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Identification of a Novel *Streptomyces chattanoogensis* L10 and Enhancing Its Natamycin Production by Overexpressing Positive Regulator ScnRII

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A novel *Streptomyces* strain, L10, which is capable of producing natamycin, was isolated from a soil sample collected from Zhejiang province, China. On the basis of phylogenetic analysis of *rpoB* gene and 16S rDNA sequences, as well as phenotypic comparison, strain L10 (CGMCC 2644) is proposed to be a previously uncharacterized strain of *S. chattanoogensis*. By screening a cosmid library of strain L10 and primer walking, a partial sequence of *scnRI* and the entire sequence of *scnRII* were obtained, which are orthologues to the pathway-specific positive regulator genes of natamycin biosynthesis in *S. natalensis*. The engineered *S. chattanoogensis* D1, generated by inserting an additional copy of *scnRII* into the chromosome of strain L10, increased its natamycin production by 3.3 fold in YSG medium and 4.6 fold in YEME medium without sucrose.

***Keywords*:** *Streptomyces chattanoogensis* L10, natamycin, pathway-specific regulatory genes, strain improvement, genetic engineering

The genus *Streptomyces* represents a group of microorganisms that is widely distributed in nature. Members of this genus are well-known producers of diverse bioactive compounds, it produces more than 70% of commercially available antibiotics. Genetic approaches have become increasing useful in determining streptomycete taxonomy. 16S rDNA sequences analysis has shown to be a powerful method for elucidating phylogenetic relationships among prokaryotic organisms and has been used to facilitate the identification of the genus *Streptomyces* (Stackebrandt *et al.*, 1992). However, the 16S rDNA sequences may be insufficient to define closely-related species and those strains belonging to the same species because of the evolutionary conservation of 16S rRNA (Woese, 1987). Furthermore, the 16S rDNA sequence alone can also be misleading because of intraspecific variation (Clayton *et al.*, 1995). Comparisons of partial sequences of the *rpoB* gene, which encodes the β subunit of RNA polymerase, have been applied to phylogenetic analysis of the genus *Streptomyces*. Studies have shown that this can be used as a complementary method to 16S rDNA analysis for determining the polyphasic taxonomy (Kim *et al.*, 2004).

Polyenes macrolide antibiotics, such as amphotericin B and nystain, are a class of antimicrobial polyene compounds that target fungi. Their chemical structures are characterized by a large macrolactone ring containing multiple conjugated double bonds and one or more mycosamine sugars. Natamycin, also known as pimaricin, is a polyene macrolide antibiotic produced by submerged fermentation of *Streptomyces* strains, such as *S. natalensis* (el-Enshasy *et al.*, 2000), *S. gilvosporeus* (Li *et al*., 2008) and *S. chattanoogensis.* In contrast to nystain and filipin, natamycin acts via a novel mode: it blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane (te Welscher *et al.*, 2008). Because of its broad spectrum of activity and the lack of development of resistance, natamcyin is widely used as an antifungal agent.

Classical methods (i.e. UV mutagenesis) and medium optimization have been used in attempts to increase natamycin production (Farid *et al.*, 2000; Li *et al.*, 2008). Recently, biosynthetic gene cluster of natamycin in *S. natalensis* ATCC 27448 was cloned (Aparicio *et al.*, 2000), making it possible to use genetic engineering to improve the strain for industrial applications (Chiang, 2004; Li and Townsend, 2006). However, strain improvement by this method requires deep understanding of biosynthesis and regulation of the production of desired compounds. Regulation of secondary metabolite production is a complex process involving multiple levels. Global regulators usually work on a high level of the regulatory hierarchy and have pleiotropic effects on secondary metabolism, such as morphological differentiation or secondary metabolite production (Bibb, 2005). The lowest level is composed of genes that only regulate a single antibiotic biosynthetic pathway. These pathway-specific regulators are usually found within the respective antibiotic biosynthesis gene clusters and have been shown to control expression of genes in the resident cluster directly and specifically. Since these regulators are not normally present in saturating amounts, increasing gene dosage of such regulator genes may enhance antibiotic production.

In the study, we describe the identification of a newly isolated natamycin-producing strain L10 and cloning of *scnRI*

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(incomplete) and *scnRII*, which comprise two putative pathway-specific positive regulators of natamycin biosynthesis in strain L10. In addition, L10 was genetically engineered to contain an additional copy of *scnRII*, which led to an increase in natamycin production.

Materials and Methods

Organisms and culture conditions

Strain L10 was isolated from a soil sample collected from Zhejiang province, People's Republic of China. The strain was maintained by cultivation on ISP2 agar at 4°C and as glycerol suspensions (20%, v/v) at -80°C. Strain L10 was deposited at China General Microbiological Culture Collection as CGMCC 2644. *Streptomyces gilvosporeus* ATCC 13326 was purchased from the American Type Culture Collection (ATCC, USA).

Cultural and morphological properties

The morphological and cultural characteristics were determined by methods used in the International *Streptomyces* Project (Shirling and Gottlieb, 1966). Cultural characteristics were observed on yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts starch agar (ISP5), Gause's agar, and Czapek's agar after 14 days of culturing at 26°C. Microscopic observations of spores and mycelia of strain L10 grown on ISP2 for 14 days were made by light microscopy (OLYMPUS BX51, Japan) and scanning electron microscopy (HITACHI S-3000N, Japan). Colors were determined according to the color chips from the ISCC-NBS Color System (http://tx4.us/nbs-iscc.htm). Tolerance to temperature and sodium chloride was tested using modified ISP2 agar plates incubated for 7∼14 days at 26°C. Media and methods used for determination of physiological features and carbon-source utilization were those described by Shirling and Gottlieb (1966) and Locci (1989).

16S rDNA and *rpoB* sequencing

The genomic DNA of strain L10 was isolated as described by Hopwood *et al.* (Kieser *et al.*, 2000), The 16S rRNA gene was amplified using primers: F; 5'-AGAGTTTGATCCTGG CTCAG-3' and R; 5'-AAGGAGGTGATCCAGCCGCA-3'. Amplifications were carried out in a MyCycler Thermal Cycler (Bio-Rad, USA) as follows: one cycle of denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, with one extension cycle at 72°C for 5 min. The *rpoB* gene was amplified by using KOD plus (Toyobo, Japan) with defined primers (Kim *et al.*, 2004). The reaction mixture was subjected to one cycle of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 45 sec, with one extension cycle at 72°C for 5 min. The amplified products were purified using BioDev Gel Extraction System B (BioDev Biotech, China), and ligated to pTA2 vector (Toyobo) for sequencing.

Phylogenetic analysis

The 16S rDNA and *rpoB* sequences of the test strain was aligned manually with sequences of related *Streptomyces* in the EMBL/GenBank/DDBJ database. Phylogeny was inferred by using three tree-making algorithms, i.e., neighbour-joining (Saitou and Nei, 1987), Fitch-Margoliash (Ficth and Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981). Evolutionary distance matrices were calculated with the Jukes-Cantor model (Jukes and Cantor, 1969), and phylogenetic trees were constructed with the PHYLIP package (Felsenstein, 1993). The topologies of resulting trees were evaluated by bootstrap analysis with 1,000 resampling. CLUSTAL X and MEGA4 were used to generate the multiple sequence alignments. TreeView was used to draw the phylogenetic trees. Root position of a tree was estimated by using the 16S rDNA of *Streptosporagium roseum* DSM 43021 and the *rpoB* of *Micromonospora echinospora* ATCC 15836 as an outgroups.

Nucleotide sequence accession number

GenBank accession number for 16S rDNA sequence of strain L10 is FJ171335, *rpoB* is FJ171334, *scnRII* is FJ418775 and *sgnRII* is FJ418776.

Recombinant DNA methods

Standard techniques for DNA manipulation were performed as described by Sambrook and Russell (2001). Restriction enzymes and T4 DNA ligase were obtained form TaKaRa (Japan). PCR products were cloned directly into pTA2 vector (Toyobo). DNA sequencing was performed on an ABI Prism™ 3730 *xl* sequencer (Applied Biosystem, USA) using the Big Dye Terminator V3.1 Cycle Sequencing kit. All sequencing and primer walking were performed at Sangon (China).

Gene amplification of the pathway-specific positive regulators in *S. chattanoogensis* L10

Based on the sequences of LAL-family (large ATP-binding

Table 1. Cultural characteristics of strain L10 on various media

Medium	Growth	Diffusible pigment	Colony color	
			Aerial mycelium	Color of reverse
Yeast-malt extract agar (ISP2)	Good	Strong orange	Grayish white	Grayish yellow brown
Oatmeal agar (ISP3)	Good	Vivid orange yellow	White	Pale yellow
Inorganic salts-starch agar (ISP4)	Moderate	Light yellow	Grayish white	Grayish yellow brown
Glycerol-asparagine agar (ISP5)	Moderate	Light yellow	White	Pale yellow
Gause's agar	Good	Pale yellow	White	Pale yellow
Czapek's agar	Good	None	White	Grayish white
Nutrient agar	Poor	None	Scant	Pale yellow

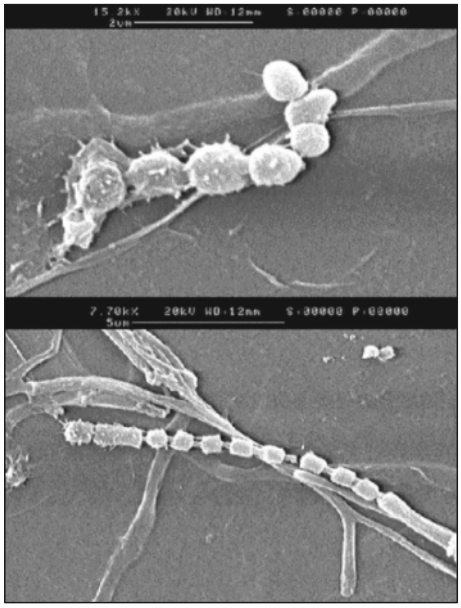

Fig. 1. Electron scanning micrograph of the spore chains of strain L10 (×15.2 k and ×7.7 k).

regulators of the LuxR family) regulators from different *Streptomyces* strains having high sequence similarity, including previously reported natamycin positive regulator, PimR (Anton *et al.*, 2004), three degenerate PCR primers: ScNRIF1; 5'-CTGCTGCTCATMCGSCTSGGC-3', ScNRIR1; 5'-GGTC TTKCCGACKCC-3', and ScNRIR2; 5'-GCGSACGCCSGTS GGRAT-3', were designed using the conserved region of these sequences, taking into account the codon bias of *Streptomyces*. The PCR reaction was performed using TaKaRa LA *Taq* (TaKaRa, Japan) and consisted of one cycle of denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec, with one extension cycle at 72°C for 10 min. The 1,884 bp products, amplified with ScNRIF1 and ScNRIR2, were cloned into pTA2 (Toyobo). To get flanking sequences, ScNRIF1 and ScNRIR2 were used to screen a cosmid library of *S. chattanoogensis* L10 by PCR.

Construction and screening of the cosmid library of strain L10

High molecular weight genomic DNA of strain L10 was partially digested with *Sau*3AI, dephosphorylated, and ligated to pHAQ31 (Zhang *et al.*, 2008), which was digested with *Nhe*I, dephosphorylated, and restricted with *Bam*HI. The liga-

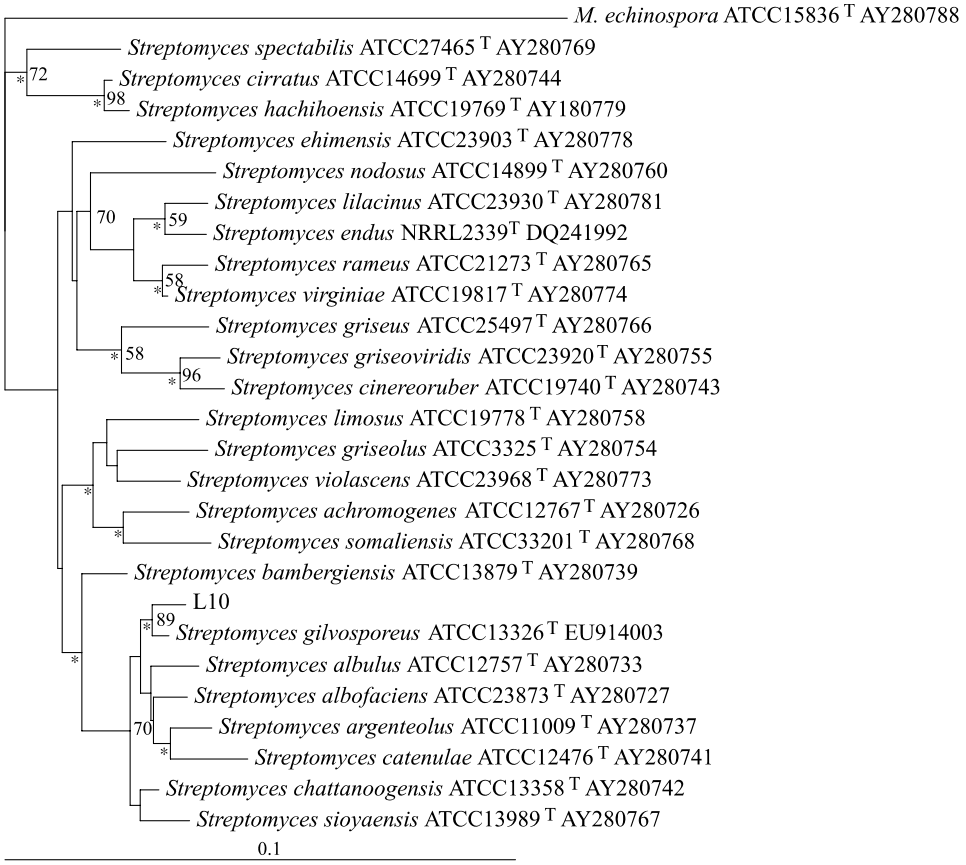

Fig. 2. Neighbour-joining tree of streptomycetes based on partial nucleotide sequences (306 bp) of the RNA polymerase β-subunit gene (*rpoB*). The tree was constructed using the neighbour-joining method. Percentages at nodes represent levels of bootstrap support from 1,000 resampled datasets. Bootstrap values less than 50% are not shown. The asterisks denote the branches that were also recovered using the Fitch-Margoliash and maximum-likelihood algorithms. *M. echinospora* ATCC 15836 was used as an outgroup.

tion mixture was packaged using MaxPlax Packaging Extract from Epicentre Technologies (USA). The packaged phages were propagated in *E. coli* DH10B cells. Individual colonies were picked and inoculated into 96-well microtiter plates containing LB broth, grown overnight, and then adjusted to contain a final concentration of 20% glycerol. These plates were stored at -80°C and served as glycerol stocks of the cosmid library.

Computer-assisted sequence analysis

Open reading frames (ORFs) were identified using FramePlot 2.3.2 (http://www.nih.go.jp/jun/cgi-bin/frameplot.pl). BLAST searches were then performed with these putative ORFs to identify orthologues.

Generation of *S. chattanoogensis* strain D1

A site-specific integration vector, pSET152 (Kieser *et al.*, 2000), containing Φ31 *int* and *attP*, were used to construct a integration recombinant plasmid. The DNA fragment of *scnRII* and its promoter was amplified by PCR using primers: ScNRII-fw1; CCTTGAATTCTTGCGGTCGGTGGTG CGGGCATTACGG and ScNRII-rv; TCCTGGATCCGCCC TGTGCCCGCTCACTTCACGAAGTCG. The resulting PCR fragment was digested with *Eco*RI and *Bam*HI, and cloned into the same sites of pSET152, resulting in the plasmid pMRD1. This plasmid was then transferred to the strain L10 via conjugal transfer from *E. coli* ET12567 (pUZ8002) using standard procedures (Flett *et al.*, 1997). Confirmation of plasmid integration was performed by PCR using oligonucleotides specific for amplification of a 749 bp fragment of the apramycin resistance gene.

Fermentation and analysis of natamcyin

Strain L10 was routinely cultured on ISP2 agar at 26°C for 7 days. Erlenmeyer flasks (500 ml) were filled with 70 ml seed medium (1.75% glucose, 1.5% peptone, 1.0% NaCl) and inoculated with strain L10 by the addition of small areas of growth cut from the agar plate. Flasks were then incubated at 30°C for 24 h on a rotating shaker (250 rpm).

Two different production media were used: YSG (2.8% soybean flour, 0.7% yeast extract, 6% glucose) and YEME without sucrose (Aparicio *et al.*, 2000). YEME medium without sucrose is a fermentation medium for laboratory use and is convenient for analysis of growth and natamycin

Fig. 3. Neighbour-joining tree of *Streptomycetes* based on nearly complete 16S rDNA sequences. The asterisks denote the branches that were also recovered using the Fitch-Margoliash and maximum-likelihood algorithms. The numbers at the nodes indicate the level of bootstrap support based on a neighbour-joining analysis of 1,000 resampled data sets. Scale bar, 0.01 substitutions per nucleotide position.

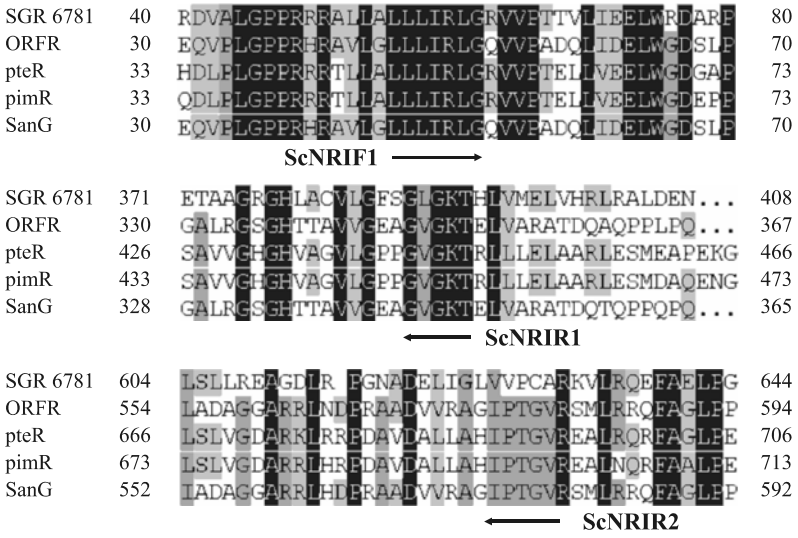

Fig. 4. Alignment of partial amino acid sequences of SGR 6781, PteR, SanG, PimR, OrfR. Identities of amino acid residues are indicated by black boxes and the similarities are by shaded boxes. SGR 6781, putative SARP-family pathway specific regulatory protein of *S. griseus* (YP_001828293); OrfR, nikkomycin regulatory protein of *S. tendae* (AJ250878); PteR, DnrI/RedD/AfsR family transcriptional regulator of *S. avermitilis* (NP_821585); PimR, activator of natamycin biosynthesis of *S. natalensis* (AJ585085); SanG, putative transcriptional activator of nikkomycin biosynthesis of *S. ansochromogenes* (AY631852).

production. YSG medium contains insoluble medium components and is used in industrial fermentation. 28 ml of production medium was inoculated with 3 ml seed culture, and flasks were incubated at 30°C for 5 days at 250 rpm.

To assay for natamycin production in the culture broths, a sample was withdrawn and the pH was adjusted to 2.5~3.0 with a 20% solution of oxalic acid. After ultrasonic extraction with twice the volume of methanol, the methanol layer was recovered by centrifugation at 4,000 rpm for 15 min. The concentration of natamycin was determined using an HPLC system (Agilent Series 1100, Agilent Technologies, USA) equipped with a Zorbax Eclipse XDB-C18 column (150×2.1 mm). The column temperature was maintained at 35°C and UV detector was set at 303 nm. The mobile phase, which had a flow rate of 0.7 ml/min, contained 0.2% KH_2PO_4 solution and methanol in the ratio 42:58.

Results and Discussion

Identification of strain L10

Strain L10, which grew on a wide variety of agar media, showed the typical morphology of *Streptomycetes* (Table 1). At maturity, the aerial mycelium formed long, flexible spore chains with spiny surface spores (Fig. 1). The organism produced a diffusible orange pigment on several media, and utilized D-glucose, D-fructose, D-manitol, inositol, sucrose, glycerol, and maltose as sole carbon sources, but not D-xylose, L-arabinose, L-rhamnose, raffinose, D-sorbitol, or sodium acetate. The strain was positive for gelatin liquefaction, starch hydrolysis and milk peptonization. Strain L10 grows in the pH range 5~12 and in the presence of 7% NaCl. These biochemical and morphological characteristics indicate that strain L10 belongs to the genus *Streptomyces.*

Table 2. Phenotypic characteristics that differentiate strain L10 from its neighbours

Characteristic	L10	*S. chattanoogensis* ATCC 13358	*S. gilvosporeus* ATCC 13326	*S. natalensis* NRRL 2651	*S. lydicus* NRRL 2433
Spore surface	Spiny	Spiny	Spiny	Spiny	Smooth
Spore chain morphology	Flexuous	Spirales	Spirales	Spirales	Spirales
Diffusible pigment	+	+	-	-	-
Starch hydrolysis	+	+	+	ND	+
Peptonzation of milk	+	-	+	ND	ND
Liquefaction of gelatin	+	-	+	ND	+
NaCl tolerance	<7%	7~10%	<3%	ND	<10%
Utilization of carbon source					
D-Xylose	-	-	ND	ND	+
L-Arabinose	-	-	-	-	+
Raffinose	-	+	-	ND	+

+, positive; −, negative; ND, not determined. Data for reference strains were taken from Shirling and Gottlieb (1972), Karwowski *et al.* (1992) and Lin *et al.* (1994).

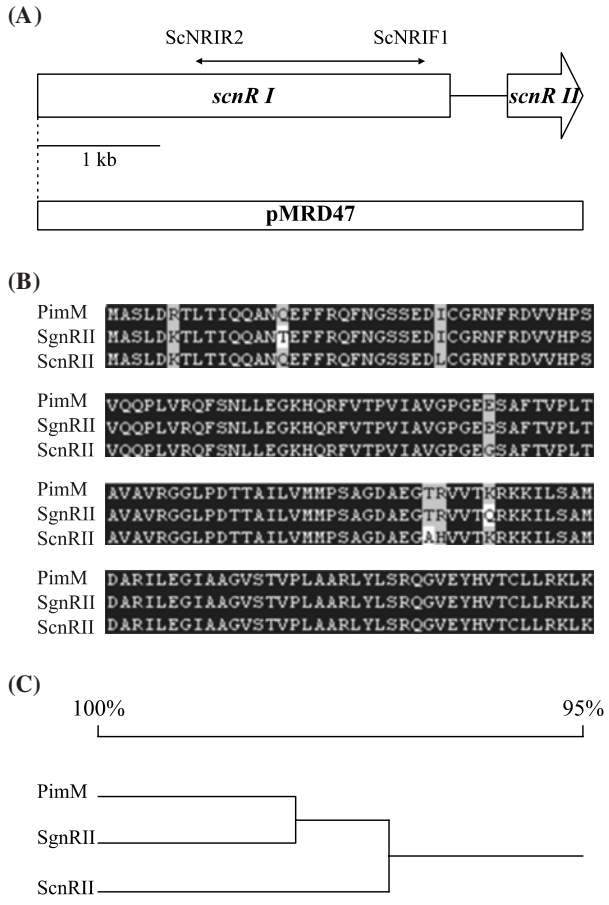

Fig. 5. (A) Gene organization of a 4,226 bp fragment from *S. chattanoogensis* L10. ScNRIF1 and ScNRIR2, forward and reverse primers for amplification of a 1,884 bp fragment within *scnRI*. The bar beneath the gene map represent one end of insert DNA of pMRD47. (B) Sequence alignment of PimM, ScnRII, and SgnRII. (C) Homologous tree of PimM, ScnRII, and SgnRII.

Furthermore, these characteristics show that this strain is most closely related to *S. chattanoogensis.*

In the phylogenetic tree of nearly complete 16S rDNA sequences, strain L10 clustered with two strains of *S. chattanoogensis* and *S. lydicus* NBRC 13058 (Fig. 4). The sequence similarity of strain L10 was 100% to *S. chattanoogensis* NBRC 12754 (1,481/1,481 bp), 99.93% (1,480/1,481 bp) to *S. lydicus* NBRC 13058 and 99.79% (1,481/1,484 bp) to *S. chattanoogensis* DSM 40002. Two other previously reported natamycin-producing *Streptomyces* strains, *S. natalensis*, and *S. gilvosporeus*, were also found to be closely related to strain L10. The phylogenetic tree using partial *rpoB* gene sequences placed strain L10 in a subclade with *S. gilvosporeus* ATCC 13326, with which it shares the highest *rpoB* gene sequence similarity (99.14%).

Closer comparison with its neighbours revealed that the L10 strain was similar to the type culture of *S. chattanoogensis* in many properties, but distinguishable from the latter with respect to features such as spore chain morphology, milk peptonization, getalin liquefaction, and the ability to utilize raffinose as sole carbon source (Table 2). Thus, it is reasonable to conclude that this strain is a new strain of the known species *S. chattanoogensis*. Accordingly, L10 strain was designated as *S. chattanoogensis* L10.

Cloning of natamycin pathway-specific regulators from *S. chattanoogensis* L10

Recent studies on the biosynthesis and regulation of natamycin in *S. natalensis* have identified two pathway-specific positive regulators, PimR and PimM (Anton *et al.*, 2004, 2007). Here, we focus our study on the cloning of their homologous genes in the newly isolated *S. chattanoogensis* L10, as they might provide an efficient way to increase production yield of natamycin. BlastP analysis revealed a group of LAL-family regulators from different *Streptomyces* strains had high sequence similarity, including previously reported natamycin positive regulator, PimR (Fig. 4). Three conserved regions were selected for primer design, taking into account the codon bias of *Streptomyces*. A band with the expected size of 1,884 bp was obtained from *S. chattanoogensis* L10 genomic DNA with the ScNRIF1 and ScNRIR1. This product was cloned and sequenced.

DNA sequence and BLASTX analysis showed that the amino acid sequence from the translated frame had high sequence similarity to the corresponding region of the previously reported natamycin positive regulator PimR. To acquire the flanking sequence for further studies, a cosmid library was constructed by use of cosmid vector pHAQ31, which contains *E. coli* ColE1 *ori*, two *cos* sequences and the *Streptomyces tsr* and *melC* genes. Primers ScNRIF1 and ScNRIR2 were used to screen the cosmid library of *S. chattanoogensis* L10 by PCR. Three cosmids (pMRD45, pMRD46, pMRD47) that gave PCR fragments of the expected size were picked for end-sequencing. Sequencing results showed that one end of the insert of pMRD47 had high sequence similarity to the sequence near the C terminus of PimR. Sequencing of an additional 4,226 bp beyond this end was performed by primer walking, and the result showed it contained a partial sequence of the gene *scnRI* and the entire sequence of *scnRII* gene (Fig. 5A), which are homologous genes to *pimR* and *pimM*.

Sequence analysis of the *scnRII* gene product (192aa) showed a strikingly high sequence identity (99%) to AURJ3M (GenBank accession number: ACD75765), a positive regulator in *S. aureofuscus.* In addition, the amino acid sequence similarity of ScnRII to PimM is 96% (186/192aa). Both proteins have a PAS sensor-binding domain at the N terminus and an HTH motif of the LuxR type at the C terminus. Previous studies showed that PimM mainly regulates genes involved in initiation and first elongation cycles of polyketide chain extension. Based on its sequence similarity to PimM, ScnRII is likely to have positive regulatory functions and follow a similar regulatory pattern.

With the same the primers used in the construction of pMRD1 (see 'Materials and Methods'), we also cloned an orthologue of *scnRII* directly from the genomic DNA of *S. gilvosporeus* ATCC 13326 and designated it as *sgnRII*. Comparative sequence analysis of SgnRII with ScnRII and PimM showed that these three proteins are highly conserved (Fig. 5B). SgnRII showed higher similarity to PimM (189/192aa) than to ScnRII (185/192aa) (Fig. 5C), which agrees well

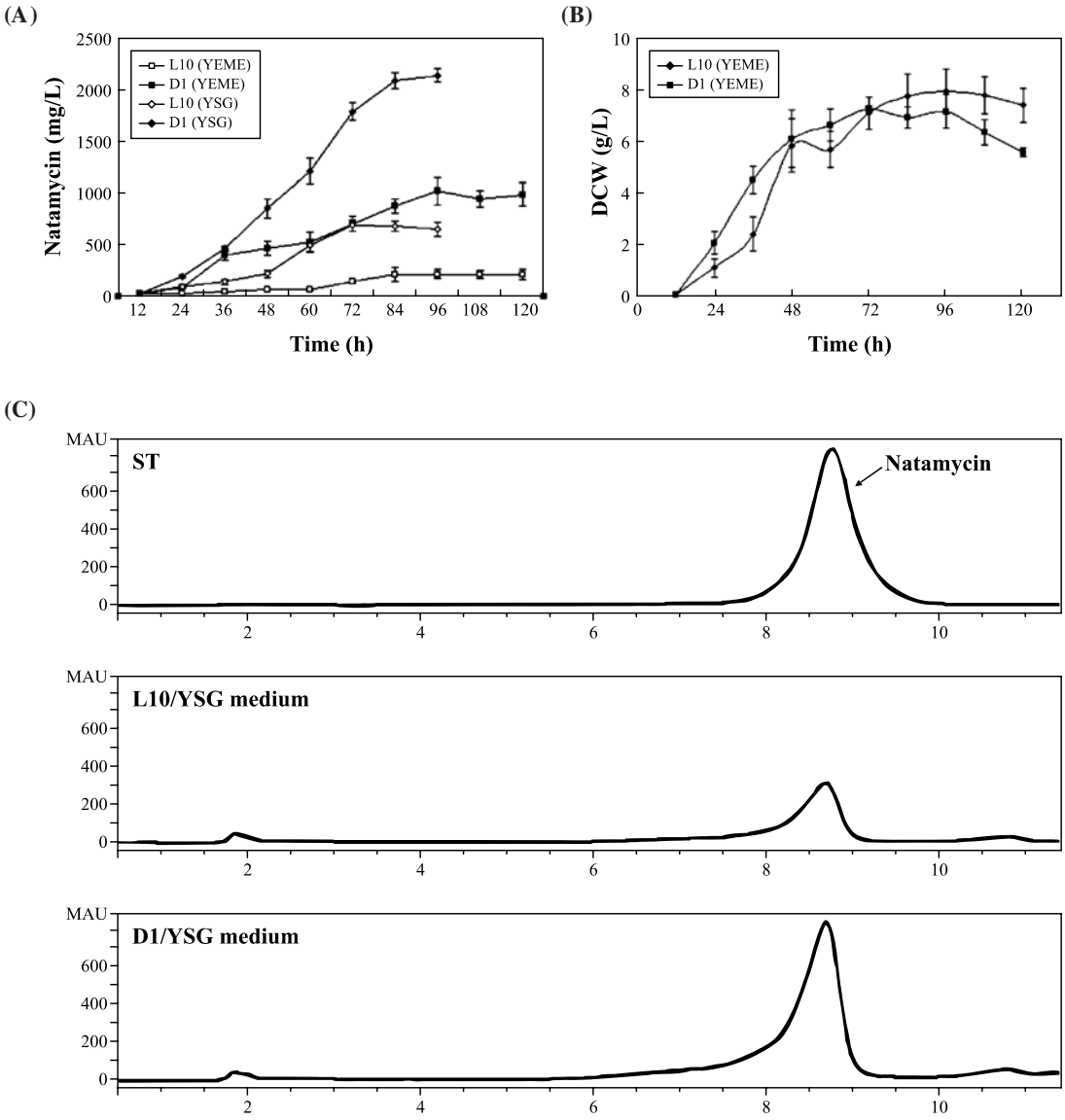

Fig. 6. (A) Effect of increasing *scnRII* gene dosage on natamycin production in YEME medium and YSG medium, data are the average of three duplicate flasks. (B) Comparison of growth curves between L10 and D1 in YEME medium. (C) Comparison of HPLC profiles between L10 and D1 at the time of highest production in YSG medium, along with standard natamycin peak (ST).

with the relationships shown in the phylogenetic tree of their 16S rDNA sequences. This result supports the close evolutionary relationship between *S. gilvosporeus* and *S. natalensis*.

Generation of *S. chattanoogensis* strain D1, with enhanced natamycin production

In view of the high sequence similarity between PimM and ScnRII, we believe that ScnRII is a positive regulator of natamycin biosynthesis in *S. chattanoogensis* L10. In this study, an integration recombinant vector (pMRD1), which contains a copy of the *scnRII* gene and its native promoter, was introduced into *S. chattanoogensis* L10 by intergeneric conjugation, resulting in strain D1. The growth and natamycin production kinetics of strain L10 and D1 in flask cultures using YEME medium without sucrose were studied (Fig. 6). Although no obvious change in growth curve was observed, a significant increase in production of natamycin was detected: natamycin production of D1 increased by 4.6 fold compared to strain L10 after 96 h-fermentation. The effect of *scnRII* duplication on natamycin production was similarly investigated in YSG medium (Fig. 6B). As the HPLC profiles comparing natamycin concentrations during peak production show (Fig. 6C), strain D1 reached 2.14 g/L at 96 h, a concentration about 3.3 fold greater than that of strain L10. We also tested the stability of the pMRD1 in the chromosome by passing *S. chattanoogensis* D1 through three generations without selective antibiotic pressure. The progeny still showed the same level of enhanced production of natamycin in YSG medium (data not shown).

Although traditional strain improvement, by introduction

of mutations, has contributed to enhanced production of many desired compounds, this approach is random and time-consuming. Moreover, after several rounds of mutagenesis and selection, generation of an even higher producing strain becomes rather difficult. Strain improvement by genetic engineering has been established as a good alternative for traditional methods. Deeper understanding of biosynthesis and regulatory network of desired products will allow us to increase compound yield. Enhanced production of antibiotics had been achieved via manipulation of pathway-specific positive regulators, including antibiotic production in a strain of *S. fradiae* that had already been subjected to empirical strain improvement (Stratigopoulos *et al.*, 2004). In the present study, two pathway-specific regulator genes *scnRII* and *sgnRII* were cloned from *S. chattanoogensis* L10 and *S. gilvoposreus* ATCC 13326. By overexpressing ScnRII in the newly isolated *S. chattanoogensis* L10, natamycin production was significantly increased. This method of increasing antibiotic production may also be applied to production enhancement in industrial strains.

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