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Original research article

Iteratively improving natamycin production in by a large operon-reporter based strategy

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ABSTRACT

Many high-value secondary metabolites are assembled by very large multifunctional polyketide synthases or non-ribosomal peptide synthetases encoded by giant genes, for instance, natamycin production in an industrial strain of . In this study, a large operon reporter-based selection system has been developed using the selectable marker gene to report the expression both of the large polyketide synthase genes and of the entire gene cluster, thereby facilitating the selection of natamycin-overproducing mutants by

whose titers have already been greatly improved by conventional iterative breeding.

Natamycin (also known as pimaricin) is a 26-member polyene macrolide antifungal antibiotic originally isolated from and also produced by several other strains, including . *h5* (Du et al., 2009), . (Lu et al., 2008), and . (Liang et al., 2008). Natamycin is the only antifungal agent recognized by the US FDA as GRAS (generally regarded as safe) (Aparicio et al., 2016), and it has been widely used by the food industry as a natural preservative to prevent mold contamination for nearly 50 years. Natamycin is also used for the treatment of topical fungal infections, such as keratitis (Ansari et al., 2013). The polyene skeleton of natamycin is synthesized by a type I polyketide synthase (PKS) assembly line from acetyl-CoA, malonyl-CoA, and methylmalonyl-CoA. The natamycin biosynthetic gene cluster of . includes five large PKS genes (*0*- *4*) and 14 genes for tailoring enzymes, transport, and regulation (Aparicio et al., 1999, 2000); the natamycin biosynthetic gene cluster ( ) from . *h5* shares high similarity with the cluster with respect to overall sequence, gene organization, and regulation (Du et al., 2011). The five PKS genes of . constitute three large transcription units, i.e., the *2-4* operon (40 kb), the stand-alone gene *1* (20 kb), and the *CGF 0* operon (8 kb) (Santos-Aberturas et al., 2011). Both and clusters contain two large transcription units with sizes approximately 20- and 40 times that of the average gene, which, for example, in . is 0.99 kb (Bentley et al., 2002).

In this study, our goals were to increase natamycin production by the industrial natamycin-producing strain . Ins1, using iterative mutagenesis and selection. Through sequence analysis of the natamycin biosynthetic gene cluster, we determined that . - Ins1 possesses very large PKS genes, and we hypothesized that the transcription and stability of the very long mRNAs of these genes constitute an unsuspected bottleneck limiting natamycin production in the strain. Accordingly, a new semi-rational method was developed to substantially improve natamycin production in . Ins1, taking into account the very long polyketide synthase genes and taking advantage of iterative random mutagenesis breeding.

2. Materials and methods

2.1. B , ,

. Ins1 was used as the starting strain, and *E h5 h5* DH5α was used as cloning host. *E.* ET12567 (pUZ8002) was used as a helper strain to mobilize plasmid into via *E.* - intergeneric conjugation (Kieser et al., 2000). The cosmid vector SuperCos1 was used in construction of the genomic library of . Ins1, using a Gigapack III XL Packing Extract kit (Stratagene). Cosmids containing the natamycin biosynthetic gene *1* were identifi

Table 1
Oligonucleotides used in this study.

Primer	Sequence (5' → 3')

was discarded, and 1.5 mL methanol: glacial acetic acid (95:5, v/v) was added to the pellet and mixed. The mixture was ultrasonicated for 20 min, centrifuged for 5 min at 7,000 rpm, and then 50 μl supernatant was pipetted into a new Eppendorf tube before diluting with 950 μl methanol: glacial acetic acid (95:5, v/v) for HPLC analysis. HPLC was conducted with an Agilent ZORBAX SB-C18 (4.6×250 nm) column at a flow rate of 0.5 mL min^{-1}, and the eluate was monitored with a UV detector at 303 nm. The mobile phase contained methanol, water, and glacial acetic acid, with a ratio of 60:40:5 (V/V/V). The natamycin standard was purchased from Lifecome Biochemistry Co., Ltd.

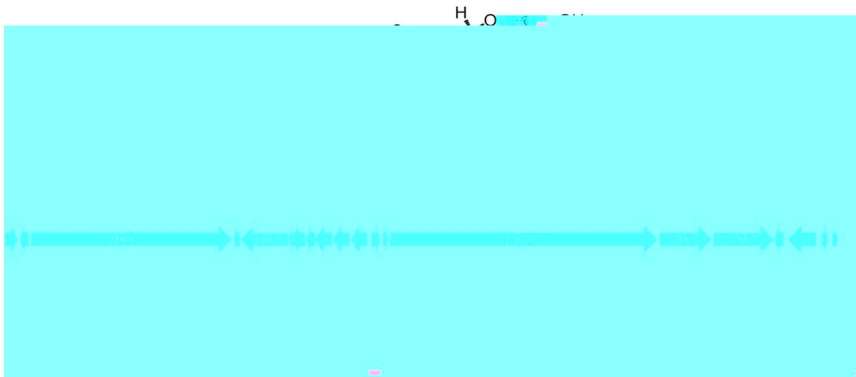

Fig. 1. Natamycin biosynthetic gene cluster from the industrial producer strain Ins1. The structure of natamycin is also shown. The two large gene/operons encoding the giant polyketide synthases are indicated by grey arrows. Thick lines indicate the inserts of cosmids identifi

2.5. I A - C

Total RNA from strains was prepared with Redzol reagent and extracted using the Total RNA extraction kit (SBS Genetech Co., Ltd.), according to the manufacturer's instructions. Genomic DNA was removed by RNase-free DNase I (Fermentas), and the concentration and quality of total RNA were determined using a Biophotometer Nanodrop (Thermo). Reverse transcription was conducted using the cDNA Synthesis Kit (Fermentas). Quantitative real-time RT-PCR (qRT-PCR) was carried out on the Applied Biosystems 7500 system using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). The PCR conditions consisted of one cycle of denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The sequences of primer pairs are listed in Table 1. The transcription of *h5 B*, 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 *h5 B,* and all qRT-PCR reactions were performed in at least triplicate.

2.6. D h5

Copy number of the natamycin gene cluster was determined by quantitative real-time PCR using single-copy chromosomal sequences as references, according to a described method (Škulj et al., 2008). The two chromosomal reference sequences were located in the cell division protein *Z* and ATP synthase subunit alpha *A*, and two intergenic sequences (*IJ* and *D 1*) in the natamycin gene cluster were selected to represent the gene cluster, using the indicated primer sets (Table 1). A 5-fold dilution series of chromosomal DNA was used as template. Relative standard curves were configured for *Z*, *A*, *IJ* and *D 1*, using a series of five, 5-fold dilutions. The amplification efficiency (E) was calculated using the equation $E=10^{(-1/slope)}$, where the slope of the relative standard curve was used. The copy number of the natamycin gene cluster (NCN) was determined using the equation $NCN=(Ec)^{Ctc}/(En)^{Ctn}$, where Ec and En were the amplification efficiencies of the chromosome gene and natamycin gene cluster, respectively, and Ctc and Ctn were Ct values for the chromosome and natamycin gene cluster, respectively. The final NCN values were averaged from three biological replicates.

2.7. D D A

The draft genome sequence of Ins1 was determined by Pacific Biosciences and Illumina HiSeq2000. DNA sequence of the natamycin gene cluster was obtained from the draft genome and deposited to NCBI under GenBank accession numbers: KX458106.

3. Results

3.1. h5 h5 S. gilvosporeus *I 1*

Ins1 was an empirically improved strain used for industrial production of natamycin in China. Genomic sequencing of Ins1 revealed a type I polyketide biosynthetic gene cluster that we named the natamycin biosynthetic gene cluster ( ) and which shares high sequence similarity with the gene cluster from (99% identity) and the gene cluster from *h5* L10 (93% identity). The deduced amino acid sequences of the 20 Sgn proteins, i.e., SgnS0, SgnS1-S4, SgnA-K, SgnL, SgnM, SgnR, and SgnT, share 99–100% sequence identity with their counterparts in the Pim proteins. There are five large PKS genes, *0-4,* in the cluster, and *1* and *2* are particularly large in size, spanning 20.4 kb and 28.6 kb, respectively. The gene position and orientation of the cluster are the same as in the and clusters, both of which contain two abnormally large transcription units corresponding to *1* (20.4 kb) and *2 3 4* (40.1 kb) of the cluster (Fig. 1, grey arrows).

3.2. D h5 - natS1-neo *-*

A semi-rational approach was designed to improve the - Ins1 strain via mutagenesis and selection, with the use of a selectable reporter specifically engineered into the gene cluster. The large PKS gene *1* in the cluster was chosen as the engineering target to be linked at its 3' end with a promoterless

reporter gene, , which encodes neomycin phosphotransferase and confers neomycin/kanamycin resistance. In the artificial 1- operon, the two open reading frames share an overlapping nucleotide at the TGA stop codon of 1 and the ATG start codon of (Fig. 2); expression of the promoterless should therefore depend on read-through transcription from the upstream 1 producing a large polycistronic messenger RNA. Essentially, the expression of would report the expression of 1. Furthermore, it was anticipated that, situated within the cluster, the reporter would serve as a marker of expression of the whole cluster and of natamycin production, assuming that the genes of the natamycin biosynthetic pathway are expressed coordinately as in many other antibiotic biosynthetic pathways (Santos-Aberturas et al., 2011; Liu et al., 2013). Thus, this cluster-situated reporter should be useful for selecting mutants with increased expression of the gene cluster and higher fermentation titers of natamycin.

3.3. C h5 - natS1-neo S. gilvosporeus I 1

To engineer the designed 1- reporter operon into the industrial strain by gene replacement, we constructed a genomic cosmid library of Ins1 and obtained four cosmid clones carrying 1 and adjacent genes by in situ Southern blot using a DNA probe and confirmed by end sequencing of the inserts (Fig. 1). Cosmid 2F1, which has a genomic DNA insert covering the entire 1 and 0 genes, was selected to proceed. The promoterless was inserted into the 3' end of 1 in pWW2 to yield the gene replacement vector pWW4. However, the attempt to construct the reporter strain using pWW4 failed, and pWW4 was subsequently found to be subject to large DNA deletions in the conjugation helper strain *E.* ET12567/pUZ8002, probably due to the presence of repeat sequences in the large PKS genes 1 and 0. To overcome this problem, a 7.8 kb I-*B* II DNA segment containing only parts of the 1- reporter operon and 0 was excised from pWW4 and inserted into the suicide vector pOJ260 to produce the new gene replacement vector pAL06 (Fig. 3A). pAL06 was then used to construct the cluster-situated, large 1- reporter operon in Ins1 by homologous recombination. The resulting streptomycin-resistant and apramycin-sensitive, double-

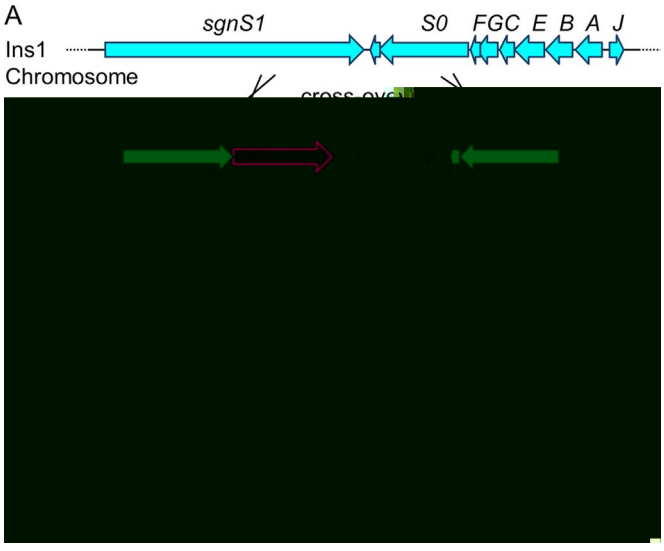

Fig. 3. Construction of the -labeled strain Lbd1 from Ins1 via homologous recombination. (A) Schematic representation of homologous recombination between the gene cluster of Ins1 and the gene replacement plasmid pAL06. P1, P2, and P3: PCR1, PCR2, and PCR3 designed for verification of the labeled strain. The locations and calculated sizes of PCR amplicons are indicated by dashed double arrows. (B) Verification of the labeled strain by PCR. Single-X strain, single crossover strain.

crossover candidate strain Lbd1 was verified by PCR (Fig. 3B), and the 1- junction in Lbd1 was confirmed by DNA sequencing analysis of the PCR products. The natamycin fermentation titer in shaking cultures of the -labeled strain Lbd1 and the parental strain Ins1 were 3.35 ± 0.16 g L^{-1} and 3.20 ± 1.01 respectively, suggesting that the -reporter engineering in Lbd1 did not affect natamycin production significantly.

3.4. h5

The -reporter-labeled strain Lbd1 was subjected to random mutagenesis followed by kanamycin selection to select for mutants with greater kanamycin resistance, which was more likely to be associated with higher natamycin production. In our initial trials, NTG-treated spores were spread on fermentation medium (NPM medium) containing kanamycin for selection of mutants. After incubation, confluent growth was observed on plates at low kanamycin concentration (1–20 mg L^{-1}), probably due to basal expression of in the starting strain, and no growth on plates with kanamycin at higher concentrations (> 20 mg L^{-1}), however no single/sporadic kanamycin resistant mutant was observed. In subsequent rounds of mutagenesis-selection, 24–48 h of preincubation of the inoculated-plates was carried out to allow the kanamycin-resistance reporter gene to be expressed, potentially at distinguishable levels among different mutants, before the kanamycin selection was applied. In addition, NPM medium was diluted by five times to delay the formation of aerial mycelium by so as to provide a longer time window for exposing the plated colonies to flooding with kanamycin solution; aerial mycelia are highly hydrophobic and are therefore refractory to flooding with the antibiotic solution. The optimized mutagenesis and selection procedure is schematically illustrated in Fig. 4.

3.5. G h5 h5-

In the first round of mutagenesis with NTG, lethality was determined to be 93%. The NTG-treated spores were incubated for 24 h until very short aerial mycelia started to develop, and then plates were flooded with kanamycin solution to give final concentrations of 5, 10, 20, 40, 50, and 80 mg L^{-1}. Single colonies were observed on plates with 40 or 50 mg L^{-1} kanamycin, and 28 Kan^R colonies (mutants) were randomly picked, cultured to obtain spores, and fermented to determine levels of natamycin production. Nine of these strains showed significant increases in natamycin production compared to the parent strain Lbd1 (Fig. 5A). The highest natamycin producer, named 1G1, yielded natamycin at 6.64 ± 1.38 g L^{-1}, a level 1.1- and 1.0-fold higher than that of the industrial strain Ins1 and the labeled strain Lbd1, respectively.

1G1 was selected for the second round of mutagenesis, and NTG-treated 1G1 spores were incubated for 24 h and flooded with kanamycin to final concentrations of 70, 80, 100, and 120 mg L^{-1}. Fourteen Kan^R mutants were obtained, and seven of these mutants produced more natamycin than 1G1 (Fig. 5B). The top producer, named 2G1, produced natamycin at 10.49 ± 0.47 g L^{-1}, which is 2.3- and 2.1-fold higher than the levels produced by Ins1 and Lbd1, respectively.

2G1 was used for the third round of mutagenesis, and NTG-treated 2G1 spores were incubated for 24 h before flooding with kanamycin to final concentrations of 100, 120, 150, 200, and 300 mg L^{-1}. Continuous growth was observed on plates with 100, 120, and 150 mg L^{-1} kanamycin, and no growth was observed on plates with 200 and 300 mg L^{-1} kanamycin. However, no isolated colonies were obtained, and therefore the mutagenized spores were instead incubated for 48 h before flooding plates with kanamycin to final concentrations of 350–700 mg L^{-1}. Forty-two Kan^R mutants were obtained, and 17 of

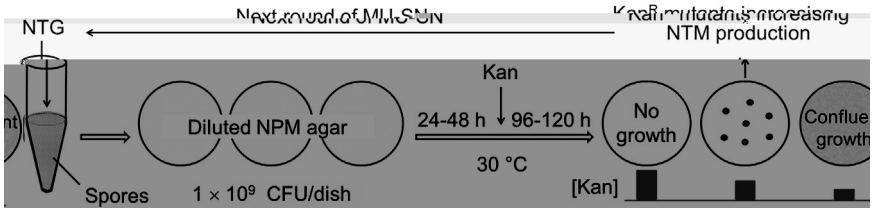

Fig. 4. Flowchart of the optimized mutagenesis-selection procedure. The NTG-treated pores, approximately 1×10^9 CFU/dish, were plated out on Petri dishes precast with solidified fermentation medium, followed by incubation and selection with kanamycin at different concentrations. The preincubation before antibiotic overlay is crucial. After further incubation for 4–5 d, sporadic kanamycin-resistant colonies were observed on some plates containing suitable kanamycin concentrations, whereas con

them produced more natamycin than 2G1. The highest producer, 3G1, yielded natamycin at 14.11 ± 0.73 g L^{-1}, which is 3.4- and 3.2-fold higher than the level produced by Ins1 and Lbd1, respectively (Fig. 5B).

The hereditary stability of Lbd1 and the high-yield strains 1G1, 2G1, and 3G1, with respect to natamycin production, were evaluated by following successive generations. After seven rounds of sporulation without antibiotic selection, the offspring produced natamycin at levels similar to those of the first generations (Fig. 6).

3.6. E *h5* sgn

To analyze the expression of the biosynthetic cluster in the labeled strain Lbd1 and the natamycin overproducers by quantitative real-time RT-PCR, these strains were cultivated in 20 mL fermentation medium. An aliquot (0.5 mL) of each was removed for extraction of total RNA at 96 h, and the remainder was continually cultured for monitoring for natamycin production and biomass. Natamycin produced by 1G1, 2G1, 3G1, or the parent strain Lbd1 showed single HPLC peak identical with that of the authentic standard (Fig. 7A). In this condition, the natamycin titers of 1G1, 2G1, and 3G1 increased successively (Fig. 7B), while the biomass did not changed significantly (Fig. 7C). Fig. 7D shows the relative gene expression of the cluster using Lbd1 as the reference. The expression of the reporter gene increased successively in 1G1, 2G1, and 3G1, and the expression of *1*, which was linked to in an operon, also increased successively. In addition, most genes, including genes within the very large *2 3 4* operon and the *CGF 0* operon, also increased in 1G1, 2G1, and 3G1 (Fig. 7D). We therefore attributed the overproducing capability to the improved expression of the genes in these mutants.

3.7. C *h5* sgn

Amplification of biosynthetic gene clusters leading to increased antibiotic production in industrial strains has been reported (Yanai et al., 2006), so we determined the copy number of the gene cluster in the natamycin overproducers by quantitative real-time PCR, using the two intergenic sequences *IJ* and *D 1* as references for the gene cluster and (encoding the cell division protein FtsZ), and *A*

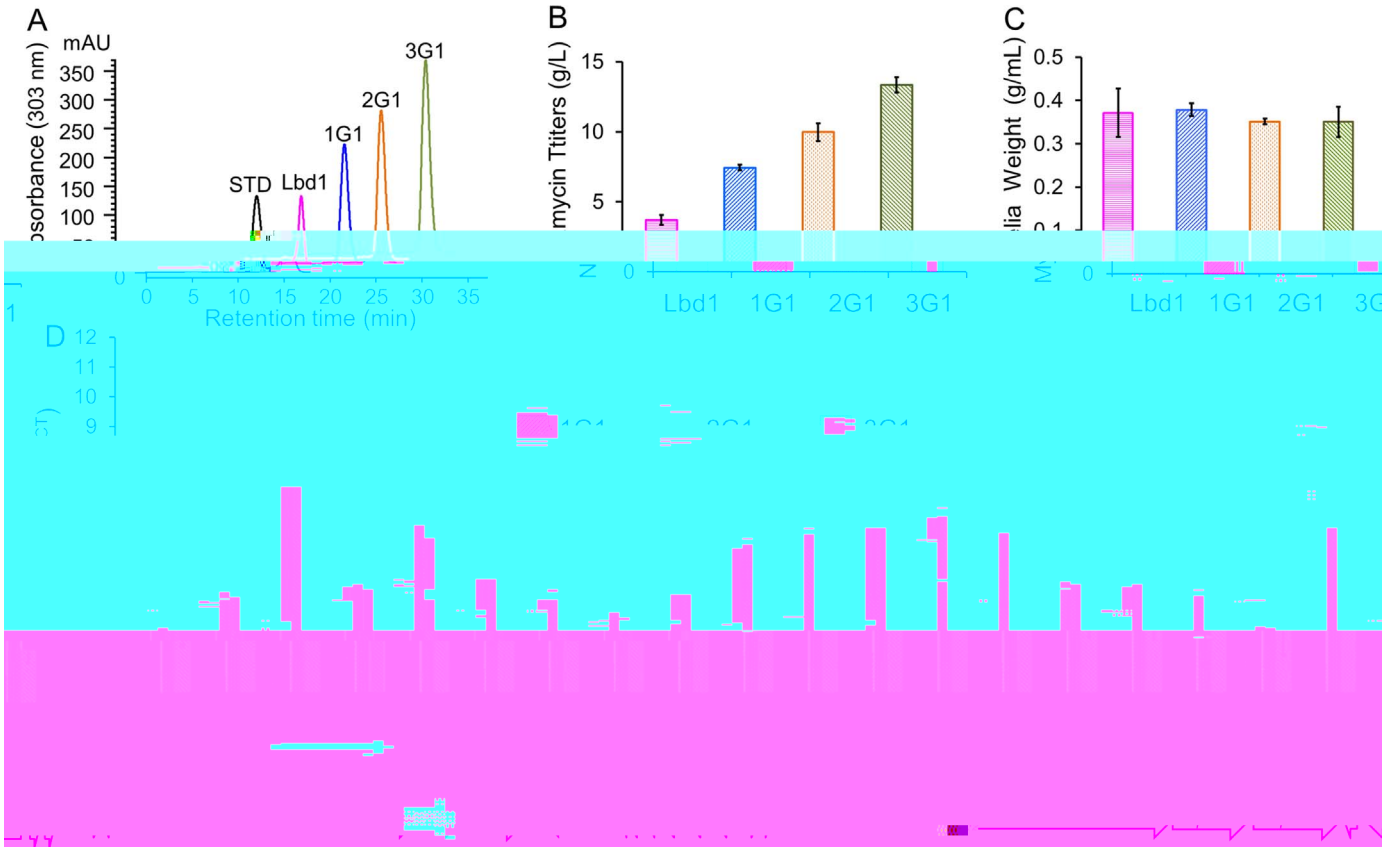

Fig. 7. Transcription analysis of the gene cluster in the overproducing mutants cultured in fermentation medium. (A) HPLC analysis of the natamycin production. (B) Natamycin titers. (C) Biomass. (D) Quantitative real-time RT-PCR. The cluster underneath shows the gene organization, with emphasis on the artificial *1*- operon (pink arrow and shadow) and the *2*, *3*, *4* operon (grey arrow and shadow). For the quantitative real time RT-PCR, Lbd1 was used as the reference and *B* was used as internal control. Each data point in (B), (C), and (D) represents the mean ± SD of ten biological replicates. STD, authentic standard of natamycin.

Table 2
Detection of copy number of the cluster in four strains.

Copy number[a]	Ins1	1G1	2G1	3G1
IJ/ *A*[b]	1.07 ± 0.50	0.95 ± 0.14	0.99 ± 0.35	1.96 ± 0.27
IJ/	1.00 ± 0.47	1.06 ± 0.15	0.95 ± 0.25	2.07 ± 0.43
D, *1*/ *A*	0.97 ± 0.31	0.81 ± 0.28	0.74 ± 0.35	1.98 ± 0.29
D, *1*/	0.91 ± 0.29	0.83 ± 0.11	0.71 ± 0.26	1.86 ± 0.38

[a] Average ± standard deviation from three biological replicates using quantitative real-time PCR.
[b] *IJ* and *D*, *1* are sequences from the cluster. *A* and represent single-copy chromosomal genes.

(encoding ATP synthase subunit alpha) as references for single-copy genes of the chromosome. 1G1, 2G1, and the original industrial strain Ins1 were all determined to be carrying one copy of the gene cluster, whereas 3G1 was found to have two copies per chromosome (Table 2). Therefore, the doubling of the gene cluster in 3G1 probably contributed to increased levels of natamycin.

4. Discussion

Reporter-based selection systems have been employed to improve the production of secondary metabolites. A pioneering example is the *F* promoter reporter-based selection system for lovastatin-overproducing mutants of (Askenazi et al., 2003). Transcriptional analyses of lovastatin producers revealed that expression of the biosynthetic gene *F* was associated with lovastatin production, and fusion of the *F* promoter to the gene that confers resistance to phleomycin resulted in a reporter-based selection system that could be employed to accelerate strain improvement in *A*. . A similar reporter-guided mutant selection (RGMS) system was developed and applied for improving clavulanic acid production in (Xiang et al., 2009). In both examples, the antibiotic-resistant reporters were fused to the promoters of biosynthetic genes, and the resulting promoter-reporters were integrated into the chromosomes at ectopic sites of the cognate biosynthetic gene clusters.

In our study, due to the concern that the expression of very large PKS genes might limit the biosynthesis of natamycin, the reporter gene was engineered into the biosynthetic gene cluster and specifically joined to a very large biosynthetic gene, *1*. Our approach allowed the expression of the reporter gene to report, i.e., serve as an indicator of, the complete transcription of the upstream giant gene and to indicate the integrity of the long mRNA of the *1*- fusion operon. The increased expression of both S1 and in all of the three production-improved strains validated this approach, and furthermore, the expression of other genes in the gene cluster was coordinately increased in the three improved strains, suggesting that the system also reflected expression of the entire gene cluster. We conducted three rounds of chemical mutagenesis and selection, and natamycin production by the best strain increased by 110%, 230%, and 340% over original levels, in the 1st, 2nd, and 3rd round, respectively. The final mutant (3G1) produced a natamycin titer of 14.1 g L^{-1}, which is 4.4 times the level produced by the starting industrial strain (3.2 g L^{-1}).

According to the relative gene expression profile (Fig. 7D), the transcript amounts of *1* (PKS), *3* (PKS), *B* (ABC transporter), *K* (mycosamine transferase), *J* (GDP-mannose 4,6-dehydratase), and *I* (type II thioesterase) were slightly increased in 1G1. In 2G1, expression of all genes was significantly increased (1.9–8.8-fold), while the expression was increased the most (8.8-

fold). This implied that the unknown mutation(s) in 2G1 improved the expression of the whole gene cluster through regulation on , which is homologous to the PAS domain positive regulator PimM (Antón et al., 2007). In 3G1, the transcript amounts of most genes were increased further, plausibly owing to the doubling of the gene cluster (Table 2). These suggested that at least three different mutations contributing to the natamycin overproduction had been incorporated into 3G1 during the course of the -guided semi-rational strain improvement.

Many important drugs and reagents are synthesized by giant multifunctional non-ribosomal peptide synthetases (NRPS) and type I PKS that are encoded by very long open reading frames, usually more than ten times the average size of approximately 1 kb for bacterial genes. For example, the PKS genes *A*, *B*, and *C* for the synthesis of rapamycin (immunosuppressant) in *h5* are 25.7 kb, 30.7 kb, and 18.8 kb in length, respectively (Schwecke et al., 1995). The NRPS genes *A*, *BC*, and *D* for the biosynthesis of daptomycin (anti-infective) in are 17.5 kb, 22.0 kb, and 7.1 kb, respectively (Miao et al., 2005). Therefore very long mRNAs have to be efficiently transcribed and sufficiently stable to achieve substantial production of the corresponding compounds. This factor constitutes an additional, unique, and often ignored bottleneck for the biosynthesis of non-ribosomal peptides and polyketides that are synthesized by modular, multifunctional megaenzymes. Large transcripts are known to be associated with some secondary metabolic pathways, for example, a >16 kb transcript encodes six cephamycin C biosynthetic genes in (Enguita et al., 1998), and a 35 kb transcript encodes seven erythromycin biosynthetic genes in *h5* *h5* (Reeves et al., 1999). The method described in this study could be applied to facilitate titer improvement of these compounds. One single large PKS/NRPS gene or the last gene of a large operon could be chosen as a target to be fused with the coding sequence with a sequence overlap of one or four nucleotides at the stop/start codon, so as to generate a reporter-labeled strain. It is not necessary to design a specific ribosome binding site for the downstream coding sequence since translational coupling from the upstream gene may re-initiate its translation in theory (Spanjaard and van Duin, 1989; Tian and Salis, 2015).

Additionally, titer-improved strains generated by this method can be resources for future investigations of mutations associated with overproduction and the mechanisms governing the synthesis and stability of very long messenger RNAs.

Acknowledgements

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