The pleitropic regulator AdpA_{ch} is required for natamycin biosynthesis and morphological differentiation in *Streptomyces chattanoogensis*

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The complete natamycin (NTM) biosynthetic gene cluster of *Streptomyces chattanoogensis* was cloned and confirmed by the disruption of pathway-specific activator genes. Comparative cluster analysis with its counterpart in *Streptomyces natalensis* revealed different cluster architecture between these two clusters. Compared with the highly conserved coding sequences, sequence variations appear to occur frequently in the intergenic regions. The evolutionary change of nucleotide sequence in the intergenic regions has given rise to different transcriptional organizations in the two clusters and resulted in altered gene regulation. These results provide insight into the evolution of antibiotic biosynthetic gene clusters. In addition, we cloned a pleitropic regulator gene, *adpA_{ch}* in *S. chattanoogensis*. Using the genetic system that we developed for this strain, *adpA_{ch}* was deleted from the genome of *S. chattanoogensis*. The \( \Delta \text{adpA}_{ch} \) mutant showed a conditionally sparse aerial mycelium formation phenotype and defects in sporulation; it also lost the ability to produce NTM and a diffusible yellow pigment normally produced by *S. chattanoogensis*. RT-PCR analysis revealed that transcription of *adpA_{ch}* was constitutive in YEME liquid medium. By using rapid amplification of 5’ complementary DNA ends, two transcription start sites were identified upstream of the *adpA_{ch}* coding region. Quantitative transcriptional analysis showed that the expression level of the NTM regulatory gene *scnRI* decreased 20-fold in the \( \Delta \text{adpA}_{ch} \) mutant strain, while the transcription of the other activator gene *scnRII* was not significantly affected. Electrophoretic mobility shift assay (EMSA) showed that AdpA_{ch} binds to its own promoter but fails to bind to the promoter region of *scnRI*, indicating that the control of *scnRI* by AdpA_{ch} is exerted in an indirect way. This work not only provides a platform and a new potential target for increasing the titre of NTM by genetic manipulation, but also advances the understanding of the regulation of NTM biosynthesis.

**INTRODUCTION**

Streptomyces are filamentous soil bacteria belonging to the order Actinomycetales, and have attracted much attention for their ability to make a variety of bioactive compounds that are used as antibiotic, antiparasitic, anticancer and immunosuppressive agents. Natamycin (NTM) is a polyene macrolide antifungal agent produced by several *Streptomyces* strains. So far, the preferred species for industrial production of NTM are *Streptomyces gilvosporeus* and *Streptomyces chattanoogensis*. NTM has been widely used as a natural food preservative to prevent mould contamination of dairy products, meats and other foods. As a drug, it is also used to treat fungal infections, such as fungal keratitis.

Classical methods of strain improvement by random mutation and selection are always time- and resource-intensive; rational strain improvement requires deep understanding of not only the biosynthesis of the desired compounds but also the processes by which biosynthesis is regulated. The regulation of secondary metabolite production is a complex process involving multiple levels. The biosynthetic gene cluster for NTM in *Streptomyces*
natale\ns has been cloned (Aparicio et al., 2000) and shown to contain 19 ORFs spanning a distance of 88.6 kb (Vicente et al., 2009), including two pathway-specific positive regulator genes, \textit{pimR} and \textit{pimM}. Both \textit{pimR} and \textit{\Delta pimM} mutants are defective in NTM production (Antón et al., 2004, 2007). Several other NTM biosynthesis-related regulators located outside the biosynthetic gene cluster have also been identified in \textit{S. natalensis}, including the two-component PhoP–PhoR system (Mendes et al., 2007) and the butyrolactone regulon (Lee et al., 2005, 2008).

An important regulator involved in morphological differentiation and secondary metabolism is AdpA (Ohnishi et al., 2005), which belongs to the AraC/XylS family. It was first discovered in \textit{Streptomyces griseus} and has also been characterized in \textit{Streptomyces coelicolor} (Takano et al., 2003). In \textit{S. griseus}, \textit{adpA} is the sole target of the A-factor receptor (ArpA). By applying microarray and electrophoretic mobility shift assay (EMSA) analysis, AdpA has been shown to directly bind 37 promoter regions to activate 72 genes throughout the chromosome, including \textit{strR}, the pathway-specific activator for streptomycin biosynthesis (Hara et al., 2009). While in \textit{S. coelicolor}, \textit{adpA} transcription does not depend on the butyrolactone system, the \textit{\Delta adpA}, mutant is conditionally defective in morphological differentiation. In \textit{S. coelicolor}, AdpA, has also been shown to be essential for actinorhodin production, though not for undecylprodigiosin biosynthesis (Takano et al., 2003). These results suggest that the mechanism of AdpA regulation of morphological differentiation and secondary metabolism varies in a strain-specific manner.

In a previous study (Du et al., 2009), we reported a new strain of \textit{S. chattanoogensis}. After optimization of media components and culture conditions, NTM production reaches a maximum of 4.5 g L\(^{-1}\) in shake-flask experiments (our unpublished data). Its high NTM production makes the strain an attractive candidate for industrial NTM production and led us to study the regulation of NTM biosynthesis in the strain. In this paper, we describe the development of a genetic system and the cloning of the NTM biosynthesis gene cluster in \textit{S. chattanoogensis}. These results will provide a platform for genetic manipulation for strain improvement. In addition, we cloned and characterized a pleitropic regulator gene, \textit{adpA\textsubscript{ch}}, in \textit{S. chattanoogensis}. By showing that NTM biosynthesis depends on AdpA\textsubscript{ch} regulation, we identified \textit{adpA\textsubscript{ch}} as a new potential target for increasing the titre of NTM by genetic engineering. Our study of \textit{adpA\textsubscript{ch}} also advances the understanding of the regulation of secondary metabolism exerted by AdpA in streptomycetes.

**METHODS**

**Bacterial strains, plasmids, growth conditions and DNA manipulation.** Bacterial strains and plasmids used in this study are listed in Table 1. \textit{S. chattanoogensis} and its derivative strains were normally maintained on YM\textsubscript{G} agar (1% malt extract, 0.4% yeast extract, 0.4% glucose, 0.2% Ca\textsubscript{3}CO\textsubscript{3} and 2% agar, pH 7.2) at 26 °C.

The medium used for examination of the morphological development of \textit{S. chattanoogensis} strains was prepared according to standard procedures (Kieser et al., 2000; Shirling & Gottlieb, 1966). \textit{Escherichia coli} strains were maintained on Luria–Bertani (LB) agar (Sambrook et al., 1989) and grown in LB liquid medium containing appropriate antibiotics when used for propagating plasmids. \textit{Saccharomyces cerevisiae} BY4741 was grown in NTM bioassays was maintained on YPD agar (2% glucose, 2% tryptone, 1% yeast extract, 1.5% agar). DNA manipulations and \textit{E. coli}–\textit{Streptomyces} conjugation were carried out according to standard procedures (Kieser et al., 2000; Sambrook et al., 1989).

**Cloning of \textit{adpA\textsubscript{ch}} in \textit{S. chattanoogensis} L10.** All oligonucleotide primers used in this study are shown in Supplementary Table S2. Primers (\textit{adpA-COF} and \textit{adpA-COR}) were selected using protein alignments of the previously characterized \textit{adpA} genes of \textit{S. coelicolor} and \textit{S. griseus}. These primers were used to amplify part of the \textit{adpA\textsubscript{ch}} gene and screen a cosmid library of \textit{S. chattanoogensis} L10 (Du et al., 2009) by PCR. The PCR product used as a probe for Southern hybridization with the BglII-digested cosmids was prepared by PCR in the presence of biotin-11-dUTP (Fermentas). The DNA fragment giving a signal was recovered and subcloned into pTA2 (Toyobo) for sequencing.

**In-frame deletion and complementation of \textit{adpA\textsubscript{ch}}.** To construct an in-frame deletion mutant of \textit{adpA\textsubscript{ch}}, a 7 kb HindIII–XbaI fragment from pMRD58 was cloned into the corresponding sites of plasmid pJTU870 (L. Bai, Jiaotong University, Shanghai, China, unpublished results), which is a derivative of pHZ1358 (Sun et al., 2002). The resulting plasmid pMRD73 was introduced into \textit{E. coli} BW25113 carrying plJ790. A mutant \textit{adpA\textsubscript{ch}} allele, in which part of the \textit{adpA\textsubscript{ch}} coding region (amino acids 2–368) was deleted, was constructed by PCR targeting using primers \textit{adpA-DEF} and \textit{adpA-DER}. These primers were used to amplify the disruption cassette \textit{aac(3)IV} (Apra) from \textit{pHY773} (Z. Qin, Institute of Plant Physiology and Ecology, CAS, Shanghai, China, unpublished results), with the resulting product carrying 5′ ends with homology to the corresponding region of \textit{adpA\textsubscript{ch}}. The PCR product was then introduced into \textit{E. coli} BW25113 (pJ790/pMRD73), and transformed cells carrying mutagenized pMRD73 were selected on LB agar containing apramycin. Plasmids extracted from the transformants were used to transform \textit{E. coli} DH5\textsubscript{z} (BT340) in order to excise the apramycin-resistance gene, as described elsewhere (Gust et al., 2003). The targeted pMRD73 was designated pMRD128. After conjugal transfer from \textit{E. coli} ET12567 carrying pUZ8002 into \textit{S. chattanoogensis} L10, exconjugants were obtained after selection for thiostrepton. Exconjugants were then inoculated onto YMG plates for two rounds of nonselective growth before selection by replica plating for thiostrepton-sensitive colonies. Deletions within \textit{adpA\textsubscript{ch}} were confirmed by Southern hybridization and PCR analysis using primers \textit{adpA-KOF} and \textit{adpA-KOR}.

**RNA isolation, RT-PCR, quantitative RT-PCR (qRT-PCR) and rapid amplification of 5′ complementary DNA ends (5′ RACE).** The total RNA of \textit{S. chattanoogensis} was isolated from the strains grown in YEME medium (0.5% tryptone, 0.3% yeast extract, 0.3% malt extract, 1% glucose, 5% PEG 6000). RNA was prepared with TRIzol reagent (BBI) according to the manufacturer’s instructions. The genomic DNA was removed by RNase-free DNase I (Takara), and the absence of genomic DNA contamination was subsequently confirmed by PCR using two different primer pairs. The RNA concentration was determined by measuring the \textit{A}_{260} in a spectrophotometer.

Two-step RT-PCR was performed; cDNA was made from 2 μg total RNA using a PrimeScript 1st Strand cDNA Synthesis kit (Takara) according to the manufacturer’s manual, and cDNA was amplified
with Takara rTaq (Takara). The PCR conditions consisted of one cycle of denaturation at 94 °C for 2 min, followed by 30–34 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with one extension cycle at 72 °C for 5 min. Amplification and detection by qRT-PCR were performed on a Roche LightCycler 480 instrument (Roche), and the synthesized cDNA was amplified with SYBR Premix Ex Taq (Takara) according to the manufacturer’s instructions. The transcription of hrdB, which encodes the principal sigma factor of RNA polymerase, was used as the internal control: all values were normalized to the corresponding transcriptional level of hrdB. All reactions were performed in triplicate.

The transcription start sites of adpAch, scnRI, and scnRIII were determined by 5’ RACE performed as suggested by the manufacturer’s recommendations of the 5’/3’ RACE kit (Roche).

**Expression and purification of AdpAch**. The coding region of adpAch was amplified from pMRD48 by PCR using primers adpA-EXF and adpA-EXR. The amplified fragment was digested with Ndel and NotI, and then inserted into the corresponding sites of the IPTG-inducible expression vector pET28a, resulting in pMRD81. After confirmation by DNA sequencing, pMRD81 was introduced into E. coli BL21(DE3). An overnight culture of E. coli BL21(DE3) harbouring pMRD81 was inoculated at 1:100 concentration in 80 ml LB broth with the same concentration of kanamycin (50 μg/ml−1). The culture was grown at 37 °C to OD600 0.4–0.6. IPTG was then added to a final concentration of 0.2 mM. After 4 h of further incubation at 30 °C, the cells were harvested by centrifugation and disrupted by sonication on ice, and the supernatant was recovered by centrifugation (13000 g for 20 min). His-tagged AdpAch was separated using nickel-nitrilotriacetic acid (Ni-NTA) His Bind Resin (Novagen) according to the manual of the supplier.

**Gel mobility shift assay**. The intergenic region (400 bp) of scnRI–scnRIII and the promoter region (408 bp) of adpAch were amplified by PCR with the primers listed in Supplementary Table S2. After digestion with EcoRI and HindIII, the amplified products were cloned into pUC19. Biotin-labelled probes used for EMSA were obtained by PCR with the 5’–biotin-labelled M13 universal primers and gel-purified. Five nanograms of the probes were incubated with appropriate amounts of purified His-tagged AdpAch at 30 °C for 25 min in a buffer containing 2 μg sheared salmon sperm DNA, 20 mM Tris-base (pH 7.5), 0.01 % BSA and 5 % (v/v) glycerol in a total volume of 20 μl. Protein-bound DNA and free DNA were resolved on 6 % acrylamide gels in 0.5 Tris-buffere DTA. EMSA gels were then electroblotted onto a Biodyne B membrane (Pall). Labelled DNA was detected by a Chemiluminescent Nucleic Acid Detection kit (Beyotime) as described by the manufacturer.

**Scanning electron microscopy**. The morphology of S. chattanoogensis strains was observed by scanning electron microscopy (Hitachi S-3000N) after 10 days of growth on YMG medium. The cut agar blocks of different strains were frozen in liquid nitrogen and sputter-coated with gold before examination.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features</th>
<th>Reference or source</th>
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<td><strong>Strains</strong></td>
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<td>Du et al. (2009)</td>
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<td>scnRI-disrupted mutant</td>
<td>This study</td>
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<td><em>S. chattanoogensis</em> ZJUD10</td>
<td>adpAch-deleted mutant complemented with adpAch and its own promoter</td>
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<td><em>E. coli</em> BW25113 (pIJ790)</td>
<td>Strain used for PCR-targeted mutagenesis</td>
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<td><em>E. coli</em> BL21I: BL21(DE3)</td>
<td>Strain for recombinant protein expression</td>
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<td>Indicator strain for NTM bioassays</td>
<td>Laboratory stock</td>
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<td>Derivative of pIJ773, containing the disruption cassette P1-FRT-aac3(IV)-FRT-P2</td>
<td>Z. Qin</td>
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<td>Cosmid from the genomic library of <em>S. chattanoogensis</em> L10</td>
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<td>adpAch and its promoter cloned in pSET152</td>
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**NTM bioassays.** NTM production on YMG agar was detected by bioassays. Agar plugs (diameter 5 mm) of *S. chattanoogensis* strains were removed from the lawns grown on YMG medium for 10 days and placed on a lawn of freshly plated *Saccharomyces cerevisiae*. Bioassay plates were first incubated at 4 °C for 12 h, and then at 30 °C for 18 h before inhibition zones were recorded.

**Fermentation.** For seed culture preparation, spores of *S. chattanoogensis* (1 × 10⁵) were inoculated into 20 ml YEME medium and incubated at 30 °C for 10–12 h on a rotary shaker (225 r.p.m.). The seed culture was then added to 100 ml of the same medium to give an OD₆₀₀ of 0.15. The main cultivation was carried out for 5 days for NTM production under the same conditions as for seed culture preparation. Quantification of NTM production was performed as described previously (Du et al., 2009).

**RESULTS**

**Cloning and analysis of the NTM biosynthetic gene cluster of *S. chattanoogensis***

The NTM biosynthetic gene cluster of *S. chattanoogensis* was identified by genomic walking using a cosmid library and DNA segments from *scnRI*, a *pimR* homologue in *S. chattanoogensis* L10 (Du et al., 2009). Sequencing of the inserts in the five cosmids obtained from the genomic library revealed 18 ORFs putatively responsible for NTM biosynthesis, spanning 86 kb of DNA sequence, as shown in Fig. 1; the deduced functions of the genes are listed in Supplementary Table S1. To distinguish them from the previously characterized NTM biosynthetic gene cluster (*pim* cluster) of *S. natalensis* (Aparicio et al., 2000), we named the cluster from *S. chattanoogensis* the *scn* cluster.

To confirm that this cluster is indeed responsible for NTM production in *S. chattanoogensis* L10, the two cluster-located regulator genes (*scnRI* and *scnRII*) in the *scn* cluster were disrupted using a PCR-targeting system (see Supplementary Methods), and both mutants showed defects in NTM production, as expected (data not shown).

The product of the ORF (*orf1*) downstream of *scnRII* has 43% identity to CalU2, which is found flanking the calicheamicin gene cluster in *Micromonospora echinospora* (Ahlert et al., 2002) and is still uncharacterized. The gene *orf2* downstream of *orf1* is predicted to encode a putative RNA polymerase sigma factor. Both *Δorf1* and *Δorf2* mutants could produce NTM normally (data not shown). These results indicate that *scnRII* is the left boundary of the *scn* cluster. For the right boundary, the predicted gene *orf3* flanking *scnD* encodes a putative methyltransferase, which is not expected to participate in NTM biosynthesis based on the structure of NTM, indicating that *scnD* is the right boundary of the NTM biosynthetic gene cluster. Domain analysis of this methyltransferase in the National Center for Biotechnology Information (NCBI) Conserved Domain Database (CDD) revealed that it belongs to the family of *S*-adenosylmethionine-dependent methyltransferases, and all of the top listed sequences were genes encoding DNA adenine methyltransferases.
Comparative cluster analysis of the \textit{scn} and \textit{pim} clusters

To assess the differences between the \textit{pim} and \textit{scn} clusters, the complete sequences of the two NTM biosynthetic gene clusters were aligned with the \textsc{mauve} program (Darling \textit{et al.}, 2004). As shown in Fig. 2, the \textit{pim} cluster contains two major strain-specific regions, which correspond to \textit{pimH} and \textit{pimT}; they are located at the two ends of the cluster. The deduced product of \textit{pimH} is an efflux pump, which is putatively involved in NTM export (Aparicio \textit{et al.}, 2000). \textit{pimT} has recently been shown to encode an amino acid exporter involved in modulating the expression of NTM biosynthetic genes via secretion of the NTM inducer PI factor (Recio \textit{et al.}, 2004). In contrast, the \textit{scn} cluster contains only one major strain-specific region, which contains a putative transposase gene (\textit{tnp}) located between \textit{scnL} and \textit{scnS1}; this gene might be a vestige of the evolution of the cluster. A probable transposase gene has also been found in the rimocidin biosynthetic gene cluster that is thought to have a common evolutionary origin with NTM clusters (Seco \textit{et al.}, 2004); these data support the idea that horizontal gene transfer plays an important role in the distribution of antibiotic gene clusters. It is worthy

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{(a) Comparison of the complete biosynthetic gene clusters of \textit{scn} and \textit{pim} using the \textsc{mauve} program. The horizontal panels depict the \textit{scn} (upper) and \textit{pim} (lower) clusters. Mean sequence similarities are proportional to the heights of the red bars, and the respective scales show the sequence coordinates in base pairs. Regions with low similarities and strain-specific regions are marked with black triangles. Arrows indicate deduced transcriptional units. (b) Gene expression analysis of the \textit{scn} cluster by RT-PCR. Analysis was carried out on \textit{S. chattanoogensis} L10, and \textit{ΔscnRI} and \textit{ΔscnRII} mutant strains. (c) Transcriptional organization analysis of the \textit{scn} cluster by RT-PCR. cDNA for RT-PCR (right-hand lanes) was prepared from \textit{S. chattanoogensis} L10 after growth for 36 h; genomic DNA (gDNA) was used as a template for controls (left-hand lanes).}
\end{figure}
of note that a gene encoding a putative tyrosine phosphatase is present in both clusters, but it was not annotated in the pim cluster by the original authors. This gene shows high homology to rimC of the rimocidin gene cluster (Seco et al., 2004). Disruption of rimC has a slight effect on the ratio between rimocidin and CE-108. The role of this protein (ScnL) in the NTM biosynthetic pathway remains to be determined.

Orthologous genes from the scn and pim clusters were located in identical relative positions and showed a very high sequence identity, which was generally around 90 % at the protein level (Supplementary Table S1). However, compared with the striking homology of the coding sequences, sequence variations, including nucleotide substitution, insertion and deletion, appear to occur frequently in intergenic regions (Fig. 2a). For instance, a 121 bp insertion occurred in the scn–scnS2 intergenic region, while an 88 bp deletion between scnI and scnJ was observed. The segmental insertion or deletion in these regions could potentially have consequences for gene regulation. To assess whether this indeed happened, gene expression analysis of the scn cluster in ΔscnRI and ΔscnRII mutants and parental strain L10 was performed by RT-PCR (Fig. 2b), since ScnRI and ScnRII have been proven to be the pathway-specific positive regulators of the scn cluster. Transcription of the hrdB gene was assessed as an internal control, and these analyses were carried out at least two times with different RNA samples. After 34 cycles of amplification, transcripts of all the genes in the scn cluster of strain L10 were clearly detected, while no transcripts were found for most of genes in ΔscnRI and ΔscnRII mutants. The transcription of scnK in both mutants was reduced, and very low level transcription of scnJ was observed in the ΔscnRI mutant strain. Interestingly, this transcription pattern was quite different from that of earlier studies of the pim cluster (Antón et al., 2004, 2007), in which pimE was hardly affected in a ΔpimM (scnRII orthologue) mutant, and the transcription of most genes still remained detectable in ΔpimR (scnRI orthologue) and ΔpimM mutants.

The organization of transcription units in the scn cluster was also assessed by RT-PCR using cDNA samples from the mycelium of L10 after 36 h of growth. Primers were designed to amplify the cDNA covering the intergenic region between two genes, and the products were then cloned and sequenced for confirmation (Fig. 2c). The deduced transcriptional organization of the scn cluster is shown in Fig. 2(a): scnA, scnB, scnE, scnC, scnG, scnF, scnS0 and scnL were encoded in a polycistronic transcript longer than 14 kb, while scnJ and scnS2S3S4 were transcribed as an operon. Transcripts covering the intergenic region between scnRI and scnK were observed, indicating that scnRI and scnK also form an operon. No amplification cDNA band linking scnS2 and scnI was detected, suggesting that scnS2S3S4 has its own promoter. Other genes (scnRII, scnS1, scnD) were thought to be transcribed as monocistronic units according to their chromosomal arrangement.

When we were revising our manuscript, a paper was published (Santos-Aberturas et al., 2011) that reported a similar transcriptional organization analysis of the pim cluster in S. natalensis; the proposed organization of transcription units is also shown in Fig. 2(a). Surprisingly, a significant difference in transcriptional organization between the two NTM biosynthetic clusters was observed. Compared with the scn cluster, pimAB, pimE and pimCGFS0 are transcribed separately, while piml and pimk also have their own promoters. These data clearly show that the change of nucleotide sequence in intergenic regions has caused a reorganization of the transcription units, resulting in altered gene regulation.

**Development of a genetic system for S. chattanoogensis**

In order to establish a genetic system for S. chattanoogensis, growth and sporulation conditions were evaluated and optimized. S. chattanoogensis grows optimally between 26 and 28 °C. Common media such as MS, R5, ISP4, ISP5, TSB and SMMS are suitable for vegetative growth, but good sporulation is only observed on YMG agar medium after 7–10 days of growth. S. chattanoogensis L10 is sensitive to apramycin, thiostrepton and spectinomycin, but resistant to even high concentrations of kanamycin (200 μg ml⁻¹). We tested several vectors for their suitability for introducing DNA fragments and for further manipulation, including replicative plasmids (pHZ1358 based on pIJ101 replicons, pKCI139 based on pSG5 replicons, pKCI1218 based on SCP2 replicons) and an integrative plasmid (pSET152 based on pUC18 site-specific integration). All these plasmids were introduced successfully into S. chattanoogensis by intergeneric conjugation. The highest number of exconjugants (1 × 10⁷) was obtained when the number of recipient was set at 5 × 10⁸, using pHZ1358 as vector. When using REDIRECT technology (Gust et al., 2003) adapted to S. chattanoogensis, conjugation was only achieved when 10⁸ spores were used as recipients. The use of this PCR-targeting system in S. chattanoogensis was demonstrated by the disruption of the two NTM regulatory genes, scnRI and scnRII (Supplementary Methods).

**Cloning of adpA<sub>ch</sub> from S. chattanoogensis**

Conserved sequences present in the adpA genes of S. griseus and S. coelicolor were used to design primers for amplifying part of the adpA homologue gene in S. chattanoogensis. Using these primers, a single 0.4 kb DNA fragment was obtained by PCR from the genomic DNA of S. chattanoogensis. After confirmation by DNA sequencing, these primers were used to screen the cosmid library of S. chattanoogensis L10 (Du et al., 2009). Three recombinant cosmids giving the expected product were selected and analysed by Southern blotting using the 0.4 kb DNA fragment as probe. A 6.9 kb BglII DNA fragment giving a positive signal was recovered and subcloned to pTA2 for
sequencing. Computer-assisted analysis of the obtained sequence revealed five complete ORFs and a partial ORF (Fig. 3a). Because the product of a 1290 nt ORF shared 92% sequence identity with both AdpA<sub>ag</sub> and AdpA<sub>ac</sub>, we designated this gene adpA<sub>ch</sub>. Downstream of adpA<sub>ch</sub>, separated by 10 nt, was an ORF (ornA<sub>ch</sub>) encoding a putative oligoribonuclease, and upstream of adpA<sub>ch</sub> was the ORF of a gene that encodes a putative universal stress protein. This arrangement of genes is similar to that found in <i>S. coelicolor</i> and <i>S. griseus</i>.

**Construction of a ΔadpA<sub>ch</sub> mutant**

In order to study the role of the adpA<sub>ch</sub> gene in <i>S. chattanoogensis</i>, an in-frame deletion mutant of adpA<sub>ch</sub> was constructed using a modification of the REDIRECT technology to avoid polar effects on the downstream ornA<sub>ch</sub> gene (see Methods). As a result, a 1101 bp fragment of the adpA<sub>ch</sub> coding region (amino acids 2–368) was replaced with an 81 bp ‘scar’ sequence remaining after FLP-mediated excision of the disruption cassette (Fig. 3a). After conjugation and two rounds of nonselective growth, five colonies were found to be thiostrepton-sensitive in the replica plate assay, all of which had similar phenotypes. Gene replacement was further confirmed by PCR (data not shown) and Southern blotting (Fig. 3b). A hybridizing band of 4.4 kb was found for strain L10, as expected, while a 3.3 kb band was detected in the disrupted mutant, which indicates that a double-crossover event had occurred. The mutant was named <i>S. chattanoogensis</i> ZJUD5.

**AdpA<sub>ch</sub> is required for morphological differentiation and NTM biosynthesis**

Sparse aerial mycelia were produced by strain ZJUD5 after long periods of incubation on MSF or YMG agar (Fig. 4a, b), while a bald phenotype was observed when grown on MM, ISP4 and ISP5 agar (data not shown). ZJUD5 failed to produce the yellow pigment on YMG agar (Fig. 4a) and other media tested. To assess NTM production on solid media, agar blocks of <i>S. chattanoogensis</i> L10 and ZJUD5 were subjected to bioassay against <i>Saccharomyces cerevisiae</i>. No NTM was produced by the ΔadpA<sub>ch</sub> mutant (Fig. 4c). To monitor production of NTM in YEME liquid culture, samples were withdrawn periodically for NTM analysis. No NTM was detected throughout the process of growth (Fig. 5a, upper panel). To verify that the mutation was responsible for the phenotype, the ΔadpA<sub>ch</sub> mutant was complemented with an integrative plasmid, pMRD158 (pSET152 carrying adpA<sub>ch</sub> with its own promoter). The resulting strain ZJUD10 recovered the ability to form spores, pigment and NTM (Fig. 4). These results indicate that AdpA<sub>ch</sub> is required for differentiation, NTM biosynthesis and the production of diffusible yellow pigment in <i>S. chattanoogensis</i>.

**Transcription of adpA<sub>ch</sub>**

Transcription of adpA<sub>ch</sub> in YEME medium was determined by semiquantitative RT-PCR. As shown in Fig. 5(a), lower panel, the transcription of adpA<sub>ch</sub> was constant during the
whole process of growth. Two transcriptional start sites (TSSs) were identified by 5′ RACE; they were located 96 and 153 nt upstream of the translation initiation codon of the adpACh gene (Fig. 5b). In S. griseus, a single TSS has been determined to be the C 263 bp upstream from the initiation codon (Ohnishi et al., 1999), while in S. coelicolor, three promoters of adpAa have been identified (Takano et al., 2003). Interestingly, none of these promoters corresponds in position to the promoters of adpACh in S. chattanoogensis, even though nucleotide sequences upstream of the coding regions are well-conserved among the three species (data not shown).

Dependence of scnRI transcription on AdpACh

It has been reported that streptomycin production in S. griseus is activated by StrR, which is the pathway-specific activator of the streptomycin biosynthetic gene cluster, and that the transcription of strR is directly controlled by AdpAg (Ohnishi et al., 1999). Since AdpAaCh also has a positive role in NTM biosynthesis, it was of interest to analyse changes in the transcription patterns of the two positive regulators of NTM biosynthesis between L10 and ZJUD5. For gene expression analysis, total RNA was prepared from S. chattanoogensis L10 and ZJUD5 after incubation for 36 h in YEME medium. Transcriptional analysis by RT-PCR showed no significant differences in the expression level of scnRII compared with RNA samples from L10. However, the expression of the scnRI gene, encoding a member of the LAL family of transcriptional regulators, was greatly decreased in ZJUD5 (Fig. 6a). The results of three trials of qRT-PCR showed that the expression level of scnRI in ZJUD5 was only about 5% of that in L10 (Fig. 6b). Since the disruption mutant of scnRI is defective in NTM production, it is safe to conclude that such low-level transcription of scnRI is at least partially responsible for the NTM-nonproduction phenotype of the ΔadpACh strain ZJUD5.

Earlier studies have shown that AdpAg represses its own transcription by cooperative binding to the promoter region containing multiple operator sites (Kato et al., 2005). Given that AdpAaCh has the same amino acid sequence forming the DNA-binding domain as AdpAg, it should also bind the same nucleotide sequence. In S. chattanoogensis, putative binding sites (site 1, site 2 and site 3) can be assigned at corresponding positions upstream of the adpACh coding sequence (Fig. 5b). The typical consensus AdpA-binding sequence, 5′-TGGCSNGWWY-3′, can also be deduced from these nucleotides, indicating that a similar autorepression mechanism of adpACh is present in S. chattanoogensis.

To find whether AdpAaCh directly activates the transcription of scnRI, we first determined the TSSs of scnRI and scnRII by 5′ RACE (Fig. 7a). Our analysis suggested that the TSS for scnRI is located 70 nt upstream of the translation start codon.
codon, and the TSS for \textit{scnRII} was identified 151 nt upstream of the translation start codon. EMSAs were then performed using purified His-tagged AdpA\textsubscript{ch} and a 0.5 kb DNA fragment covering the promoter region of \textit{scnRI} was used as probe (probe P1). As a positive control, a 0.5 kb probe (probe P2) containing all AdpA\textsubscript{ch}-binding sites upstream of the \textit{adpAch} gene was used. As shown in Fig. 7(b), strong shift signals were obtained as expected when using probe P2, while no shift band was found with probe P1. These results showed that AdpA\textsubscript{ch} cannot bind the promoter of \textit{scnRI}, suggesting that the control of AdpA\textsubscript{ch} over \textit{scnRI} is exerted in an indirect way.

**DISCUSSION**

In this study, we cloned the NTM biosynthetic gene cluster from the recently identified NTM producer \textit{S. chattanoogensis} var. L10. Compared with the \textit{pim} cluster from \textit{S. natalensis}, the presence of a transposase gene located in the \textit{scn} cluster was unexpected, and an orphan DNA methyltransferase flanking this cluster was also observed. In prokaryotes, most of the DNA methyltransferases are coupled with cognate restriction endonucleases, forming restriction–modification (R–M) systems that protect hosts from ‘foreign’ invaders such as transposons and transfectants.
bacteriophages. Prior methylation of the cluster sequence by DNA methyltransferase during DNA transfer could afford protection against the R–M system of the host and maintain the integrity of the incoming material. A probable transposable element and a similar flanking methyltransferase are also present in the amphotericin gene cluster, which is thought to have an ancestor in common with the NTM clusters. These data support the idea that horizontal gene transfer has played important roles in the distribution of antibiotic gene clusters, and strongly suggest that the scn cluster of *S. chattanoogensis* L10 was acquired by lateral gene transfer. Analogues of *pimH* and *pimT* are absent in the scn cluster. Two different ABC transporters have been found in the two clusters, and they are believed to associate to form heterodimers that export NTM. Homologues of these genes are also located in the biosynthetic gene clusters for nystatin and amphotericin, and all of these clusters are thought to have common evolutionary origins. The gene *pimH*, which is also absent from other polyene macrolide biosynthetic gene clusters (Aparicio et al., 2003), has been suggested to encode a putative efflux pump, functioning as an alternative transport system. However, its role in the export of NTM remains unclear. In *S. natalensis*, PimT is an amino acid exporter involved in secretion of the NTM inducer PI factor, which to date has only been found in this host (Aparicio et al., 2003). A Δ*pimT* mutant shows a 65% decrease in NTM production. Given that *pimH* and *pimT* are located at both ends of the *pim* cluster, and that they are not essential for the function of the NTM gene cluster, it is possible that they were lost or acquired during the strain-specific evolution process.

Compared with the highly conserved coding regions in the *pim* and *scn* clusters, the sequences of the intergenic regions containing promoters showed much more variation, and it is interesting to note that the evolutionary changes in the nucleotide sequences between these two clusters have resulted in the reorganization of the transcription units and a consequent difference in gene regulation, since the transcriptional pattern of the cluster genes in the Δ*scnRI* and Δ*scnRII* mutants was different from that of Δ*pimR* and Δ*pimM* mutants. The change in the transcriptional units is characterized by the formation of larger operons in the *scn* cluster. For instance, *scnI* and *scnJ* are co-transcribed, while their counterparts, *pimI* and *pimJ*, are transcribed separately. The promoters of *pimI* and *pimJ* have been identified recently (Santos-Aberturas et al., 2011), and

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**Fig. 6.** Transcriptional analysis of *scnRI* and *scnRII* in *S. chattanoogensis* L10 and ZJUD5 by RT-PCR (a) and qRT-PCR (b). The oligonucleotides used are listed in Supplementary Table S2. The RNA samples were from 36 h cultures grown in YEME medium. Error bars in (b), ±SD for three replicates of each sample.

**Fig. 7.** Gel mobility shift assay of the *adpA<ch>* promoter and the *scnRI* promoter with AdpA<ch>-His protein. The biotin-labelled probes used were P1 and P2 (a). The boxed G and T indicate the TSSs of *scnRI* and *scnRII*, respectively. The boxed numbers indicate the positions of the three putative AdpA<ch>-binding sites. (b) The amounts of AdpA<ch>-His were 50 ng (lanes 2) and 150 ng (lanes 3). As a control, no AdpA<ch>-His was added in lanes 1.
both are transcriptionally activated by PimM. Furthermore, binding sites for PimM have been found in the promoter regions of \textit{piml} and \textit{pimf}. ScnRII has exactly the same length as PimM, with only six amino acid substitutions, and the two proteins have identical amino acid sequences in the DNA-binding domains, indicating that they should recognize similar binding sequences. We found a conserved binding site in the promoter region of \textit{scnI}, while the ScnRII-binding site for \textit{scnI} was lost due to the segmental deletion in the intergenic region of \textit{scnI} and \textit{scnC}. This change seems to provide a more economical way for the host to control and initiate the production of NTM, since fewer ScnRII molecules are needed. Since increasing the gene dosage of \textit{scnRII} or \textit{pimM} could enhance NTM production (Antón \textit{et al.}, 2007; Du \textit{et al.}, 2009), it is an interesting hypothesis to think about an enhanced NTM yield for the \textit{scn} cluster compared with the \textit{pim} cluster. Up to now, the evolutionary analysis of antibiotic gene clusters has mainly focused on the phylogenetic analysis of synthases such as polyketide synthase (PKS) and NRPS; our research provides new insights into the evolution and distribution of these gene clusters.

In the NTM cluster (\textit{scn}) of \textit{S. chattanoogensis}, three genes were found to have a rare leucine codon, TTA. Two of these genes are the pathway-specific regulator genes \textit{scnRI} and \textit{scnRII}, both of which contain two TTA codons. The third gene is the NTM biosynthetic structural gene \textit{scnS1}, which has only one TTA. These results suggest that NTM production should be controlled by \textit{bldA}, which encodes the only tRNA for UUA (Chater & Chandra, 2008). We found that genes homologous to \textit{adpA} and its downstream gene \textit{ornA} are present in all the available streptomycete genomes in GenBank (our unpublished data). Besides the \textit{adpA} genes characterized so far, all the \textit{adpA} genes contain a TTA codon, which suggests that the dependence of AdpA upon the translational regulation system exerted by \textit{bldA} is a common feature of streptomycetes. However, accumulating evidence reveals that the regulatory position of AdpA varies among species. As part of the butyrolactone signalling cascade in \textit{S. griseus}, the AdpA\textsubscript{x} regulon has been investigated extensively, and a diverse set of downstream targets has been identified. Unlike in \textit{S. griseus}, in which AdpA\textsubscript{x} activates streptomycin formation by directly binding to the promoter region of \textit{strR}, AdpA\textsubscript{ch} in \textit{S. chattanoogensis} appears to activate NTM biosynthesis in an indirect manner. Additionally, the transcription of \textit{adpA}_{ch} is not affected by the butyrolactone system in \textit{S. chattanoogensis} (our unpublished data).

Another interesting finding of this study is that of the two pathway-specific regulator genes, only \textit{scnRI} was greatly influenced by the disruption of AdpA\textsubscript{ch}, while no significant changes in the expression level of \textit{scnRII} were observed. An earlier study (Antón \textit{et al.}, 2004) and our unpublished data reveal that all genes of the NTM biosynthetic gene cluster are dramatically decreased in expression in the \textit{scnRI} (\textit{pimR}) disrupted mutant, and that these mutants are defective in NTM production. The trace expression level of \textit{scnRI} observed in the $\Delta$AdpA\textsubscript{ch} mutant may not be sufficient to activate the NTM cluster, resulting in an NTM-nonproducing phenotype. However, we cannot exclude the possibility that other uncharacterized AdpA\textsubscript{ch}\textendash dependent regulatory systems are involved. It has been proposed elsewhere that the control of NTM biosynthesis exerted by PimR is independent of that exerted by PimM. Our results indicate that the regulation of these two pathway-specific activators by higher-level regulators may also be independent.

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**REFERENCE**


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