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SlnM gene overexpression with different promoters on natamycin production in *Streptomyces lydicus* A02

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Abstract Natamycin is an important polyene macrolide antifungal agent produced by several *Streptomyces* strains and is widely used as a food preservative and fungicide in food, medicinal and veterinary products. In order to increase the yield of natamycin, this study aimed at cloning and overexpressing a natamycin-positive regulator, *slnM2*, with different promoters in the newly isolated strain *Streptomyces lydicus* A02, which is capable of producing natamycin. The *slnM* gene in *S. lydicus* is highly similar to gene *pimM* (*scnRII*), the pathway-specific positive regulator of natamycin biosynthesis in *S. natalensis* and *S. chattanoogensis*, which are PAS-LuxR regulators. Three engineered strains of *S. lydicus*, AM01, AM02 and AM03, were generated by inserting an additional copy of *slnM2* with an ermEp* promoter, inserting an additional copy of *slnM2* with dual promoters, ermEp* and its own promoter, and inserting an additional copy of *slnM2* with its own promoter, respectively. No obvious changes in growth were observed between the engineered and wild-type strains. However, natamycin production in the engineered strains was significantly enhanced, by 2.4-fold in strain AM01, 3.0-fold in strain AM02 and 1.9-fold in strain AM03 when compared to the strain A02 in YEME medium without sucrose. These results indicated that the ermEp* promoter was more active than the native promoter of *slnM2*. Overall, dual promoters displayed the highest transcription of biosynthetic genes and yield of natamycin.

Keywords Dual promoters · Natamycin yield · Pathway-specific regulatory gene · *SlnM* gene · *Streptomyces lydicus*

Introduction

Actinomycetes produce a wide variety of bioactive compounds with important medical and agricultural applications. Polyene macrolides are a large family of potential antifungal antibiotics produced by soil actinomycetes. Biosynthesis of polyene macrolides usually includes assembly of a polyene macrocyclic ring, followed by several steps of oxidation and glycosylation [17]. The antifungal mechanism of polyene macrolides relies on the specific interaction of polyenes with the main fungal sterol, ergosterol, and leads to leakage of cellular materials [40]. Recent studies show that natamycin (also known as pimaricin), an important macrolide antibiotic, produced by several *Streptomyces* species, including *S. natalensis* [2], *S. gilvosporeus* [20], *S. lydicus* [25] and *S. chattanoogensis* [8], can inhibit the growth of fungi via immediate inhibition of amino acid and glucose transport across the plasma membrane [41]. Due to its broad spectrum of antifungal activity and the lack of development of resistance, natamycin is widely used in the treatment of fungal keratitis, as a food preservative [3, 18, 30] and as an antifungal agent in agriculture [25].

Several strategies have been employed to improve natamycin production, including optimization of fermentation conditions, traditional breeding methods, genome shuffling and genetic engineering [26]. In particular, genetic engineering has been successfully applied in improving natamycin production and also in deepening the understanding of regulation of natamycin biosynthesis. Regulation of secondary metabolite production is a complex process involving global regulators, as well as pathway-specific

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regulators. Global regulators, usually situated outside biosynthetic gene clusters, have pleiotropic effects on the secondary metabolism. Contrarily, pathway-specific regulators are usually found within specific biosynthetic gene clusters, and they specifically and directly regulate a single antibiotic biosynthetic pathway [16, 22].

The natamycin biosynthetic gene clusters of *S. natalensis* ATCC 27448 and *S. chattanoogensis* L10 have been cloned. Comparative cluster analysis of the two strains revealed different architecture between these two clusters, providing insight into the evolution of antibiotic biosynthetic gene clusters [3, 9]. Overexpression of the activators PimM [1], phosphopantetheinyl transferases (PPTases) [12] and pleiotropic regulator SngA [19], or deletion of the phoR–phoP two component systems [27], pleiotropic regulator AdpAch [9], and γ-butyrolactone autoregulator receptor SngR [18] result in improvement of natamycin production. Moreover, the activator PimR modulates natamycin production by controlling PimM expression levels [10, 34], and PimM controls the expression of the genes *pimK*, *pimS2S3S4*, *pimI*, *pimJ*, *pimAB*, *pimE*, *pimS1* and *pimD* in the natamycin gene cluster through direct binding to the promoters of these genes [33].

Several studies have suggested that overexpression of a positive pathway-specific regulator leads to higher production of the corresponding antibiotic. An increased gene expression could be achieved by improving the copy number or replacing the native promoter with a strong promoter from a heterologous strain [28, 35, 37, 42]. In our study, two novel *S. lydicus* strains, A01 and A02, which are capable of producing natamycin, were isolated, respectively, from the soil of vegetable and forest fields in suburbs of Beijing (China). Wild-type strain A02 produced 1.3-fold higher natamycin than A01 and had a yield of about 2.5 g l^{-1} in shake flask fermentation and 6.91 g l^{-1} in 30-l fermentation, indicating that it is a potential candidate for natamycin production at the industrial scale. We focused our study on increasing natamycin production in A02 by gene manipulation. Different levels of natamycin production from A01 and A02 were obtained. The *slnM* genes (*slnM1* in A01 and *slnM2* in A02) were cloned and sequenced from A01 and A02, using a polymerase chain reaction (PCR)-based approach with primers that were designed according to the sequences of *pimM* in *S. natalensis* and *scnRII* in *S. chattanoogensis*. These genes code for pathway-specific positive regulators of natamycin biosynthesis and belong to the PAS-LuxR family of transcriptional activators. All polyene macrolides known to date are regulated by PAS-luxR regulators. All of these regulators, from different polyene gene clusters, share a peculiar architecture that combines a N terminal PAS sensory domain with a C terminal HTH motif of the LuxR type for DNA binding, and they display the same regulatory pattern responsible for polyketide chain construction [32, 39]. *SlnM1* and *slnM2* showed a high sequence identity (99 %). The two genes were expressed at different levels in A01 and A02. We characterized *slnM* in the regulation of natamycin biosynthesis and examined the effects of overexpressing *slnM2* under control of the ermEp* promoter, the native promoter and dual promoters, on natamycin production in *S. lydicus* strain A02.

Materials and methods

Strains and plasmids

The bacterial strains A01 (CGMCC accession no. 1653; China General Microbiology Culture Collection Center) and A02 (CGMCC accession no. 1654) were isolated from the soil of suburban vegetable and forest fields, and identified as *Streptomyces lydicus* based on the 16S rDNA sequences and phenotypic comparison [25, 38]. The GenBank accession number for 16S rDNA of *S. lydicus* strain A01 is EF532323.1 and for *S. lydicus* strain A02 is FJ481127.1.

The strains were grown at 29 °C on potato dextrose agar (PDA) slants for spore formation. *Escherichia coli* strain DH5α was used as a host for genetic manipulation. The non-methylating *E. coli* strain ET12567 (pUZ8002) was used for DNA conjugal transfer from *E. coli* to *S. lydicus*. Conjugation and regeneration were performed as described by Kitani et al. [15] and Paranthaman et al. [29]. Plasmid pUC19 was used for routine cloning and subcloning experiments. The integration vector pSET152 [5] containing Φ31 *int* and *attP* was used to introduce a single copy of *slnM* into *S. lydicus*. The ermEp* promoter segment ligated to pSET152 generated the vector pIB139 [42]. When necessary, media were supplemented with antibiotics (apramycin was added at 100 μg ml^{-1} to LB medium, at 60 μg ml^{-1} to selection medium and at 30 μg ml^{-1} to the fermentation medium, as required). Synthesis of oligonucleotide primers and DNA sequencing of PCR products were performed by Invitrogen Biotechnology (China).

Amplification and sequencing of the *slnM* gene in *S. lydicus*

DNA manipulations of *S. lydicus* were performed as described by Kieser et al. [13]. DNA fragments carrying the *slnM* genes without their promoters, from *S. lydicus* A01 and A02, were amplified with the degenerate primers MF 5′-ATGGCGAGCCTTGATARAACRTT-3′ and MR 5′-TCACTTCACGAAGTCGTCCAC-3′, designed according to the intact gene sequences of *pimM* in *S. natalensis*

and *scnRII* in *S. chattanoogensis*. The PCR reaction was performed using TaKaRa ex Taq (TaKaRa, Japan) and consisted of one cycle of denaturation at 96 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 61 °C for 30 s and 72 °C for 45 s, with one extension cycle at 72 °C for 10 min. The two 579-bp products were sequenced and then cloned into the vector pMD18-T, which resulted in the plasmids pMD*slnM1* and pMD*slnM2*. Each amplified product was re-sequenced after cloning into plasmid vectors to ensure fidelity. GenBank accession numbers for *slnM1* of strain A01 and *slnM2* of strain A02 are JX993819 and JX993818, respectively.

Introduction of the *slnM2* gene under different promoters into the *S. lydicus* A02 strains

The primers MF1 5′-GGAATTCCATATGATGGCGAGC CTTGATAAAACGTT-3′ and MR1 5′-GGAATTCTCAC TTCACGAAGTCGTCCAC-3′ (the underlined nucleotides were added to introduce *Nde*I and *Eco*RI sites for cloning) were used to clone the *slnM2* gene without its promoter from *S. lydicus* A02 and generated a 579-bp amplicon. The PCR fragment was digested with *Nde*I/*Eco*RI and ligated into *Nde*I/*Eco*RI-digested pIB139 to generate pIBM01, which harbors the ermEp* promoter and the intact *slnM2* gene.

The primers MF2 5′-GGAATTCCATATG(TGCAGGA TATC)CGGTCGGAGGTGCGGG- CATGAC-3′ and MR2 5′-GGAATTC(GCTCTAGA)TCACTTCACGAAGTCGTC CAC-3′ were used to clone the *slnM2* gene with its own promoter from *S. lydicus* A02 [the underlined nucleotides were added to introduce *Nde*I/*Eco*RI (*Eco*RV/*Xba*I) sites for cloning] according to Du et al. [8]. The primers were designed according to the sequence of *scnRII* in the *S. chattanoogensis* natamycin biosynthetic gene cluster (GenBank accession no. HQ386234.1). A 998-bp segment was either digested with *Nde*I/*Eco*RI and ligated into *Nde*I/*Eco*RI-digested pIB139 generating pIBM02 or was digested with *Eco*RV/*Xba*I and ligated into *Eco*RV/*Xba*I-digested pSET152 generating pIBM03. Plasmid pIBM02 carried the dual promoters, ermEp* promoter and its native promoter, while the plasmid pIBM03 carried the *slnM2* gene under its native promoter. Each amplified product was sequenced to ensure fidelity after cloning into plasmid vectors. The three integration recombinant vectors (pIBM01, pIBM02 and pIBM03) were introduced into *S. lydicus* A02 by intergeneric conjugation, together with their empty plasmids: pIB139 (negative control of pIBM01 and pIBM02) and pSET152 (negative control of pIBM03). The exconjugants were screened for apramycin resistance at 60 μg ml^{-1}. The genotype of the exconjugants was confirmed by PCR analysis with specific primers for the apramycin resistance gene aac (3) IV according to Li et al. [21].

Isolation of total RNA

Streptomyces lydicus wild-type and recombinant strains were inoculated into YEME medium without sucrose. The mycelia were collected after growth for 36 and 72 h, flash-frozen in liquid nitrogen and ground into a fine powder. RNA was extracted using RNeasy Mini kit (TianGen, Beijing, China) according to the manufacturer's instructions. The RNA preparations were treated with DNase I (TianGen) to eliminate possible chromosomal DNA contamination.

Gene expression analysis by reverse transcriptase PCR (RT-PCR)

RT-PCR was performed on ribonuclease (RNase)-free deoxyribonuclease (DNase I)-treated total RNA by using the avian myeloblastosis virus (AMV) reverse transcriptase TaKaRa RNA PCRTM Kit (Takara). One μg of total RNA from each sample was used in a 20-μl RT reaction with the following program: 72 °C for 10 min, 42 °C for 50 min and 99 °C for 5 min. The cDNA (0.6 μl) was used as template for amplification of natamycin biosynthesis genes in a 20-μl PCR reaction. Different PCR cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) were performed using *TaKaRa Ex Taq*® (Takara, Dalian, China). The following primers were designed for *slnI* and *slnJ* according to the sequences of *scnI* and *scnJ* in *S. chattanoogensis* (GenBank accession no. HQ386234.1): IF 5′-CCACGGCGG AAGGGCTCT-3′ and IR 5′-CGGTGTAGTCGCTGCG GAT-3′, and JF 5′-CAGCCCGAACTCGTCACC-3′ and JR 5′-CCTGCCCGTAGTCCTCCC-3′. Primers were designed to generate PCR products of 519 bp for *slnI* and 577 bp for *slnJ*. Primers MF1 and MR1 were used for the *slnM2* gene.

Semiquantitative RT-PCR analysis was performed to ensure that the produced DNA had not reached the plateau value; RT-PCRs for each tested transcript were carried out by using different cycle numbers (25, 28 and 30). The *hrdB* gene, which encodes the major RNA polymerase sigma factor in *Streptomyces* spp. [7], was used as internal control with specific primers (forward, 5′-TACTGCGCAGCCT CAACCAG-3′, and reverse, 5′-GCCGATCTGCTTGAG GTAGTC-3′). Each amplified product was sequenced to verify its identity.

Fermentation and analysis of natamycin

For natamycin production, spores of *S. lydicus* (5×10^7) from a PDA agar slant were inoculated into 50 ml of a seed culture medium (2 % glucose, 1.5 % peptone, 0.75 % yeast extract, 1.0 % NaCl) and incubated at 29 °C for 24 h on a rotary shaker (250 rpm). Two different production media were used: YEME medium without sucrose [3] was used

for growth and natamycin production analysis, and YSG medium was used for industrial natamycin production for *S. lydicus* A02 [8]. The seed culture was added to 50 ml of the YEME medium without sucrose and then diluted to an OD_{600} of 0.1. Equal volumes of seed culture were added to 50-ml amounts of YSG medium and the flasks were incubated at 29 °C and 250 rpm for 5 days. Natamycin production was assessed according to Lee et al. [18] with a small modification; 0.5 ml culture was extracted with 4.5 ml methanol. The methanol layer was recovered by centrifugation at 8,000 rpm for 5 min. The concentration of natamycin was determined by HPLC (Japan Analytical Industry Co., Ltd., Japan) using a C18 reverse-phase column (LC-9101-JAIGEL-ODS, Japan), with a mobile phase of 70 % methanol (Fisher, USA) at a flow rate of 1 ml min^{-1}, and monitored at 303 nm with a UV detector (JAI UV 3702, Japan). Samples were analyzed in 10-μl volumes in a 25-μl injection loop.

Analysis of antifungal activity

Five Oxford cups were placed on an agar plate containing 25 ml of PDA inoculated with *Fusarium oxysporum* f. sp. *conglutinans* spore suspension and individually loaded with 100 μl (100× diluted) fermentation supernatant of *S. lydicus* grown in YEME medium without sucrose for 3 days. After incubation at 28 °C for 5 days, the inhibition diameters of *F. oxysporum* f. sp. *conglutinans* were measured. Each assay was performed three times.

Statistics

All experiments were conducted in triplicate, and the average values are reported. Key results were repeated three times to establish their validity, determined using analysis of variance (ANOVA), and expressed by their mean ± standard deviation (SD). Variation among treatment means were compared using Duncan's multiple range test ($P < 0.05$).

Results

Cloning of natamycin-positive regulator *slnM*

A pair of degenerate primers was designed according to *pimM* (AM493721) and *scnRII* (FJ418775) to amplify the *slnM1* and *slnM2* genes in strains A01 and A02. The entire sequences of *slnM1* and *slnM2* with the expected size of 579 bp were obtained from *S. lydicus* A01 and A02. BLASTX analyses of the DNA and amino acid sequence (192 aa) showed high sequence similarity to each other (99 %), the same similarity to the *scnRII* gene in *S. chattanoogensis* and a similarity of 96 % to *pimM* in *S. natalensis*.

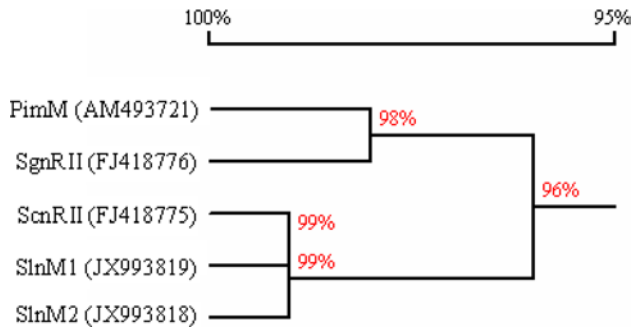

Fig. 1 Homologous tree of SlnM1 (JX993819), SlnM2 (JX993818), ScnRII (FJ418775), PimM (AM493721) and SgnRII (FJ418776)

The putative protein has a PAS sensor-binding domain at the N terminus and a HTH motif of the LuxR type at the C terminus. Based on its sequence similarity to *scnRII* and *pimM*, *slnM* was expected to share a similar regulatory pattern with *scnRII* and *pimM*, and to have a positive regulatory function on natamycin production in *S. lydicus* A02. Comparative sequence analysis of SlnM1, SlnM2, ScnRII, PimM and SgnRII (FJ418776, the positive regulator of natamycin production in *S. chattanoogensis* according to Du et al. [8]) showed that these five proteins are highly conserved. SlnM1 and SlnM2 showed higher similarity to ScnRII (192 aa) than to PimM or SgnRII (185/192 aa) (Fig. 1).

Differential natamycin production and *slnM* gene expression in *S. lydicus* A01 and A02

Both strains A01 and A02 proved to produce high levels of natamycin, but A01 displayed a lower natamycin yield than A02, and morphological differences were also evident. Strain A01 could not grow well on YEME medium without sucrose. HPLC analysis of the fermentation products revealed that strain A02 produced nearly 2.5 g l^{-1} natamycin in YSG medium, about 22 % more than strain A01 (Fig. 2a).

Total RNA used for the RT-PCR analysis was prepared from mycelia grown for 3 days in YSG medium. The transcriptional levels of *slnM* were clearly higher in A02 than in A01 (Fig. 2b). By considering the similar structural characteristics of *slnM* to the pathway-specific positive regulators *scnRII* and *pimM,* and the correlation between *slnM* gene expression and natamycin production, we predicted that *slnM* could be a positive regulator of natamycin biosynthesis in *S. lydicus*.

Overexpression of the *slnM* gene under different promoters increased natamycin production

Because *slnM* encodes a positive regulator, an increased expression of *slnM* might enhance the natamycin

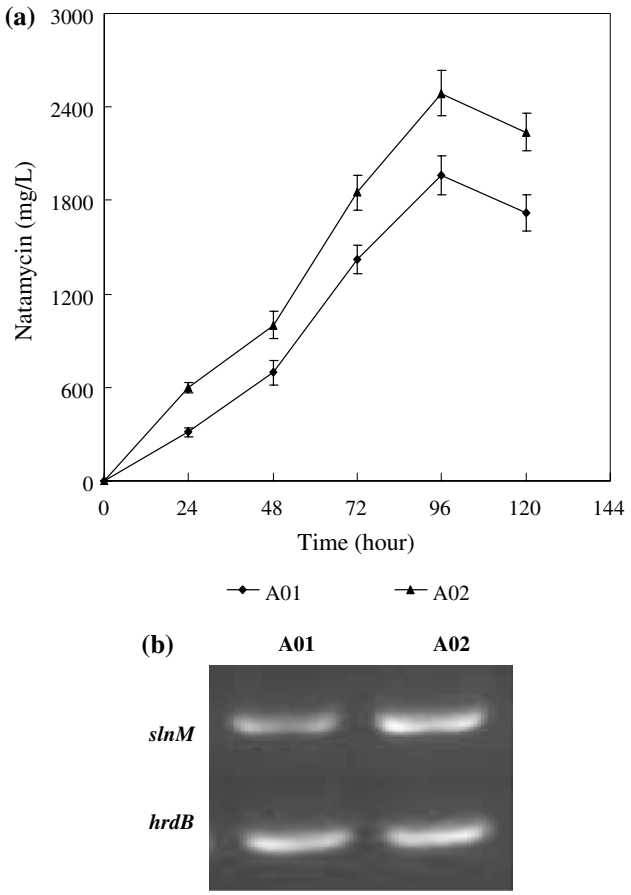

Fig. 2 Natamycin production and *slnM2* gene expression analysis of the *S. lydicus* A01 and A02. **a** Time course of natamycin production of *S. lydicus* A01 and A02. Data are mean ± SD of three independent experiments. **b** *slnM2* gene expression analysis of *S. lydicus* A01 and A02. Total RNA used as a template was prepared from the mycelium after 48 h of cultivation in YSG medium, and *hrdB* gene was used as an internal control

production in *S. lydicus.* Based on this, the effects of overexpression of *slnM2* on natamycin yield and cell growth were investigated.

Three recombinant vectors including pIBM01, with *slnM2* under the ermEp* promoter, pIBM02, carrying *slnM2* with dual promoters and pIBM03, with *slnM2* under the native promoter, were introduced into *S. lydicus* A02 by inter-generic conjugation, generating *S. lydicus* AM01, AM02 and AM03 (Fig. 3).

The effects of *slnM2* gene duplication under different promoters on natamycin production were investigated. Growth and natamycin production of wild-type strain A02, three recombinant strains AM01, AM02 and AM03, and corresponding vector control strains A02 (pIB139) and A02 (pSET152) were studied in flask cultures using YEME medium without sucrose and YSG medium. As shown in Fig. 4a, mycelial growth and natamycin production by the vector control strains were similar to that of strain A02 in both media. The strains AM01, AM02 and AM03 had no significant effect on mycelial growth compared to strain A02, whereas they had a clear enhancing effect on natamycin production. Strain AM02 increased natamycin production 3.0-fold compared to strain A02 after 72 h-fermentation in YEME medium without sucrose (Fig. 4b). In YSG medium (Fig. 4c), the highest natamycin production of strain AM02 was 5.3 g l^{-1} in shake flask fermentation for 4 days, which represents a 2.1-fold increase compared to that of the wild-type strain A02 (2.5 g l^{-1}). Strains AM01 and AM03 produced respectively 2.4- and 1.9-fold more natamycin, compared to strain A02 in YEME medium without sucrose, but their production was lower than that of strain AM02, indicating that the overexpression of *slnM2* driven by different promoters varied the natamycin yield.

Transcriptional analysis of natamycin biosynthetic genes *slnI* and *slnJ* in different constitutive overexpression strains

Total RNA was prepared from *S. lydicus* wild-type A02 and the engineered strains AM01, AM02 and AM03 after growth for 36 and 72 h in YEME medium without sucrose, and it was used as a template for gene expression analysis by RT-PCR.

Transcripts of *slnM2* gene and two genes, *slnI* and *slnJ* (GenBank accession nos. ACD75765 and ACD75765), orthologous to *pimI* (discrete thioesterase putatively involved in the removal of non-productive decarboxylated extender acyl groups from the polyketide synthase) [14] and *pimJ* (GDP-mannose dehydratase) controlled by *pimM* of *S. natalensis* were analyzed in *S. lydicus* strains. In order to make a proper comparison, the RT-PCRs were performed using different amplification cycles (25, 28 and 30). These analyses were carried out at least three times for each primer pair. We found very weak transcripts for any of the three genes after 25 cycles. After 28 cycles, an appropriate comparison was possible, whereas the genes reached a plateau value after 30 cycles.

Transcription of these three genes after 28 cycles under different sampling times were clearly detectable in strains AM01, AM02 and AM03 from the early exponential phase, where smaller amount of transcript was detected in the wild-type strain A02 (Fig. 5). Furthermore, the dual promoter-driven strain AM02 showed the highest transcriptional level. The strain AM01 with the ermEp* promoter had a higher expression level than strain AM03 with its own promoter, but all of these overexpression strains displayed a stronger transcription compared to the wild-type strain, indicating that inserting another copy of *slnM2* under the control of a different promoter (ermEp* or native promoter) enhanced natamycin production and expression of natamycin biosynthesis genes, but in varying degrees.

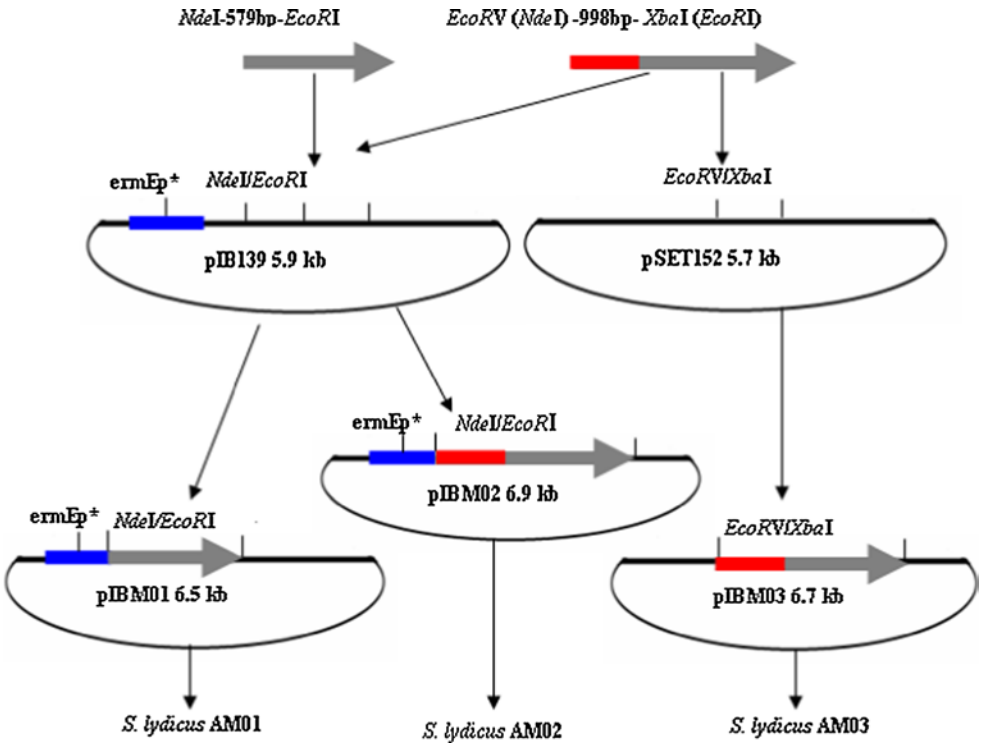

Fig. 3 Schematic representation of the strategy used for duplication of *slnM2* gene dosage under the control of different promoters (ermEp*, native promoter and dual promoter). *Gray broad arrow* indicates *slnM2* gene. The *blue box* represents ermEp* promoter, and the *red box* represents the native promoter for *slnM2*. Restriction enzyme sites *Nde*I/*Eco*RI, *Nde*I/*Eco*RI and *Eco*RV/*Xba*I were used to generate pIBM01, pIBM02 and pIBM03. The engineered strains AM01, AM02 and AM03 were generated by conjugation from non-methylating *E. coli* strain ET12567 (pUZ8002) to *S. lydicus* (color figure online)

Antifungal activity of *S. lydicus*

Inhibitory activities against *F. oxysporum* f. sp. *conglutinans* by the fermentation broth containing natamycin produced by strains AM01 (9 mg l^{-1}), AM02 (11.5 mg l^{-1}), AM03 (7.3 mg l^{-1}) and A02 (3.8 mg l^{-1}) under the same conditions were studied and the diameters of inhibition zones were measured (Fig. 6). The antifungal activity of the fermentation supernatant of all the engineered strains was increased, which was remarkable. Consistent with our observation that the strain AM02 produced threefold more natamycin than the wild-type strain A02, the inhibitory effect of the fermentation supernatant of *S. lydicus* AM02 on *F. oxysporum* was about 3.4-fold that of the wild-type A02.

Discussion

The natamycin pathway-specific regulator *pimM* in *S. natalensis* belongs to the PAS-LuxR regulators. Inactivation of the *pimM* by gene replacement of the HTH DNA-binding domain in the chromosome of *S. natalensis* resulted in complete loss of the natamycin production, and the complementation by intact pimM restored natamycin production in the mutant [1]. Later work demonstrated that PimM is a positive regulator and its mode of action for natamycin biosynthesis [33].

Overexpression of the pathway-specific positive regulator *pimM* and *scnRII* (ortholog to *pimM*) led to 1.5-fold and 4.6-fold higher natamycin production, respectively, compared to that of the wild-type strains from *S. natalensis* and *S. chattanoogensis* in YEME medium without sucrose [1, 8]. In this study, the gene *slnM2* was cloned from *S. lydicus* A02. BLASTX analysis showed that the amino acid sequence from the translated framework had 99 % similarity to ScnRII and AURJ3 M (GenBank accession no. ACD75765), a positive regulator for biosynthesis of aureofuscin in *S. aureofuscus*, and a similarity of 96 % (186/192 aa) to PimM. Similarly to PimM and AURJ3M, SlnM2 has a PAS sensor-binding domain at the N terminus and an HTH motif of the LuxR type at the C terminus. Thus, *slnM2* might be a pathway-specific positive regulator of natamycin production in *S. lydicus* A02. Overexpression of *slnM2* enhanced transcription of some genes of the natamycin clusters, which further increased natamycin production.

Ensuring the appropriate increase of a positive pathway-specific regulator gene dosage, by increasing the copy number or by replacing its native promoter with a strong promoter of the positive regulator should enable a higher production of the corresponding antibiotics in *Streptomyces* spp. This is the case for *ccaR* in the production of cephamycin and clavulanic acid [31] and *mtmR* in the production of mithramycin [24]. The vector carrying the strong heterologous ermEp* promoter of *S. erythraea* improved the yield of triketide lactone by 100-fold as compared to the level produced by the same plasmid where actII-ORF4 was expressed from its own promoter in *S. cinnamonensis* [4]. Three approaches were used to improve the expression

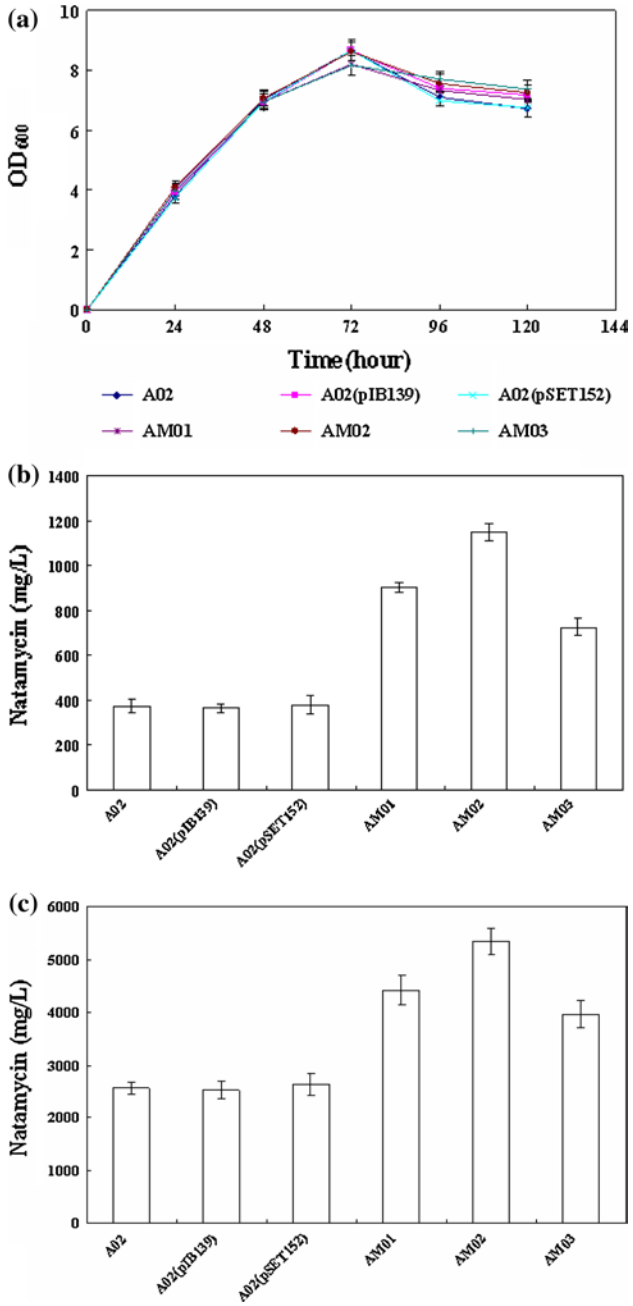

Fig. 4 Effect of increasing *slnM2* gene dosage on natamycin production and cell growth of *S. lydicus* strains in YEME medium and YSG medium. **a** Comparison of growth curves among A02, A02 (pIB139), A02 (pSET152), AM01, AM02 and AM03 in YEME medium without sucrose; growth was monitored by measuring the optical density at 600 nm (OD_{600}). **b**, **c** Natamycin production of *S. lydicus* strains in YEME medium without sucrose and in YSG medium. Data are mean ± SD of three independent experiments

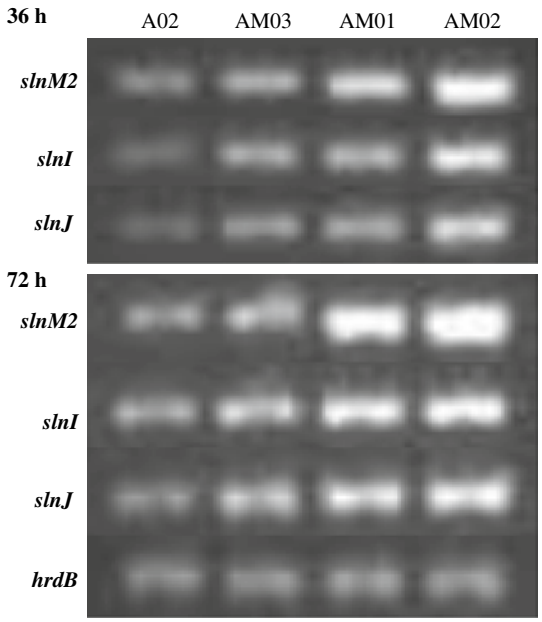

Fig. 5 Effect of *slnM2* overexpression on the transcripts of gene *slnI* and *slnJ*; a semi-quantitative RT-PCR analysis of *slnM2*, *slnI* and *slnJ* transcript levels in wild-type and *slnM2*-overexpressing strains of *S. lydicus*. *HrdB* gene was used as the internal control. Cells were collected from cultures grown on YEME medium without sucrose for 36 and 72 h

of *slnM2* in order to increase the yield of natamycin in *S. lydicus* A02. Our results showed that the three engineered strains exhibit higher production of natamycin and transcriptional levels of *slnI* and *slnJ* genes compared to that of the wild-type strain. Among the natamycin cluster of *S. natalensis*, the transcriptional levels of *pimI* and *pimJ* were controlled by *pimM*, and their transcription was positively related to that of *pimM* gene [1, 33]. Our investigations indicated that the transcription levels of *slnI* and *slnJ* were positively correlated with natamycin production and transcriptional levels of the *slnM2* gene, in agreement with Antón et al. [1].

Increasing the copy number of *slnM2* by inserting an intact *slnM2* gene with its own promoter into the wild-type strain produced a 1.9-fold increase in natamycin production, compared to that of the wild-type strain. Replacing the native *slnM2* promoter with ermEp* improved the natamycin yield to 2.4-fold and showed a substantial increase in natamycin production in *S. lydicus*. Moreover, the overexpression of *slnM2* under dual promoters permitted the achievement of a 3.0-fold higher yield, as compared to the wild strain, suggesting that the expression of the activator gene under its native promoter alone may not give optimal antibiotic production. The ermEp* promoter from a heterologous actinomycete strain, drives a more effective transcription than the homologous promoter of *slnM2* in *S. lydicus* A02, but the combination of both promoters resulted in a more robust expression of *slnM2* gene and a higher yield of natamycin than either single promoter. Previous studies showed that genes driven by double promoters exhibited higher gene expression or a much wider host range for gene

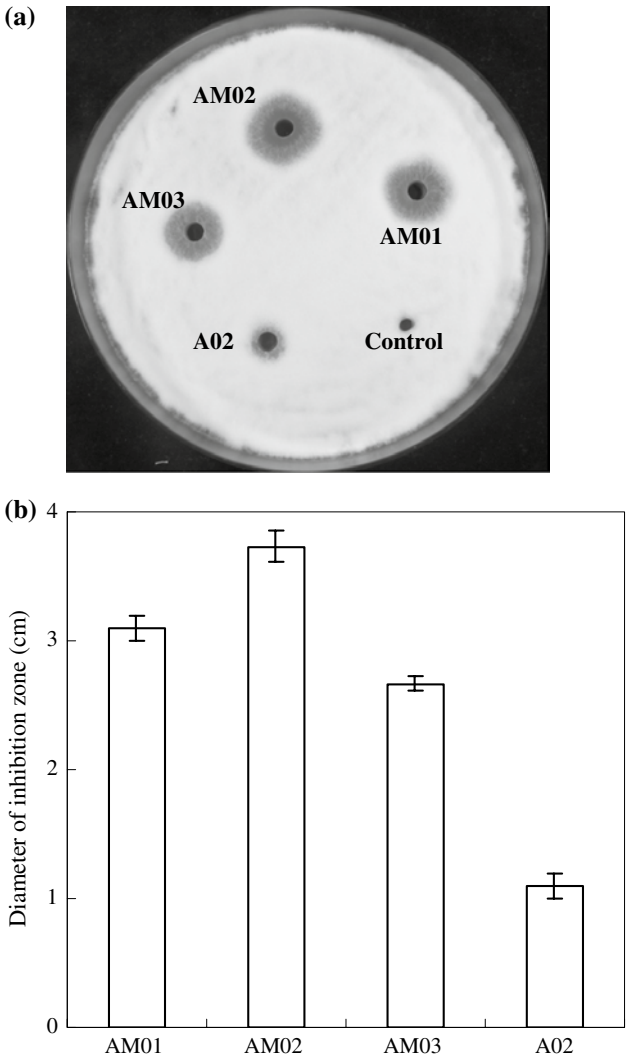

Fig. 6 **a** Inhibition zones produced by the fermentation broth containing natamycin produced by strains AM01, AM02, AM03 and A02 under the same conditions. Each hole with 100 μl (100×) diluted 3-day-old fermentation supernatant of *S. lydicus* in YEME medium without sucrose and the control hole with 100 μl YEME medium without sucrose. **b** Statistical analysis of inhibition zones. Data are mean ± SD of three independent experiments

expression than by a single promoter in microorganisms and plants [6, 23, 36].

All of the PAS-luxR regulators from different polyene gene clusters share a particular architecture and display the same regulatory pattern for polyketide chain construction [32]. For example, introduction of heterologous regulators of the PAS/LuxR class, such as amphRIV, nysRIV or pteF, into the chromosome of a strain of *S. natalensis* ΔpimM restored the production of natamycin. Introduction of a single copy of pimM into the amphotericin producing strain *S. nodosus*, or into the filipin producing strain *S. avermitilis*, boosted the production of both polyenes [32]. All of the exconjugants overexpressing six nystatin-like *Pseudonocardia* polyene (NPP) pathway-specific regulators (three of them belong to a typical LAL-type transcriptional family) in the rare actinomycete *Pseudonocardia autotrophica* individually resulted in the improvement of NPP production [11].

All of these results confirmed the functional conservation of orthologous PAS-luxR regulators not only in *Streptomyces*, but also in other actinomycetes. The dual promoter's strategy used in our study would provide a beneficial way for further exploring how to enhance the efficiency of the gene expression and improve the productivity of secondary metabolites in actinomycetes.

In *S. chattanoogensis* L10, overexpression of the pathway-specific positive regulator *scnRII* led to a higher production of natamycin, about 4.6-fold in YEME medium without sucrose and 3.3-fold in YSG medium compared to the wild strain, respectively [8]. The engineered *S. chattanoogensis* D1 produced about 2.14 g l^{-1} natamycin in an industrial production medium in laboratory fermentation. The increase of natamycin production influenced by overexpression of the *slnM2* gene in *S. lydicus* was lower than that in *S. chattanoogensis*, though the strain AM02 showed significantly higher yield than the engineered *S. chattanoogensis* D1. The wild-type strain *S. lydicus* A02 had a significantly higher capacity of natamycin production (2.5 g l^{-1}) than the wild-type of *S. chattanoogensis* L10 (about 0.6 g l^{-1}). This may be due to the maximum threshold regulation ability of *slnM2* for natamycin production or to the biosynthetic capacity of *S. lydicus* A02. Natamycin production of the engineered strain *S. lydicus* AM02 yielded 5.3 g l^{-1} after 4 days growth in a shake flask. The production capacity in pilot fermentation studies should be stronger than that of strain A02 previously achieved (6.9 g l^{-1}). This indicated that strain AM02 is a potential producer of natamycin for larger scale industrial production.

Recently, *S. lydicus* has been reported to produce natamycin [25], but there has been no report about the genetic operation and regulatory mechanism of the natamycin biosynthesis. In this study, we cloned the pathway-specific positive regulator of natamycin biosynthesis in *S. lydicus* and compared the regulatory role of *slnM2* with different promoters on natamycin biosynthesis in *S. lydicus* A02, and this would accelerate research on the mechanisms of the natamycin biosynthesis.

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