. 7b, while SHJG8833DBD bound the promoter of other genes, we did not detect binding to its own promoter, indicating that SHJG8833 is not autoregulatory, consistent with the fact that the expression of SHJG8833 is not altered in Δ8833 (Fig. 6b, c).

Comparison of the DNA sequence bound by SHJG8833DBD revealed a putative conserved sequence (CCCGnnnCCgG) in the probes (Fig. 7d). The conserved sequence in Probe 2 (containing the intergenic region between SHJG8826 and SHJG8827) is in fact within the coding sequence of SHJG8826, at a position 83 bp upstream of the stop codon of SHJG8826 or 120 bp upstream of the putative start codon of SHJG8827. Similarly, the conserved sequence in Probe 5 (covering the intergenic region between SHJG8829 and SHJG8830) is located within the coding sequence of SHJG8829, at a position 5 bp upstream of the stop codon.
of SHJG8829 or 58 bp upstream of the putative start codon of SHJG8830. Though no shift was observed for Probe 4 (the intergenic region between SHJG8828 and SHJG8829), a sequence similar to the consensus was detected in the promoter of SHJG8829 by sequence analysis using CLUSTAL W (Chenna et al., 2003). Thus, altogether five potential sites for SHJG8833 were detected in the gene cluster, with three sites in promoters (for SHJG8826, SHJG8828 and SHJG8829) and the other two sites in coding sequences (SHJG8826 and SHJG8829).

**DISCUSSION**

A gene cluster identical to that encoding cyclothiazomycin from *S. hygroscopicus* 10-22 was identified in *S. hygroscopicus* 5008 by genome sequencing (Wu et al., 2012). In this study, this cluster was shown to be functional based on the isolation of cyclothiazomycin produced by *S. hygroscopicus* 5008. Of the three regulatory genes that are within or flanking the cluster, SHJG8833, which encodes a regulatory protein of the LAL family, is critical for cyclothiazomycin biosynthesis. SHJG8833 regulates thiopeptide antibiotic biosynthesis by activating seven consecutive structural genes of the biosynthetic pathway. Regulators of the LAL family are mostly pathway-specific and have been implicated in the regulation of several macrolide antibiotics. Mutation of either NysRI, NysRII or NysRIII, the three LAL-type regulators in the nystatin pathway, leads to abrogated or significantly decreased nystatin production in *S. noursei* (Sekurova et al., 2004). Expression analyses suggested that these regulators have differential control over antibiotic biosynthesis (Sekurova et al., 2004). Deletion of a DNA segment containing fsrRII and

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**Fig. 7.** Binding of SHJG8833DBD to its target. (a) Location of probes used in EMSAs. Numbers between two genes indicate the length (bp) of the putative intergenic region. (b) Binding of SHJG8833DBD to the probes. The arrows indicate the shifted bands. (c) Specific binding of SHJG8833DBD to the SHJG8828 promoter. The labelled probe was incubated with no protein (lane 1); 0.9 µg protein (lane 2); 0.9 µg protein and 10-fold, 30-fold or 50-fold excess of unlabelled specific probe (lanes 3 to 5). (d) Alignment of SHJG8833DBD binding sites. Numbers indicate the position of the last base shown relative to the TSP (SHJG8826, SHJG8826p or SHJG8829) or to the predicted stop codon (SHJG8826 and SHJG8829). Positions conserved in three, four or five sequences are indicated by lower case, underlined or bold letters, respectively. N, any nucleotide.
fscRIII, each encoding a LAL-type regulator in the gene cluster for polypeptide antibiotic FR-008, blocked FR-008 production in Streptomyces sp. FR-008 (Chen et al., 2003). PikD is another characterized LAL-type regulator that is critical for pikromycin synthesis, whose deletion resulted in complete loss of antibiotic production in Streptomyces venezuelae (Wilson et al., 2001). LAL regulators characterized so far generally demonstrate a positive role in the regulation of metabolite production by controlling the transcription of gene(s) involved in the biosynthesis of the corresponding antibiotic.

Though pathway-specific LAL regulators are involved in the regulation of antibiotic biosynthesis in Streptomyces, the molecular mechanism underlining this function is not clear yet. It is generally presumed that the HTH motif is responsible for the ability of LAL regulators to bind DNA and activate the expression of target genes upon ATP hydrolysis, which is catalysed by its nucleotide triphosphate (NTP)-binding motif. Expression assays using reporter gene revealed as targets several gene promoters for NysRI, NysRII and NysRIII (Sekurova et al., 2004) and for PikD (Wilson et al., 2001), yet the nature of the sequence associated with the binding of LAL regulators has not been recognized. Recently, an imperfect palindromic sequence was revealed as the binding site of PcaO, an atypical LAL-type regulator critical for the beta-ketoadiapate pathway associated with PCA degradation in Corynebacterium glutamicum (Zhao et al., 2010). In this study, we found that the DNA-binding domain of SHJG8833 is able to interact with the upstream sequence of four genes in the SHJG8833 regulon. A consensus sequence consisting of two imperfect direct repeats (CCCGnnnCCgG) was identified for binding by SHJG8833. As expected, a sequence similar to the consensus is present in the promoter of SHJG8826, the first gene of operon SHJG8826-8827. However, an additional binding site for SHJG8833 is mapped also in the internal sequence of SHJG8826, at a position close to its stop codon, implying there might be dual levels of control over the SHJG8826 operon by SHJG8833. Similarly, a sequence similar to the consensus is present in the promoter of SHJG8828, the first gene of operon SHJG8828-8832. Unexpectedly, a binding site was revealed in the promoter of SHJG8829 by sequence analysis, though this region was not bound by, or had the least affinity to, SHJG8833 in vitro. It is possible that flanking sequences may be required to assist the binding of SHJG8833, as is reported for MprA (He et al., 2006; Pang et al., 2007). Surprisingly, another binding site is present internal to the SHJG8829 coding sequence at a position close to its stop codon, which was bound by SHJG8833 in EMSAs. As the binding sites internal to SHJG8826 and SHJG8829 are close to the start codon of the downstream genes, these sites might be used by SHJG8833 to control the expression of SHJG8827 or SHJG8830, respectively. However, since no obvious TSP was located for these two genes, it is likely that these sites might be used as an additional means of control over the expression of SHJG8826 and SHJG8829 by SHJG8833.

Most secondary metabolites, especially antibiotics, are the products of complex biosynthetic pathways that are expressed in a growth phase-dependent manner, either on solid or in liquid cultures (Bibb, 2005). The initiation of antibiotic biosynthesis is controlled at several levels genetically (Liu et al., 2013). The most basic and specific control involves genes encoding regulators that work only on one biosynthetic pathway. The regulatory effect of pathway-specific regulators are usually confined to genes in the same cluster. The best-studied pathway-specific regulators include ActII-ORF4 (Fernández-Moreno et al., 1991), RedD (Feitelson et al., 1985; Narva & Feitelson, 1990), DnrI (Stutzman-Engwall et al., 1992) and AlpV (Aigle et al., 2005). Multiple pathway-specific regulators often exist in a gene cluster, forming a regulatory cascade (Cundiffe, 2008; Pang et al., 2004). However, based on the fact that only structural genes, and not the two regulatory genes SHJG8837 and SHJG8838, were downregulated in Δ8833, SHJG8833 appears to be a pathway-specific regulator that functions at a very basic level.

The gene SHJG8838 does not have a counterpart in strain 10-22 and its role in the regulation of cyclothiazomycin biosynthesis is unclear, as the in-frame deletion of most of its coding sequence did not affect cyclothiazomycin production under the conditions tested. The role of SHJG8837 still needs to be confirmed. An SHJG8837 mutant strain with a deletion of only its C-terminal sequence, retaining an intact N-terminal HTH, was used in this study and the gene may have still been functional. Therefore, despite the absence of a detectable phenotype in the mutant, we could not exclude the possibility that SHJG8837 has a role in the biosynthesis of cyclothiazomycin. Further work is needed to address these questions and whether either of these other regulators has a role in the regulation of SHJG8833.

By searching the sequence flanking the gene cluster in strain 5008, potential transposase genes were identified, one on the left (SHJG8821) and three on the right (SHJG8840–42) flank of the cluster. Similarly, genes predicted to encode transposases were also found at both sides of the clt cluster (cltL and cltQ) in strain 10-22, implying that the two clusters might be a result of horizontal gene transfer. The identical genes and organization between these two clusters also suggests that they might have originated from a common ancestor, and that biosynthesis of cyclothiazomycin in strain 10-22 was regulated in a similar way to that in strain 5008.

In conclusion, we demonstrated that SHJG8833 regulates seven consecutive structural genes of the cyclothiazomycin pathway in S. hygroscopicus 5008, and that SHJG8833 is critical for the cyclothiazomycin biosynthesis. These studies will provide new insights into the mechanism of thiopptide antibiotic regulation.

ACKNOWLEDGEMENTS

We sincerely thank Susan T. Howard for her critical reading of the manuscript. This work was supported by grants from the Independent
Innovation Foundation of Shandong University, IIFSDU (2012ZD031) and the Key Laboratory of Microbial Technology.

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