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Short Communication

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TAR cloning and integrated overexpression of 6-demethylchlortetracycline biosynthetic gene cluster in *Streptomyces aureofaciens*

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Abstract

6-Demethylchlortetracycline (6-DCT), a tetracycline antibiotic produced by *Streptomyces aureofaciens*, is a crucial precursor employed for the semi-synthesis of tigecycline, minocycline, and amadacyclin (PTK 0796). In this study, the 6-DCT biosynthetic gene cluster (BGC) was cloned from genomic DNA of a high 6-DCT-producing strain, *S. aureofaciens* DM-1, using the transformation-associated recombination method. An extra copy of the 6-DCT BGC was introduced and integrated into the chromosome of *S. aureofaciens* DM-1. Duplication of the 6-DCT BGC resulted in a maximum increase of the 6-DCT titer by 34%.

Key words: 6-demethylchlortetracycline, *Streptomyces aureofaciens*, biosynthetic gene cluster, transformation-associated recombination

Introduction

Streptomyces aureofaciens is a Gram-positive actinomycete that produces four tetracycline antibiotics, chlortetracycline, tetracycline, 6-demethylchlortetracycline (6-DCT), and 6-demethyltetracycline [1]. 6-DCT was first reported by McCormick in 1957, and it has become an important antibiotic, exhibiting activity against a wide range of Gram-positive and Gram-negative bacteria, including organisms such as *Chlamydiae*, *Mycoplasma*, and *Rickettsiaceae*. Furthermore, 6-DCT is also an industrial material for the production of semi-synthetic tetracyclines [2,3]. Thus far, the highest-yielding industrial producer of 6-DCT was obtained by continuous 'random mutagenesis and screening' procedures, and further yield improvement using this strategy is limited. However, with a better understanding of the synthesis and regulation of natural secondary metabolites in *Streptomyces* and the development of various molecular biological tools, metabolic engineering has been developed as an effective approach to improve the production of various secondary metabolites.

The genes involved in the synthesis of secondary metabolites are usually present in a linear cluster. Overexpression of synthetic genes individually or in groups has been demonstrated to enhance the yield of target products. In recent years, many antibiotic biosynthetic gene clusters (BGCs) have been cloned, either for the improved production of antibiotics or for the investigation of functional genes [4–6]. For instance, the production of nikkomycin X and nikkomycin Z was increased by 4- and 1.8-fold, respectively, after introduction of an extra copy of the nikkomycin BGC into the genome of a producer strain [7]. However, large-size DNA region cloning usually depends on genomic DNA library construction, which is time-consuming and requires screening to identify the desired cosmid/fosmid clones. Hence, the cloning of BGCs is a challenging task. However, homologous recombination-assisted cloning approaches, such as RecE-mediated homologous recombination [7–9] and transformation-associated recombination (TAR) [10–12], have been developed and demonstrated to be effective approaches for direct cloning of targeted gene clusters from genomic DNA.

Taking advantage of the highly efficient recombination capacity of *Saccharomyces cerevisiae*, TAR cloning is an innovative approach useful for rapid capturing and heterologous expression of large

chromosomal regions [13]. It uses the yeast cell as a host, in which a DNA target of interest and a linearized TAR cloning vector preloaded with homologous regions form a large circularized plasmid via *in vivo* homologous recombination [14]. The genetic target of interest can be readily amplified, propagated with the replication of the TAR cloning vector, and can be easily prepared for any use, such as homologous expression or reprogramming of secondary metabolite BGCs.

The *ctc* gene cluster which encodes chlortetracycline was initially obtained by cosmid library construction and screening. Recently, Zhu *et al.* [15] identified a 43.9-kb *ctc* gene cluster by screening a fosmid library of the chlortetracycline industrial producer *S. aureofaciens* F3. Our previous work demonstrated the high similarity between the 6-DCT BGC of *S. aureofaciens* DM-1 (Genebank accession number: CP020567) and the *ctc* gene cluster of *S. aureofaciens* F3 (Genebank accession number: HM627755.1).

Here we report the implementation of TAR for direct capture of the 6-DCT BGC from *S. aureofaciens* DM-1 genomic DNA. A strain with a duplicated 6-DCT BGC was obtained by delivering a *Φ*C31-int-based cluster plasmid into *S. aureofaciens* DM-1. Overexpression of the integrated 6-DCT biosynthetic cluster led to a 34% increase in 6-DCT production.

Materials and Methods

Strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in **Table 1**. *Streptomyces aureofaciens* DM-1 and its derivatives were grown at 30°C on ISP2 medium (yeast extract 4.0 g/l, malt extract 10.0 g/l, glucose 4.0 g/l, and agar 20 g/l) for spore preparation. Strain DM-1 was cultivated in liquid ISP2 medium (without agar) on an orbital shaker (250 rpm) at 28°C for total DNA isolation.

Escherichia coli strains EPI300 and DH5α were used for routine DNA cloning. *Escherichia coli* ET12567/pUZ8002 was used for conjugal transfer between *E. coli* and *S. aureofaciens* DM-1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium supplemented with necessary antibiotics. Kanamycin (Kan, 50 μg/ml), apramycin (Apr, 50 μg/ml), chloramphenicol (25 μg/ml), and nalidixic acid (15 μg/ml) were used for the selection of *E. coli* or *Streptomyces* recombinant strains.

Reagents and enzymes

PCR products and restriction enzyme digestion products were purified using an AxyPrep DNA Gel Extraction Kit or an Agarose Gel DNA Extraction Kit (Axygen, Union City, USA). Restriction enzymes were purchased from Thermo Scientific (Waltham, USA). Oligonucleotides were obtained from GenScript (Nanjing, China). Plasmids pCAPDM and pCAPDMA were isolated and purified using an E.Z.N.A.s BAC/PAC DNA Kit (Omega Bio-Tek, Winooski, USA). Other reagents used in TAR cloning experiments are described in a previous report [12].

Preparation of genomic DNA fragments for TAR

Streptomyces aureofaciens DM-1 was grown in ISP2 liquid medium. Genomic DNA was isolated by standard procedures [19]. Approximately 150 μg of genomic DNA was digested with 200 U of *NsiI*/*Mfe*I, which does not cut the 6-DCT gene cluster, in an overnight reaction at 37°C. The digested genomic DNA fragments were precipitated and washed with 70% ethanol. The resulting DNA pellet was dissolved in 100 μl of 20% Tris-EDTA buffer (2 mM Tris-HCl and 0.2 mM EDTA, pH 8.0).

Construction of the gene cluster capture vector pCAPLR

The primers Rarms-long and Rarmas-long and Larms-long and Larmas-long were used to amplify two homologous arms by PCR analysis (1468 and 1471 bp long, respectively) corresponding to flanking regions of the 6-DCT BGC from DM-1 genomic DNA. The primer Rarms-long contains a *Pme*I restriction site. The homologous

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Source
E. coli		
DH5α	F$^-$80Φ*dlacZ*DM15Δ(*lacZYA-argF*)*U169deoR recA1 endA1 hsdR17*(rk$^-$ mk$^+$) *supE44 λ$^-$thi$^-$1gyrA96 relA1*	GIBCO-BRL
EPI300	F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ80*dlacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK λ- rpsL (StrR) nupG trfA tonA*	Epicenter
ET12567	*dam*, *dcm*, *hsdS*, *cat*, *tet*, *tra*, *neo*, *RP4*	[16]
S. cerevisiae		
VL6-48	Host strain for *in vivo* homologous recombination: *MAT alpha*, *his3-D200*, *trp1-D1*, *ura3-52*, *lys2*, *ade2-101*, *met14*, *psi* + *cir0*	ATCC MYA-3666
S. aureofaciens		
DM-1	The parental strain that produces 6-DCT	NORTH CHINA PHARMACEUTICAL CO., LTD
DM-1-10	DM-1 containing plasmid pCAPDMA	This study
DM-1-11	DM-1 containing plasmid pCAPA	This study
Plasmids		
pCAP01	*ARSH4/CEN6*, *pUC ori, aph(3)II, φC31 int-attP, oriT (RP4)*	[17]
pSET152	*pUC19ori*, *ΦC31 int/attP*, *aac(3)IV*, *lacZα*, and *oriT RK2*	[18]
pCAPA	A plasmid derived from pCAP01, adding a screen part(*aac(3)IV*) from pSET152	This study
pCAPLR	pCAP01with two homologous arms containing the upstream and downstream DNA fragments of the 6-DCT BGC	This study
pCAPDM	pCAPLR carrying the captured 6-DCT BGC	This study
pCAPDMA	A plasmid derived from pCAPDM, adding a screen part (*aac(3)IV*) from pSET152	This study

arms were then cloned simultaneously into *Kpn*I-digested pCAP01, yielding the gene cluster-specific capture vector pCAPLR. Prior to direct TAR cloning, the 'circular' construct was digested with *Pme*I, generating a linear capture vector flanked by 1.5-kb capture arms at each end.

Direct cloning of the 6-DCT gene cluster using TAR

Direct TAR cloning of the 6-DCT gene cluster from genomic DNA was performed similarly to the protocol previously described [11,12]. *Streptomyces cerevisiae* strain VL6-48 was grown in 50 ml YPD medium supplemented with adenine (100 mg/l) at 30°C with shaking until the $OD_{600\,nm}$ reached 0.6–1.0. The preparation of spheroplasts was carried out as reported previously [12]. After incubation at 30°C for 40 min, the spheroplasts were transformed with 2–3 μg of DM-1 genomic DNA fragment and 1 μg linearized 6-DCT gene cluster-specific capture vector pCAPLR. The transformed spheroplasts were mixed with 10 ml SD-Trp top agar (SD-Trp containing 3% agar), equilibrated at 50°C and overlaid on SD-Trp agar. The plates were incubated at 30°C for 3 days. Transformants that appeared on the plates were picked with toothpicks and transferred onto new SD-Trp agar plates, which were incubated for 3 days at 30°C. To screen for the directly cloned 6-DCT gene cluster in *S. cerevisiae* clones, genotyping of multiple transformants was carried out by colony PCR using primers Halo-up/Halo-down, Larm up-4/Larm down-4, and Rarm up-4/Rarm down-4, respectively. Cells confirmed by colony PCR were first cultivated in 4 ml YPD medium and collected by centrifugation for 5 min at 1000 *g*, and were then suspended in 1 ml SPE solution containing 10 μl Zymolyase-20T (**Supplementary Table S1**) and incubated at 37°C for 1 h before extraction of plasmids.

Then, plasmids bearing the 6-DCT gene cluster were obtained using an E.Z.N.A.s BAC/PAC DNA Kit, and subsequently transferred into *E. coli* EPI300 by electroporation to facilitate the harvest and sequencing of the 6-DCT BGC plasmids. After electroporation, Kan^r *E. coli* clones were randomly picked and analyzed by colony PCR. The plasmids purified from Kan^r *E. coli* transformant colonies were confirmed by PCR screening using primers listed in **Supplementary Table S2** and restriction analysis with *Bam*HI. The yielded construct was designated as pCAPDM.

To conveniently select both *E. coli* ET12567 and *Streptomyces* recombinants, we added an Apr resistance gene (*aac(3)IV*, Apr^r) to pCAPDM. The *aac(3)IV* gene was first amplified from plasmid pSET152 using primers apra-pcaPs/apra-pcaPas, and then ligated into *Nhe*I-digested pCAPDM using the One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China), yielding pCAPDMA.

All constructed vectors were confirmed by restriction enzyme digestion analysis and DNA sequencing. The plasmids pCAPDMA and the control plasmid pCAPA were introduced individually into the parental strain *S. aureofaciens* DM-1 through conjugal transfer, generating the corresponding overexpression strains DM-1-10 and DM-1-11 (**Table 1**). Strain DM-1-11 was used as a negative control.

Fermentation of *S. aureofaciens* and analysis of 6-DCT production

Streptomyces aureofaciens strains were grown on ISP2 medium at 30°C for 6 days and then inoculated into seed medium [g/l: corn starch 30, soybean flour 30, peptone 5, yeast extract 2, soybean oil 16, $(NH_4)_2SO_4$ 2, KH_2PO_4 1.5, $CaCO_3$ 10]. The pH was adjusted to 5.4–5.6 prior to $CaCO_3$ addition and sterilization. For *S. aureofaciens* fermentation in Erlenmeyer flasks, the cultures were incubated at 28°C in 30 ml of seed medium in 250-ml Erlenmeyer flasks on an orbital shaker (250 rpm). After 46–48 h, 3 ml of preculture was inoculated into 30 ml of fermentation medium [g/l: corn starch 40, glucose 10, soybean flour 30, soybean oil 12, corn steep liquor 4, L-Lysine hydrochloride 8, NaCl 4, $(NH_4)_2SO_4$ 3, $CaCO_3$ 10]. The pH was adjusted to 6.0 prior to $CaCO_3$ addition and sterilization.

For the analysis of 6-DCT production, solid oxalic acid was used to adjust the pH of the fermentation samples to 1.8–2.0, then the samples were extracted with an equal volume of acetone for 1 h. The culture filtrates were harvested by centrifugation and analyzed by HPLC (Waters 2487; Milford, USA) using a YMC-Triart C18 column (4.6 mm × 250 mm, 5 μm). For HPLC detection, a mixture of water (containing 0.01 M oxalic acid and 0.08 M ammonium oxalate):acetonitrile:methanol (10:3:2; v/v/v) was used as the mobile phase, with a flow rate of 1.0 ml/min. The eluate was monitored at 350 nm, and the column temperature was 30°C. Production of 6-DCT was calculated from a standard curve.

Results

TAR cloning of the 6-DCT BGC

The 6-DCT BGC spans a 43.5-kb region containing 36 open reading frames (ORFs), 28 of which are involved in 6-DCT biosynthesis. It was found that unique restriction sites *Mfe*I and *Nsi*I flanked the 6-DCT BGC. They were chosen to digest the genomic DNA to obtain a nucleotide fragment containing the entire BGC. The plasmid pCAP01 contains three distinct modules. One module is necessary for propagation in *E. coli* and *S. cerevisiae*, another module is necessary for conjugal transfer between the *E. coli* donor and *Streptomyces*, as well as ΦC31-derived integrase module useful for integration into the *attB* site that is ubiquitous in the *Streptomyces* genome [18]. Two regions inside the *Mfe*I and *Nsi*I cleavage sites, corresponding to the upstream and downstream regions of the 6-DCT BGC, were amplified by PCR and cloned into pCAP01 to generate the pathway-specific capture vector pCAPLR (**Fig. 1**). pCAPLR was confirmed by restriction enzyme digestion (**Fig. 2A**). After transformation of *S. cerevisiae* VL6-48 spheroplasts with the linearized vector and *Nsi*I/*Mfe*I restriction enzyme-digested genomic DNA, 4 of the 164 *S. cerevisiae* transformants were identified as positive clones by PCR using specific primers (**Fig. 2B**). The plasmids isolated from BGC-positive yeast colonies were transferred into *E. coli* EPI300 by electroporation for amplification. The 6-DCT BGC cloning plasmid pCAPDM extracted from *E. coli* EPI300 was confirmed by PCR (**Fig. 2C**) and restriction digestion (**Fig. 2D**). The Kan resistance gene of pCAPDM was replaced by an Apr resistance gene (*aac(3)IV*) to produce pCAPDMA, which facilitated screening after conjugation with *Streptomyces* spores.

Duplication of the 6-DCT BGC in *S. aureofaciens* DM-1

pCAPDMA was introduced into the 6-DCT-producing parental strain *S. aureofaciens* DM-1 by conjugative transfer. The empty vector pCAPA was separately introduced to generate control cells. The resulting transformants, designated as *S. aureofaciens* DM-1-10 (pCAPDMA) and DM-1-11 (pCAPA), were inoculated on ISP2 plates supplemented with Apr for clone purification and fermentation.

These transformants were confirmed by PCR amplification using the pCAP01 skeleton-specific primers blatgas/Rarms-long and pset152 up/Larmas-long to avoid signals generated from the original 6-DCT BGC. The plasmid pCAPDMA and genomic DNA

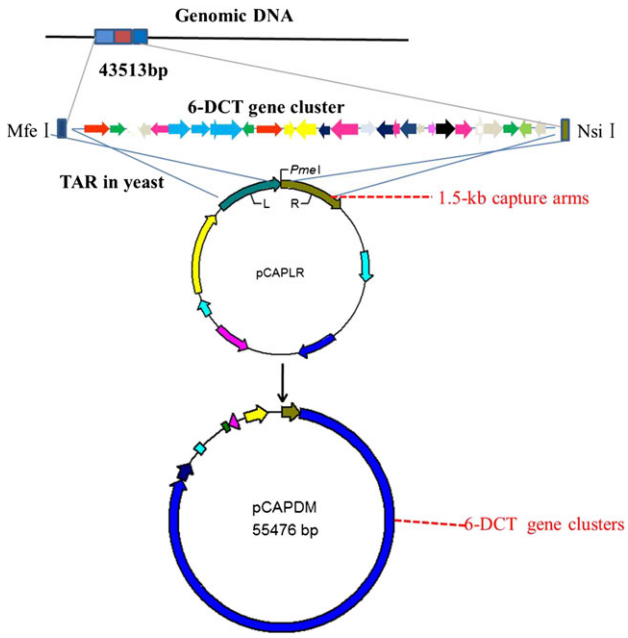

Figure 1. Schematic illustration of direct cloning of the 6-DCT BGC using the TAR method The relevant genomic DNA fragment of *S. aureofaciens* DM-1 consists of 36 ORFs; genomic DNA was digested with *Nsi*I/*Mfe*I to generate the complete 6-DCT BGC-containing DNA fragment. Spheroplasts of *S. cerevisiae* VL6-48 were transformed with the enzymatically digested genomic DNA mixture and the 6-DCT pathway-specific capture vector pCAPLR, which was linearized by *Pme*I before TAR cloning. In yeast cells, homologous recombination occurred between 1.5-kb arms in the vector pCAPLR and the genomic DNA fragment, and thus the 44-kb genomic region containing the 6-DCT locus was directly captured into the vector, yielding the replicable circular construct pCAPDM.

of strain DM-1-11 were used as positive and negative controls, respectively. Fragments of 2.0 kb and 2.5 kb were amplified from genomic DNA of the six transformants and plasmid pCAPDMA, but not from *S. aureofaciens* DM-1-11 (**Fig. 3**), demonstrating that pCAPDMA was successfully integrated into the genome of *S. aureofaciens*. One of the *S. aureofaciens* DM-1-10 clones was chosen for further investigation.

Overproduction of 6-DCT by overexpressing the 6-DCT BGC in *S. aureofaciens* DM-1

The 6-DCT production of *S. aureofaciens* DM-1-10, which contains a duplicated 6-DCT BGC, was assessed by fermentation. In parallel, parental *S. aureofaciens* DM-1 and its derivative DM-1-11 (with the empty plasmid pCAPA integrated into the DM-1 genome) were used as controls. Fermentation broth of the *S. aureofaciens* strains was sampled at different time points (3, 4, 5, 6, 7, and 8 days) and their 6-DCT production was analyzed by HPLC. Introduction of an extra copy of the BGC (in strain DM-1-10) resulted in a 34% increase in 6-DCT production, from 489 mg/l to 655 mg/l (**Fig. 4A**). Strain DM-1-11 exhibited a slight increase in 6-DCT production over DM-1 (489–518 mg/l) (**Fig. 4A**), possibly due to an unknown vector effect.

To investigate the production stability of the double-cluster recombinant strain, DM-1-10 was incubated in ISP2 medium without Apr selection. After 5 subcultures, 12 colonies were randomly selected and then cultured in medium supplemented with 50 μg/ml Apr for 5 days. All of them were Apr-resistant and able to produce approximately 34% increase in the production of 6-DCT compared with the parental strain DM-1 (**Fig. 4B**). These results demonstrated that the transformants harboring one extra copy of 6-DCT BGC were genetically stable.

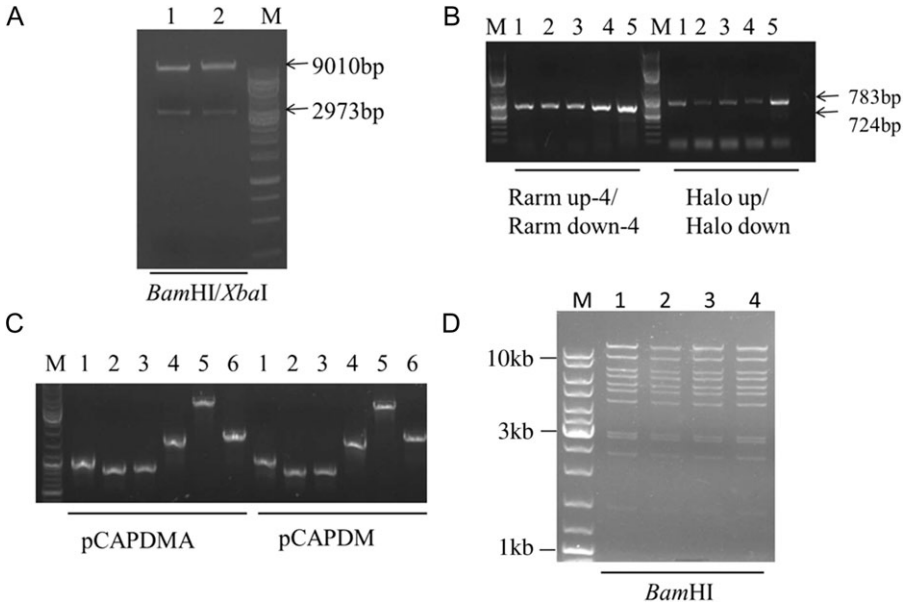

Figure 2. PCR and restriction analysis of plasmids and strains used in this study (A) Digestion of the capture vector pCAPLR isolated from two randomly picked clones. M represents 1 kb DNA ladder (Thermo Scientific). (B) Colony PCR of *S. cerevisiae* VL6-48 transformants. Four transformants (lanes 1, 2, 3, and 4) were confirmed by PCR from 164 *S. cerevisiae* VL6-48 colonies. The PCR product from genomic DNA of *S. aureofaciens* DM-1 was used as a control (lane 5). (C) PCR analysis of plasmids pCAPDMA and pCAPDM. Primer pairs apra-pcaPs/apra-pcaPas, Halo-up/Halo-down, Larm up-4/Larm down-4, Rarms-long/Rarmas-long, Rarm-up/Rarm-down, and Larm-up/Larm-down were, respectively, used to amplify 897-bp, 800-bp, 800-bp, 1471-bp, 3409-bp and 1580-bp DNA fragments with pCAPDMA and pCAPDM as the templates. (D) Restriction map of the TAR-cloned plasmid pCAPDM. The expected sizes of *Bam*HI-digested pCAPDM fragments are: 13 kb, 11 kb, 7.1 kb, 6.4 kb, 5.8 kb, 5.1 kb, 4.7 kb, 3.1 kb, 2.8 kb, 2.4 kb, 1.4 kb, and 929 bp.

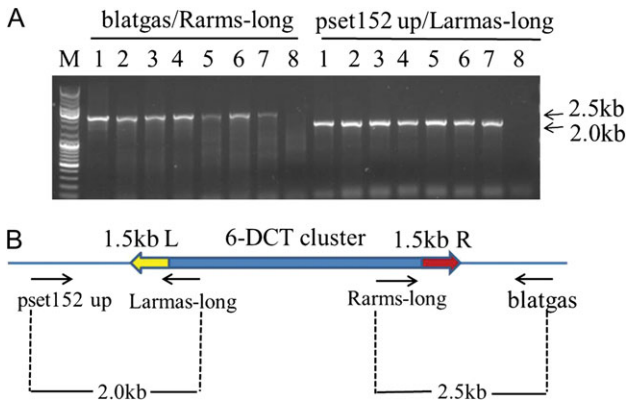

Figure 3. PCR analysis of the integration of pCAPDMA into the genome of *S. aureofaciens* DM-1 to create strain DM-1-10 (A) M, 1 kb marker; lanes 1–6, amplification from six apramycin-resistant transformants obtained after conjugal transfer; lane 7, amplification from plasmid pCAPDMA (positive control); lane 8, amplification from genomic DNA of *S. aureofaciens* DM-1-11 (negative control). (B) Schematic diagram of PCR amplification method to confirm positive clones of *S. aureofaciens*. Primer pairs pset152 up/Larmas-long and blatgas/Rarms-long were used to amplify 2.0-kb and 2.5-kb DNA fragments from the genomic DNA of engineered strain DM-1-10.

Discussion

Yield improvement of secondary metabolites, especially medically and industrially important compounds, is one of the most significant goals for pharmacy as well as basic research. Although numerous methods have been developed to improve production, such as optimizing promoters, reprograming transcriptional regulation, and overexpression of key pathway genes [20], most approaches have limitations. Due to the ubiquity of the phage ΦC31-derived *attB* site in actinomyces genomes, duplication of whole biosynthetic pathways has been proved to be an feasible and effective strategy to increase the yields of secondary metabolites [7,21–23]. Additionally, plasmid cloning of BGCs has many potential applications, including rapid refactoring of known BGCs and heterologous expression of large biosynthetic loci for the exploration of novel secondary metabolites [17] in tractable hosts [9,24,25]. Random DNA library construction, PCR amplification [26], and direct DNA synthesis are the generally used cloning methods to obtain large BGCs. However, these approaches have various limitations. For instance, random library cloning relies on laborious library construction and screening. PCR amplification and assembly procedures are expensive and require deep sequencing to confirm the fidelity. Recently reported tools, such as Red/ET technology [8], Gibson assembly [27], and TAR [10,11,25], offer direct cloning of targeted gene clusters from genomic DNA. In this study, we successfully employed restriction enzyme digestion-assisted TAR technology to clone the entire 6-DCT BGC into an integrative vector, pCAP01. The resulting plasmid was integrated into the chromosome of *S. aureofaciens* DM-1, thus leading to overexpression of the entire 6-DCT BGC.

It has been demonstrated that cutting near the targeted genomic region increases the efficiency of TAR cloning [28]. However, restriction enzyme digestion-assisted TAR cluster capture still has its drawbacks. Unique restriction enzyme sites which flank the cluster of interest and do not occur in the target region are usually unavailable or are far away from the ends of the target region, especially in larger clusters. A recently developed CRISPR-Cas9-assisted TAR method explored the precise double strand breaking ability of

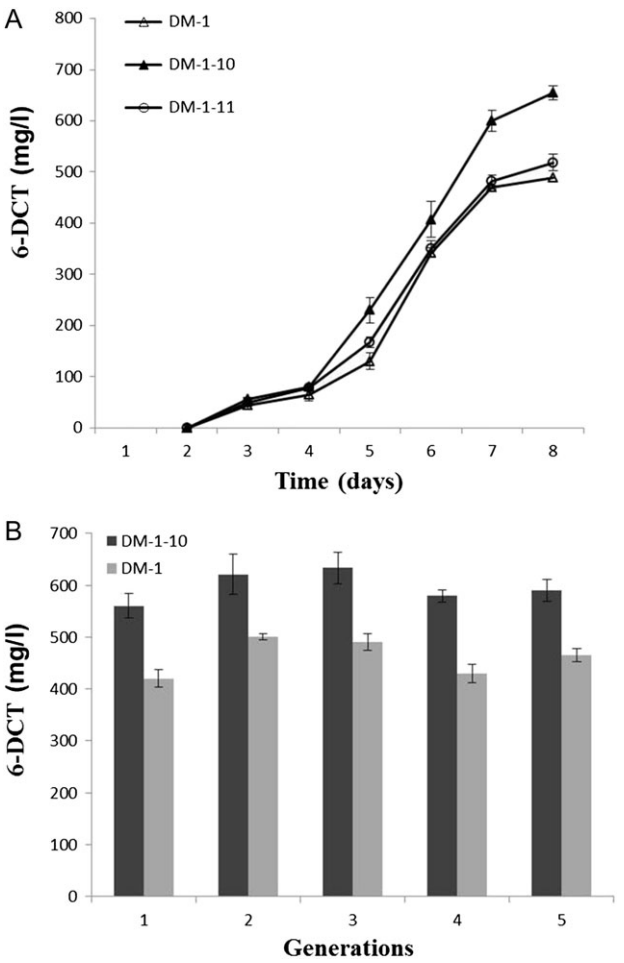

Figure 4. 6-DCT production and production stability of a *S. aureofaciens* strain containing an extra copy of the 6-DCT BGC (A) 6-DCT production profiles of strain DM-1-10 compared with DM-1 (parental) and DM-1-11 (negative control). Fermentation samples were collected at six time points, and fermentations were performed in triplicate. (B) Production stability of DM-1-10 in five continuous passages. Fermentation samples were taken on the eighth day, and fermentations were performed in triplicate.

sgRNA-guided Cas9 to cut the target region [14]. With the *in vitro* enzyme reactions, Cas9 is able to cut at almost any position of interest when provided with reprogrammed sgRNA, which will markedly reduce the size of target fragments for TAR cloning and facilitate the capture procedures.

In summary, we demostrated that the 6-DCT production of the recombinant strain with the duplicated BCG was increased by 34% relative to the parental strain, suggesting that overexpression of the whole cluster could improve 6-DCT synthesis. Based on the current cluster plasmid, many metabolic engineering strategies, such as manipulation of transcriptional regulators [20,29], increasing precursor supplies [30], optimizing promoters of key pathway genes [31], and enhancing the transcription level of genes encoding antibiotic transporters to reduce end-product toxic effects and feedback inhibition [32], may be combined to hopefully further optimize 6-DCT biosynthesis.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

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References

1. Nakano T, Miyake K, Ikeda M, Mizukami T, Katsumata R. Mechanism of the incidental production of a melanin-like pigment during 6-demethylchlortetracycline production in *Streptomyces aureofaciens*. *Appl Environ Microbiol* 2000, 66: 1400–1404.
2. Sum P, Ross AT, Petersen PJ, Testa RT. Synthesis and antibacterial activity of 9-substituted minocycline derivatives. *Bioorg Med Chem Lett* 2006, 16: 400–403.
3. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001, 65: 232–260.
4. Peschke U, Schmidt H, Zhang HZ, Piepersberg W. Molecular characterization of the lincomycin-production gene cluster of *Streptomyces lincolnensis* 78-11. *Mol Microbiol* 1995, 16: 1137–1156.
5. Murakami T, Sumida N, Bibb M, Yanai K. ZouA, a putative relaxase, is essential for DNA amplification in *Streptomyces kanamyceticus*. *J Bacteriol* 2011, 193: 1815–1822.
6. Murakami T, Burian J, Yanai K, Bibb MJ, Thompson CJ. A system for the targeted amplification of bacterial gene clusters multiplies antibiotic yield in *Streptomyces coelicolor*. *Proc Natl Acad Sci USA* 2011, 108: 16020–16025.
7. Liao GJ, Li J, Li L, Yang HH, Tian YQ, Tan HR. Cloning, reassembling and integration of the entire nikkomycin biosynthetic gene cluster into *Streptomyces ansochromogenes* lead to an improved nikkomycin production. *Microb Cell Fact* 2010, 9: 6.
8. Fu J, Bian XY, Hu SB, Wang HL, Huang F, Seibert PM, Plaza A, *et al*. Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. *Nat Biotechnol* 2012, 30: 440–446.
9. Bian XY, Huang F, Stewart FA, Xia LQ, Zhang YM, Müller R. Direct cloning, genetic engineering, and heterologous expression of the syringolin biosynthetic gene cluster in *E. coli* through Red/ET recombineering. *ChemBioChem* 2012, 13: 1946–1952.
10. Kouprina N, Larionov V. Innovation—TAR cloning: insights into gene function, long-range haplotypes and genome structure and evolution. *Nat Rev Genet* 2006, 7: 805–812.
11. Kouprina N, Noskov VN, Koriabine M, Leem SH, Larionov V. Exploring transformation-associated recombination cloning for selective isolation of genomic regions. *Methods Mol Biol* 2004, 255: 69–89.
12. Kouprina N, Larionov V. Selective isolation of genomic loci from complex genomes by transformation-associated recombination cloning in the yeast *Saccharomyces cerevisiae*. *Nat Protoc* 2008, 3: 371–377.
13. Kim JH, Feng ZY, Bauer JD, Kallifidas D, Calle PY, Brady SF. Cloning large natural product gene clusters from the environment: piecing environmental DNA gene clusters back together with TAR. *Biopolymers* 2010, 93: 833–844.
14. Lee NCO, Larionov V, Kouprina N. Highly efficient CRISPR/Cas9-mediated TAR cloning of genes and chromosomal loci from complex genomes in yeast. *Nucleic Acids Res* 2015, 43: e55.
15. Zhu T, Cheng XQ, Liu YT, Deng ZX, You DL. Deciphering and engineering of the final step halogenase for improved chlortetracycline biosynthesis in industrial *Streptomyces aureofaciens*. *Metab Eng* 2013, 19: 69–78.
16. MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH, MacNeil T. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* 1992, 111: 61–68.
17. Yamanaka K, Reynolds KA, Kersten RD, Ryan KS, Gonzalez DJ, Nizet V, Dorrestein PC, *et al*. Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A. *Proc Natl Acad Sci USA* 2014, 111: 1957–1962.
18. Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces spp*. *Gene* 1992, 116: 43–49.
19. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. *Practical Streptomyces Genetics*. Norwich: John Innes Foundation, 2000.
20. Olano C, Lombó F, Méndez C, Salas JA. Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. *Metab Eng* 2008, 10: 281–292.
21. Thykaer J, Nielsen J. Metabolic engineering of β-lactam production. *Metab Eng* 2003, 5: 56–69.
22. Baba S, Abe Y, Suzuki T, Ono C, Iwamoto K, Nihira T, Hosobuchi M. Improvement of compactin (ML-236B) production by genetic engineering in compactin high-producing *Penicillium citrinum*. *Appl Microbiol Biotechnol* 2009, 83: 697–704.
23. Li L, Zhao YW, Ruan LJ, Yang S, Ge M, Jiang WH, Lu YH. A stepwise increase in pristinamycin II biosynthesis by *Streptomyces pristinaespiralis* through combinatorial metabolic engineering. *Metab Eng* 2015, 29: 12–25.
24. Keasling JD. Synthetic biology and the development of tools for metabolic engineering. *Metab Eng* 2012, 14: 189–195.
25. Feng Z, Kim JH, Brady SF. Fluostatins produced by the heterologous expression of a TAR reassembled environmental DNA derived type II PKS gene cluster. *J Am Chem Soc* 2010, 132: 11902–11903.
26. Karas BJ, Suzuki Y, Weyman PD. Strategies for cloning and manipulating natural and synthetic chromosomes. *Chromosome Res* 2015, 23: 57–68.
27. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 2009, 6: 343–345.
28. Leem SH, Noskov VN, Park JE, Kim S, Larionov V, Kouprina N. Optimum conditions for selective isolation of genes from complex genomes by transformation-associated recombination cloning. *Nucleic Acids Res* 2003, 31: e29.
29. Yin SL, Wang WS, Wang XF, Zhu YX, Jia XJ, Li SS, Yuan F, *et al*. Identification of a cluster-situated activator of oxytetracycline biosynthesis and manipulation of its expression for improved oxytetracycline production in *Streptomyces rimosus*. *Microb Cell Fact* 2015, 14: 46.
30. Zabala D, Braña AF, Flórez AB, Salas JA, Méndez C. Engineering precursor metabolite pools for increasing production of antitumor mithramycins in *Streptomyces argillaceus*. *Metab Eng* 2013, 20: 187–197.
31. Liu S, Wang M, Du G, Chen J. Improving the active expression of transglutaminase in *Streptomyces lividans* by promoter engineering and codon optimization. *BMC Biotechnol* 2016, 16: 75.
32. Dairi T, Aisaka K, Katsumata R. A self-defense gene homologous to tetracycline effluxing gene essential for antibiotic production in *Streptomyces aureofaciens*. *Biosci Biotechnol Biochem* 1995, 59: 1835–1841.