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Development of a synthetic oxytetracycline-inducible expression system for streptomycetes using de novo characterized genetic parts

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Abstract

Precise control of gene expression using exogenous factors is of great significance. To
develop ideal inducible expression systems for streptomycetes, new genetic parts,
oxytetracycline responsive repressor OtrR, operator \textit{otrO} and promoter \textit{otrBp} from
\textit{Streptomyces rimosus}, were selected \textit{de novo} and characterized \textit{in vivo} and \textit{in vitro}.
OtrR showed strong affinity to \textit{otrO} ($K_D = 1.7\times10^{-10}$ M) and oxytetracycline induced
dissociation of the OtrR/DNA complex in a concentration-dependent manner. Based
on these genetic parts, a synthetic inducible expression system Potr* was optimized.
Induction of Potr* with 0.01-4 µM of oxytetracycline triggered a wide-range
expression level of \textit{gfp} reporter gene in different \textit{Streptomyces} species. Benchmarking
Potr* against the widely used constitutive promoters \textit{ermE*} and \textit{kasOp*} revealed
greatly enhanced levels of expression when Potr* was fully induced. Finally, Potr*
was used as a tool to activate and optimize the expression of the silent jadomycin
biosynthetic gene cluster in \textit{Streptomyces venezuelae}. Altogether, the synthetic Potr*
presents a new versatile tool for fine-tuning gene expression in streptomycetes.

Keywords

Inducible expression system, streptomycetes, genetic parts, wide-range strength,
biosynthetic gene cluster, natural product

Introduction

The Gram-positive streptomycetes are well-known producers of an immense diversity
of biologically active compounds, which have been applied in human medicine,
animal health, and plant crop protection. In the past decades, a suite of genetic tools such as plasmids and constitutive promoters have been specifically developed for streptomycetes. These tools have greatly facilitated genetic manipulations of this genus. However, only a limited number of inducible expression systems are available for the precise control of gene expression in streptomycetes.

Inducible expression systems are versatile genetic tools for controlling gene expression upon induction with chemical or physical inducers, and they are typically used for the functional characterization of genes and for the production of medically or industrially important proteins. In many cases, inducible expression systems have also been employed to optimize the levels of pathway expression in order to achieve economically viable titers, yields and productivities of products of interest. However, the existing inducible expression systems have their limitations and could not meet the need of synthetic biology to precise control gene expression in streptomycetes. The thiostrepton-inducible ptipA was the first inducible expression system developed from Streptomyces lividans. It depends on the presence of the activator TipAL but also relies on the resistance gene tsr, and it often show high level of leaky expression. Other inducible expression systems developed for streptomycetes include the ε-caprolactam-inducible expression system based on the nitrilase promoter/operator PnitA/NitR from Rhodococcus rhodochrous, the synthetic tetracycline (Tc)-inducible expression system based on the repressor TetR/operator tetO from Escherichia coli and the cumate-inducible system from Pseudomonas putida. While the PnitA/NitR inducible expression system is
excellent for protein overproduction from high copy number plasmids, it is unclear
whether this system will also perform well to control expression in routine genetic
analysis and metabolic pathway engineering\textsuperscript{17}. Importantly, the inducers
\(\epsilon\)-caprolactam and cumate are degradable in some \textit{Streptomyces} species\textsuperscript{16,19}, which
will limit their applications. In addition, the above-mentioned genetic parts for
inducible expression systems are not perfectly compatible in streptomycetes, which
are characterized by large and high-GC content genomes and intricate regulatory
networks\textsuperscript{20}. The regulatory networks of streptomycetes result in background noise
when using these genetic parts\textsuperscript{17,18}. For example, each streptomycetes genome
contains over 100 TetR family regulators, and thus TetR homologs SCO0253 perturbs
the work of the synthetic Tc-inducible expression system developed by TetR
repressor and \textit{tetO} operator in \textit{Streptomyces coelicolor}\textsuperscript{17}. Therefore, although several
inducible expression systems have been reported for streptomycetes\textsuperscript{15-18}, there is still
a definitive lack of tightly inducible expression system suitable for elaborate studies
in streptomycetes.

Ideally, an inducible expression system should be completely turned off when
repressed, and tunable to different strengths when induced. In addition, the inducer
should be easily available and have little pleiotropic effects on growth\textsuperscript{9}. \textit{Streptomyces}
species exhibit marked regulatory capacity and flexibility\textsuperscript{20}. They typically have
more than 8000 protein-coding genes, and >10\% of the coding genes are predicted to
be transcription factors\textsuperscript{20}. The native regulators and operators in the genomes of
streptomycetes are therefore rich repositories of genetic parts that fulfill the
requirements for the development of inducible expression systems.

In the present study, we aimed to find unique genetic parts that respond to small molecules in streptomycetes and to develop ideal inducible expression systems. Interestingly, regulatory genes situated in biosynthetic gene clusters (BGCs) that direct secondary metabolite biosynthesis do often interact with the products of that pathway and regulate the transcription of biosynthetic genes and/or resistance genes. For example, ActR, KijA, LanK, SimR, and VarR have all been reported to bind the end products or intermediates of the corresponding BGCs and induce the expression of divergently adjacent exporter genes. The regulatory mechanism is similar to the Tc-inducible repressor (TetR) and exporter (TetA) from the E. coli transposon Tn10. The well-characterized repressor TetR and operator tetO have been widely used as genetic parts to construct inducible expression systems in prokaryotes, mammalian cells and other organisms (such as Drosophila).

Here we therefore focused on the pairs of antibiotic exporter and regulatory genes from BGCs of streptomycetes to identify the candidate genetic parts. By combining the rareness of the regulators and the availability of the inducers, the oxytetracycline (OTC)-inducible regulator OtrR, operator otrO and promoter otrBp from Streptomyces rimosus were selected and characterized de novo. Using these well-characterized genetic parts, a synthetic and tightly controlled inducible expression system Potr* with wide-range OTC-inducible strength was developed. Potr* showed negligible leaky expression levels in the absence of the inducer OTC and high levels of induced expression in Streptomyces venezuelae, Streptomyces
coelicolor and Streptomyces albus. Furthermore, Potr* was used to activate the silent
jadomycin BGC and to optimize the production of this antibiotic in *S. venezuelae*
ISP5230, exemplifying the usefulness of this system in eliciting the production of
silent and possibly cryptic antibiotic-like natural products in streptomycetes.

**Results and Discussion**

**Search for parts in genomes of streptomycetes**

To develop a strict inducible expression system with minimum interference from
derived from the genomes of streptomycetes, pairs of antibiotic exporter and regulator
genes in the genomes of streptomycetes were selected to investigate candidate genetic
parts. For ideal genetic parts, the inducers should be highly stable and easily available.
Aromatic polyketides, synthesized by type II polyketide synthases in streptomycetes,
are recalcitrant to be degraded in these producers\(^3\). Therefore, we searched all type
II polyketide BGCs with identified products from the genomes of streptomycetes, and
extracted sequences of exporters as well as the divergently arranged putative
regulators (Table 1). The regulators were identified as TetR, ArsR, MarR or DeoR
family (Table 1). Previous studies have reported that intrinsic large numbers of TetR
homologs may perturb the inducible expression system developed through other TetR
family regulators\(^1,7\), therefore this family regulators were excluded from this study.
Considering the availability and cost of the inducers, we selected genetic parts from
the commercialized type II polyketide BGCs. Only OTC and chlortetracycline (CTC)
meet the criteria of being readily available as well as low cost. The putative regulators
of OTC and CTC exporters (OtrR and CtcS) belong to MarR family, which have many less members (approximate 20-30 members) compared with the TetR family in streptomycetes. To avoid the interference of native regulators in streptomycetes, the sequences of OtrR and CtcS were used as queries to conduct a BLASTp search against the local protein database containing the sequences of 569,791 proteins from streptomycetes deposited in UniProt. Highly homologous orthologs of OtrR and CtcS (> 30% identities) were not observed in most genomes of streptomycetes, except that of their native hosts, *Streptomyces monomycini* and *Streptomyces griseoflavus*. The results suggest that OtrR and CtcS are suitable candidates with minimum interference from other endogenous regulators in streptomycetes. Since OTC is known to be less toxic to microbial cells than CTC, we decided to investigate the regulatory mechanism of the OTC exporter to identify inducible genetic parts (Fig. 1).

**Characterization of the regulatory mechanism of OtrR**

The MarR family transcriptional regulators exist as dimers and bind palindromic sequences within target promoters, resulting in either transcriptional repression or activation\(^1\). To confirm the regulatory role of OtrR on the putative target promoter otrBp, a Lux reporter system was constructed in heterologous host *E. coli* (Fig. 2a). The lux genes were directly controlled by otrBp, endowing the strains with the ability of bioluminescence (Fig. 2b). When an OtrR expression plasmid was transferred into *E. coli* harboring the lux reporter plasmid, the bioluminescence was severely repressed (Fig. 2b), indicating that OtrR could repress the expression of otrBp. Furthermore, when OTC was added, the bioluminescence was restored in a
dose-dependent manner (Fig. 2b). These results suggest that OtrR can sense and respond to OTC to de-repress the transcription of otrBp. More importantly, the concentration of OTC needed to completely unlock otrBp is approximately 2.5 µM, which is a concentration much lower than the minimal inhibitory concentration (MIC, approximate 10-30 µM).

To further confirm the direct interaction of OtrR with otrBp, OtrR was expressed and purified from E. coli (Supplementary Fig. S1) and an electrophoretic mobility shift assay (EMSA) was performed with a probe of otrBp (P’otrB). As shown in Fig. 2c, OtrR could bind P’otrB in a concentration-dependent manner. The binding was shown to be specific, with no retardation was observed for the negative control probe of hrdB promoter (P’hrdB) (Supplementary Fig. S2). These results demonstrate that OtrR represses otrBp by direct interaction. The responses of OtrR/P’otrB complexes to OTC were also analyzed by EMSA in vitro. We observed that OtrR and P’otrB complexes would dissociate in the presence of OTC, and this dissociation effect also occurred in an OTC concentration-dependent manner (Fig. 2d). This data along with the results obtained from the in vivo Lux reporter system undoubtedly indicate that OtrR represses the transcription of otrBp by direct binding, and OTC releases this repression effect by attenuating the binding of OtrR, thus inducing the transcription of otrBp.

**Determination of OtrR operator otrO and the core promoter of otrBp**

To develop a new OTC-inducible expression system, it is necessary to identify the
operator of OtrR. DNase I footprinting was performed to locate the binding sites of
OtrR in the otrBp region using a capillary sequencer. As shown in Fig. 3a, a fully
protected region was observed in the presence of OtrR, and this region encompasses
two 6-nt inverted repeats (GACAAG) with 2-bp spacing (Fig. 3b). To further confirm
whether the identified region containing the palindromic sequence is the operator of
OtrR (designated as otrO), we amplified the probes with (PR) or without (PL) the
palindromic sequence (Supplementary Fig. S3a) and tested their binding with OtrR by
EMSA. The results showed that the retardation was only observed with the PR probe
(Supplementary Fig. S3b and c). Furthermore, the binding ability of the 20-bp probe
(P20) containing the palindromic sequence with OtrR was examined using EMSA,
and the retardation was readily detected (Supplementary Fig S3d). These results
suggest that this palindromic sequence is the core region of otrO. To determine the
equilibrium dissociation constant ($K_D$) between the interaction of OtrR and otrO,
surface plasmon resonance (SPR) was performed. The sequence containing otrO was
labeled with biotin and immobilized onto a streptavidin sensor chip, and subsequently
OtrR was injected. The sensorgrams showed increased binding with the increasing
OtrR concentrations (Fig. 3c). The best fit for OtrR yielded $K_D$ value of 0.17 nM. The
$K_D$ of interaction between OtrR and otrO is similar to that of the interaction between
Tc-inducible repressor TetR and operator tetO, which have been successfully used as
genetic parts for the development of inducible expression systems and the
construction of genetic circuits in many organisms. Therefore, the new genetic
parts OtrR and otrO show good potential for widespread application. Notably, OtrR,
containing only 165 amino acids, is smaller than TetR, which will enable a much
easier and economical application in other organism. In addition, the operator of OtrR
is very unique. A search using otrO sequence did not give closely matched sequences
in the genomes of streptomycetes, suggesting that OtrR and otrO are ideal parts that
do not interplay with host DNA or regulators in streptomycetes.

To determine the loci of the otrO relative to the the transcriptional start site (TSS) of
otrBp, rapid amplification of 5’-cDNA ends (5’-RACE) was performed (Fig. 3d).
Then the putative -10 and -35 regions were identified based on the determined TSS
(Fig. 3d), which shares high nucleotide sequence identity with the core promoter
recognized by the essential sigma factor HrdB. After defining the core region of
otrBp, otrO site was found to overlap with -35 region of otrBp (Fig. 3d).

**Development of the OTC-inducible expression system for streptomycetes**

OtrR and otrBp were identified from *Streptomyces* species, and thus the native
otrR-otrBp inducible expression system (designated as Potr) should be compatible in
streptomycetes. The performance of Potr was firstly evaluated in *S. venezuelae*, which
grows in a diffuse and homogenous manner in a variety of liquid media. Such
growth characteristics facilitate accurate data normalization. Strain *S. venezuelae*
WVR2006::pIJ-Potr was incubated in MYM medium supplemented with OTC at
concentrations ranging from 10 nM to 6 µM. We observed that GFP fluorescence
increased with increasing concentrations of OTC and Potr was fully induced at 4 µM
OTC (Fig. 4a). Surprisingly, the fully induced fluorescence was stronger than that
driven by the widely used constitutive promoters $kasOp^*$ and $ermEp^*$ (Fig. 4b), suggesting that the activity of $otrBp$ was very strong. Recently, Bai et al. has shown that $kasOp^*$ exhibited the strongest activity among all the tested constitutive promoters in streptomycetes using a flow cytometry-based quantitative method. At the fully induced state, we observed that the activity of native $otrBp$ was stronger than that of $kasOp^*$ (Fig. 4b). Hence, the activity of $otrBp$ does not require further optimization. However, we observed that the leaky expression without OTC induction was higher compared with the control (Fig. 4b). Thus, Potr should be engineered to reduce leaky expression.

Zhang et al. reported that the integration of the same operators at two locations, flanking the -35 and the -10 regions of the target promoter, can achieve tighter control of the promoter activity. To reduce the leaky expression of Potr, we engineered the inducible expression system (designated as Potr*) by inserting another operator downstream of the TSS of $otrBp$ (Fig. 4c). After examining the GFP fluorescence of $S. venezuelae$ WVR2006::pIJ-Potr* (map of pIJ-Potr*, see Supplementary Fig. S4), we observed that Potr* showed nearly the same GFP-response curve as Potr to OTC (Fig. 4d), whereas the leaky expression was significantly reduced to a level similar to the control (Fig. 4e).

To examine the activity of Potr* induced by different doses of OTC at the transcription level, real-time quantitative PCR (RT-qPCR) was performed to assess the transcript levels of the $gfp$ gene in $S. venezuelae$ WVR2006::pIJ-Potr*. The results showed that the transcript abundance of $gfp$ increased in an OTC.
concentration-dependent manner (Fig. 5a), suggesting that the inducible activity of Potr* was sensitively controlled at the transcriptional level in S. venezuelae.

To evaluate the growth inhibitory effects by OTC, the growth of S. venezuelae was assayed in the presence OTC. The results indicate OTC does not have significant effects on growth at lower than 6 µM (Supplementary Fig. S5). As a concentration less than 4 µM OTC is sufficient to induce Potr or Potr* to a fully expressed level, the inducer can be used at a concentration where the growth is hardly affected.

**Evaluation of the performance of Potr* in different Streptomyces species**

To evaluate the inducible behavior of Potr* in other Streptomyces species, the production of GFP fluorescence under the control of Potr* was further evaluated in S. coelicolor M1146::pIJ-Potr* and S. albus J1074::pIJ-Potr*. It was shown that the expression of gfp was sensitively induced in a OTC concentration-dependent manner in the two hosts; the leaky expression of gfp was negligible without OTC and the fully induced output of gfp was much higher than that of the constitutive promoters kasOp* and ermEp* (Fig. 5b and c). When grown in liquid culture, S. coelicolor and S. albus form large pellets or clumps, meaning that the GFP fluorescence assay might have margins of error in the data normalization of these strains. Therefore, we further performed RT-qPCR to more accurately evaluate the transcription levels of gfp reporter gene in S. coelicolor M1146::pIJ-Potr* and S. albus J1074::pIJ-Potr* after induction using different OTC concentrations. The results showed that the transcript levels of the gfp gene also increased in an OTC concentration-dependent manner in
the two *Streptomyces* strains (Fig. 5d and e), consistent with the results obtained in *S. venezuelae*. The highly consistent results generated in different *Streptomyces* species indicate that Potr* is a tightly controlled inducible expression system with wide-range strength in streptomycetes.

**Application of Potr* in activation and optimization of the silent jadomycin BGC**

The potential for the production of high-value secondary metabolites is even larger than previously realized in streptomycetes. However, accessing this rich resource to identify new compounds through the activation of cryptic pathways is still a challenge. We hypothesized that a cryptic gene cluster under the control of an inducible expression system will facilitate the identification of corresponding product, as the production will increase with the inducer in a concentration-dependent manner. To demonstrate this application, we designed a plug-and-play plasmid pIW01 (Supplementary Fig. S6) using the newly developed inducible expression system Potr*. This plasmid was integrated into the genome of *S. venezuelae* ISP5230 to drive the expression of silent jadomycin BGC (Fig. 6a). As expected, jadomycin B production gradually increased with the OTC inducer (Fig. 6b), while the control without OTC induction showed almost no production, similar to the results of wild type *S. venezuelae* ISP5230. These results indicate that the inducible expression system can be applied in the activation and identification of silent and possibly cryptic natural products in streptomycetes.

To improve the production of jadomycin, the expression levels of jadomycin BGC
were optimized by adding different doses of OTC at different growth time points. Accordingly, jadomycin B production under different induction conditions was examined. It was found that an induction dose of 0.75 µM OTC and time of 4 h was optimal (Fig. 6c). The use of an inducible expression system to optimize the production levels of secondary metabolites in streptomycetes is a promising approach for the improvement of antibiotics production. Previously, overexpression of BGCs or activators through constitutive strong promoters is a preferred approach. A potential disadvantage of these approaches is that the constitutive expression of BGCs leads to the synthesis of the products at early growth stage, which could reduce the growth of host cell and concomitantly decrease the overall production level. The strategy of fine-tuning the expression of BGCs could coordinate the biomass growth and desired product accumulation. Our results suggest that the inducible expression system Potr* can be applied as an optimizing tool in rational engineering.

Overall, new genetic parts (repressor OtrR, operator otrO and promoter otrBp) were identified and characterized de novo. They have been shown to fulfill the requirements of inducible expression system. Then the synthetic inducible expression system Potr* was developed using these genetic parts, and it showed tightly tunable strength though the inducer OTC in streptomycetes. Potr* was also designed as a tool to activate silent gene clusters and to optimize the production of secondary metabolites. The present work suggests that the synthetic inducible expression system Potr* will play important roles on fine-tuning gene expression in streptomycetes and the newly characterized genetic parts will have potential to be used in the control of
gene expression and construction of genetic circuits in synthetic biology.

Methods

Bacterial strains and culture conditions

The strains and plasmids used in the present study are listed in Supplementary Table S1. *E. coli* JM109 and DH5α were used for cloning and bioluminescence assays, respectively; BL21(DE3) was used for the recombinant expression of OtrR protein. *E. coli* strains were grown in Luria-Bertani (LB) medium containing ampicillin (100 µg/ml), hygromycin (50 µg/ml), kanamycin (25 µg/ml) or chloramphenicol (25 µg/ml) when necessary. For spore preparations, *Streptomyces coelicolor* M1146, *Streptomyces albus* J1074, *Streptomyces rimosus* M4018 and their derivatives were maintained on mannitol-soya flour (MS) agar plates, while *Streptomyces venezuelae* ISP5230, *S. venezuelae* WVR2006 and its derivatives were grown on maltose-yeast extract-malt extract (MYM) agar plates. For DNA and total RNA preparation, *S. rimosus* M4018 was grown in TSB medium. For GFP reporter assays, the derivatives of *S. coelicolor* M1146 and *S. albus* J1074 were grown in supplemented minimum medium (SMM), while the derivatives of *S. venezuelae* WVR2006 were grown in MYM liquid medium.

Construction of plasmids

All primers used in the present study are listed in Supplementary Table S2. For the construction of pET-OtrR, *otrR* was amplified from the genomic DNA of *S. rimosus* 4018 with primer pair OtrRF1/OtrRR1. The product was digested with NdeI/XhoI and

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inserted into the corresponding sites of pET-23b. For the construction of pLux-otrBp, 

*otrBp* was amplified from genomic DNA of *S. rimosus* 4018 with primer pair PBF1/PBR1. The product was digested with BamHI/XhoI and inserted into the corresponding sites of pCS26-Pac$^{43}$. For the construction of pOtrR, *otrR* was amplified using the primer pair OtrRF2/OtrRR2, and a fragment of plasmid backbone was amplified from pACYC184 using the primer pair 184F/184R. The two fragments were assembled using the In-Fusion cloning kit (Clontech) to generate pOtrR. For the construction of pIJ-Potr, a 637-bp fragment containing *otrR* and *otrBp* was amplified from *S. rimosus* 4018 genomic DNA using the primer pair PBF2/PBR2, and enzymatically assembled with pIJ8660::BsaI-sfgfp$^{35}$ digested by BsaI to generate pIJ-Potr$^{44}$. To construct pIJ-Potr*, a fragment of plasmid backbone was amplified from pIJ-Potr using the primer pair IJF2/IJR2 and subsequently self-ligated through enzymatic assembly to generate pIJ-Potr*. For the construction of plug-and-play plasmids, a fragment of the plasmid backbone was amplified from pIJ-Potr* using the primer pair IJF3/IJR3 and subsequently self-ligated to remove the sequences of *attP* and integrase gene to generate pIW. A fragment of plasmid backbone was amplified from pIW using the primer pair IWF1/IWR1 to remove the *gfp* gene, followed by self-ligation through enzymatic assembly to generate pIW01. For the construction of pIW01-jad, 5’-partial *jadJ* was amplified from *S. venezuelae* ISP5230 genomic DNA using the primer pair JF/JR, and a fragment of plasmid backbone was amplified from pIW01 using the primer pair IWF/IWR. Subsequently, the two fragments were enzymatically assembled to generate pIW01-jad.
Expression and purification of OtrR

The plasmid pET-OtrR was introduced into *E. coli* BL21 (DE3) for recombinant expression of OtrR. These proteins were purified as previously described.

EMSA

A probe for *otrBp* was amplified using the primers PBF4/PBR4 (Table S2). The probe was incubated with varying concentrations of purified OtrR at 25°C for 30 min in 20 µl of buffer containing 20 mM Tris-base (pH 7.5), 2 mM dithiothreitol, 5 mM MgCl₂, 0.5 mg/ml calf BSA and 5% glycerol. After incubation and electrophoresis, the non-denaturing 4% polyacrylamide gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen) for 30 min in TBE (89 mM Tris-base, 89 mM boric acid, and 1 mM EDTA, pH 8.0), and photographed under an ultraviolet trans-illuminator using Bio-Rad GelDoc XR.

Luciferase assays \textit{in vivo} using Lux reporter systems

The plasmid pLux-otrBp and control pCS26-Pac were introduced into *E. coli* DH5α. Subsequently, pOtrR was introduced into DH5α harboring pLux-otrBp to examine the effects of OtrR on *otrBp*. For luciferase assays, approximately $10^6$ cells were inoculated into 5 ml of LB for 12 h. Then the bioluminescence of *E. coli* cultures was measured using an EnSpire Multimode Reader (PerkinElmer).

DNase I footprinting assay

DNase I footprinting assays were performed through fluorescent labeling and...
capillary analyses. Briefly, DNA fragments were prepared through PCR using fluorescence-labeled primers PBF5/PBR4. After purification from agarose gel, the labeled DNA fragments (120 ng) and respective concentrations of proteins were added to a final reaction volume of 50 µl, and incubated at 25°C for 20 min. Then the fragments were digested with DNase I (Promega) for 1 min at 25°C and terminated using stop buffer (Promega). After phenol-chloroform extraction and ethanol precipitation, the samples were loaded onto an Applied Biosystems 3730 DNA genetic analyzer (Applied Biosystems) together with the internal-lane size standard ROX-500 (Applied Biosystems). A dye primer-based sequencing kit (Thermo) was used to precisely determine the sequences after aligning the results of the capillary electrophoresis reactions. The electrophoregrams were subsequently analyzed with the GeneMarker v1.8 software.

**Determination of TSS**

5′-RACE experiments were conducted to map the transcriptional start points of *otrBp* according to the manufacturer's instructions (RLM-RACE kit, Ambion). The total RNA was extracted from *S. rimosus* M4018. GSPB1 was used as special outer *otrB* primer, and GSPB2 was used as special inner *otrB* primer, respectively.

**SPR assays**

The SPR assays were conducted using a Biacore 3000 System (GE Healthcare). The experiments were performed in PBS buffer (9 mM PBS, pH 7.4, 1% DMSO and 0.05% Tween 20) with a flow rate of 30 µl/min at 25°C. The probe was obtained after
annealing the biotin-labeled forward primer SPRF and the unlabeled reverse primer SPRR, and then immobilized on a streptavidin sensor chip. OttrR was diluted to different concentrations in PBS buffer and subsequently injected. At the end of each cycle, 0.4% SDS was used to regenerate the surface. The data fitting for the binding model was conducted using BIA evaluation 4.1 software (GE Healthcare).

Quantitative measurement of GFP fluorescence

For *S. venezuelae*, 1 ml of the 24-h seed cultures of the recombinant strains was inoculated into 50 ml of MYM medium for 6 h, then induced by different doses of OTC for 18 h. 200-µl aliquots of the cultures were washed twice with PBS (pH 7.2) and re-suspended with 1 ml PBS, then GFP fluorescence was detected (excitation at 485 nm; emission at 512 nm, Synergy H4 Multi-Mode Reader). All fluorescence values were normalized to cell growth (OD$_{600}$). For *S. coelicolor* and *S. albus*, approximately 2×10$^8$ spores were inoculated into 50 ml of SMM medium for 48 and 42 h, respectively. Subsequently, the cells were washed twice with PBS (pH 7.2). The pellets were re-suspended in 1 ml R buffer (20 mM Tris-HCl, 0.5 M NaCl, and 10% (v/v) glycerol, pH 7.4) and subjected to ultra-sonication to generate a cell extract$^3$. The fluorescence intensity was quantified after normalizing the fluorescence intensity to the protein concentration in the 200-µl sample$^3$. Each value and error bar represents the average and standard deviation of three experimental replicates, respectively.

Transcriptional analysis using RT-qPCR
Total RNA was isolated from *Streptomyces* samples using a standard procedure at the same time as GFP fluorescence assay. The RNA samples were then treated with RNase-free DNase (Promega) and checked by PCR to eliminate the possibility of chromosomal DNA contamination. First-strand cDNA of *gfp* gene was performed using a Superscript III first-strand Synthesis System (Invitrogen) with 500 ng of total RNA according to the manufacturer’s instructions. All cDNA synthesis reactions included a replicate reaction without reverse transcriptase to ensure the complete removal of contaminating DNA from the RNA samples. The primers of *gfp* and *hrdB* are listed in Supplementary Table S2. RT-qPCR was performed using the ABI 7500 Detection System and SYBR Green PCR Master Mix (Applied Biosystems). The results were analyzed using ABI 7500 software v2.0.1 and the relative expression levels of the target genes were normalized to endogenous *hrdB* levels. All samples were analyzed in triplicate.

**Activation of jadomycin BGC and optimization of jadomycin production**

The plasmid pIW01-jad was introduced into *E. coli* ET12567 (pUZ8002) and conjugated into *S. venezuelae* ISP5230. Recombinant strains were incubated in 96 deep-well plates after the introduction of OTC at different time points and dose at 30°C with shaking (900 rpm), then the production of jadomycin B was examined. The HPLC condition for the detection of jadomycin B was the same as previously described.

**Supporting Information Available**

Supplementary Figures, Tables and Sequences. This material is available free of
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Author information

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Author Contributions

K.Y., W.W. and C.C. conceived the project. W.W., T.Y. and Y.L. performed the experiment. S.L., S.Y., C.C. and K.S. assisted with the primary data analysis, W.W., K.Y. and C.C. wrote the manuscript.

Notes: The authors declare no competing interest.

Acknowledgement

This work was financially supported by grants from the National Natural Science Foundation of China [Nos. 31400034 and 31570031], the Ministry of Science and Technology of China [No. 2013CB734001] and the Beijing Natural Science Foundation [No. 5154032]. W. W. is an awardee of Youth Innovation Promotion Association of CAS [2016087].

References


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**Figure legends and tables**

**Figure 1** Position and arrangement of *otrR* and *otrB* genes in the OTC BGC.

**Figure 2** Identification of the repression effect of OtrR and the de-repression effect of OTC. (a) Schematic representation of the Lux reporter system. (b) OtrR represses the bioluminescence controlled by *otrBp* and OTC induces the bioluminescence *in vivo*. All values are presented in relative light units (RLU) and represented as the means ± SD from at least three independent experiments. (c) EMSA of the interaction between the P-*otrB* and OtrR. Each lane contains 6 ng of P-*otrB* and different amounts of OtrR. (d) Dissociation effect of the DNA-binding activity of OtrR in the presence of OTC. Each lane contains 6 ng of P-*otrB*, 0.15 μM OtrR and different amounts of OTC.

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**Figure 4** Development of the inducible expression system Potr*. (a) Expression levels of Potr in response to different doses of OTC measured by GFP fluorescence. The inducer concentrations were 0, 0.01, 0.02, 0.04, 0.08, 0.2, 0.4, 0.8, 1.6, 2, 3, 4, 5, and 6 \(\mu\)M OTC. The values are presented as the means ± SD of three independent experiments. (b) Activity of the on and off state of Potr. 4 \(\mu\)M OTC was used to fully induce the activity of Potr. (c) Core promoter sequences of the native Potr and the developed Potr*. The TSS is indicated with an asterisk. The presumptive -10 and -35 regions are indicated with boxes, and the operator otrO is underlined. (d) Expression levels of Potr* in response to different doses of OTC measured by GFP reporter. The same OTC concentrations as in A were used. (e) Activity of the on and off state of Potr*. 4 \(\mu\)M OTC was used to fully induce the activity.

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**Figure 6** Application of Potr* for the activation of the silent jadomycin BGC and the optimization of jadomycin production. (a) Schematic representation of the strategy to activate or optimize the expression of the jadomycin BGC. pIW01 is the plasmid containing the inducible expression system Potr* and the multiple cloning site (MCS) for inserting the target fragment. (b) The activation and identification of the product of silent jadomycin BGC through induction using gradient doses of OTC. (c) Heat map showing the production of jadomycin B for all combinations of different OTC doses and induction time points.
Table 1 Pairs of antibiotic exporter and regulatory genes of in type II polyketide synthesis clusters.

<table>
<thead>
<tr>
<th>Streptomyces species</th>
<th>Type II polyketide</th>
<th>Transporter</th>
<th>Regulator/family</th>
<th>Effector(^a)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. coelicolor</em></td>
<td>Actinorhodin</td>
<td>ActII-ORF2</td>
<td>ActII-ORF1/TetR family</td>
<td>(S)-DNPA; kalafungin</td>
<td>M64683</td>
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<tr>
<td><em>S. sp. CM020</em></td>
<td>Alnumycin</td>
<td>AlnT1</td>
<td>AlnR8/MarR family</td>
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<td><em>S. maritimus</em></td>
<td>Enterocin</td>
<td>EncT</td>
<td>EncS/TetR family</td>
<td>NA</td>
<td>AF254925</td>
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<tr>
<td><em>S. griseoflavus</em></td>
<td>Gilvocarcin</td>
<td>GilJ</td>
<td>Gill/DeoR family</td>
<td>NA</td>
<td>AY233211</td>
</tr>
<tr>
<td><em>S. cyanogenus</em></td>
<td>Landomycin</td>
<td>LanJ</td>
<td>LanK/TetR family</td>
<td>landomycin A and B</td>
<td>AF080235</td>
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<tr>
<td><em>S. glaucescens</em></td>
<td>Tetraenomycin</td>
<td>TcmA</td>
<td>TcmR/TetR family</td>
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<td>M80674</td>
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<td><em>S. antibioticus</em></td>
<td>Oviedomycin</td>
<td>OvmE</td>
<td>Orf3/ArsR family</td>
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<td><em>S. rimosus</em></td>
<td>Oxytetracycline</td>
<td>OtrB</td>
<td>OtrR/MarR family</td>
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<td>CtcR</td>
<td>CtcS/MarR family</td>
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<tr>
<td><em>S. steffisburgensis</em></td>
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<td>SfrB</td>
<td>StfRIII/MarR family</td>
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<tr>
<td><em>S. fradiae Tü2717</em></td>
<td>Urdamycin</td>
<td>UrdJ</td>
<td>UrdK/TetR family</td>
<td>NA</td>
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<tr>
<td><em>S. antibioticus</em></td>
<td>Simocyclinone</td>
<td>SimX</td>
<td>SimR/TetR family</td>
<td>Simocyclinone D8 and C4</td>
<td>AF324838</td>
</tr>
</tbody>
</table>

\(^a\)NA indicate the unknown effector.
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45x24mm (300 x 300 DPI)
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Title: "Development of a synthetic oxytetracycline-inducible expression system for streptomycetes using de novo characterized genetic parts"

Author(s): Wang, Weishan; Yang, Tongjian; Li, Yihong; Li, Shanshan; Yin, Shouliang; Styles, Kathryn; Corre, Christophe; Yang, Keqian

35x15mm (300 x 300 DPI)