Supplementary information

A tunable and reversible thermo-inducible bio-switch for streptomycetes

Lanxin Lv[1,3], Yudie Fu[1,3], Shuo Liu[1,3], Yuxin Zhang[1], Meiyan Wang[1], Yinhua Lu[2] and Guoqing Niu[1,*]

[1] College of Agronomy and Biotechnology, Southwest University, Chongqing 400715, China.

[2] College of Life Sciences, Shanghai Normal University, Shanghai, 200234, China

[3] These authors contributed equally: Lanxin Lv, Yudie Fu, and Shuo Liu.

* Guoqing Niu

Email: niu062376@swu.edu.cn

Keywords: RheA repressor, thermal-inducible bio-switch, CRISPR/Cas9, *Streptomyces*.

This PDF file includes:

Supplementary Methods

Culture conditions

Unless otherwise specified, *Streptomyces* stains were cultivated at 28 °C, and *E. coli* strains were maintained at 37 °C. When necessary, antibiotics were applied at concentrations as follows: 100 μg/mL ampicillin, 100 μg/mL apramycin, 50 μg/mL kanamycin, 100 μg/mL hygromycin, and 25 μg/mL chloramphenicol in Luria-Bertani (LB) medium for *E. coli*; 50 μg/mL apramycin, 50 μg/mL hygromycin, and 25 μg/mL nalidixic acid in MS medium for *Streptomyces*.

Construction of Plasmids

For the construction of pSET152::*RS01-neo*, a 959 bp fragment covering the coding region of *rheA* with the intergenic region between *rheA* and *hsp18* was PCR-amplified from genomic DNA of *S. albidoflavus* J1074 with primer pair RS01 F/R. The *t0* transcriptional terminator was PCR-amplified with primer pair t0 F/R from pIJ8660[1]. The terminator was then assembled with the 959 bp fragment using overlap extension PCR with primers t0 F and RS01 R, and the resulting amplicon was cut with *Xba*I. The promoter-less neomycin phosphotransferase gene (*neo*) of transposon Tn5 origin was PCR-amplified with primer pair neo orfF/R from pUC119::*neo*, and cut with *Eco*RI. Prior to PCR amplification, the neo orfF primer was phosphorylated with T4 polynucleotide kinase to facilitate subsequent ligation. The two fragments were ligated together with *Xba*I/*Eco*RI double-digested pSET152 in a three-piece ligation reaction to generate pSET152::*RS01-neo*. A similar strategy was used for the construction of pSET152::*TRS01-neo* except that the native promoter of *rheA* was replaced by the constitutive *hrdB* promoter and the *tfd* transcriptional terminator. Generation of pSET152::*TRS02-neo* was accomplished with the

insertion of an additional *rheO* immediately after the transcription start site (TSS) of *hsp18* promoter in pSET152::*TRS01-neo*.

For the construction of pSET152::*TRS02-gusA*, the coding region of *gusA* was PCR-amplified with primer pair gusA F/R from pSET152-P_{hrdB}-*gusA*[2]. The resulting amplicon was used to replace the *neo* cassette in pSET152::*TRS02-neo* to generate pSET152::*TRS02-gusA*. The construction of pSET152::*TRS02-egfp* was achieved with a similar strategy except that the coding region of *egfp* was PCR-amplified with primer pair egfp F/R from pIJ8660[1].

For the construction of pKCcas9::StrepT-switch-d*dptABC*, StrepT-switch was used to replace the *tipA* promoter in pKCcas9dO[3], resulting in the generation of pKCcas9-StrepT-switch. Next, two fragments, covering appropriately 1.0 kb of homologous upstream and downstream of *dptABC* within the daptomycin biosynthetic gene cluster, were amplified by PCR from genomic DNA of *S. roseosporus* NRRL 15998 with primer pairs dptUp F/R and dptDn F/R. The amplicons were assembled together and inserted into pKCcas9-StrepT-switch *via* a homologous recombination technology following the manufacturer's instructions (ClonExpress MultiS One Step Cloning Kit, Vazyme Biotech Co., Ltd.). A similar strategy was used for the construction of pKCcas9::*kasO**-KI StrepT-switch except that the constitutive *kasO** promoter was inserted between the upstream and downstream homologous fragments.

Plasmids for ZouA-mediated DNA amplification were constructed as described previously[4] with modifications. In brief, two fragments containing *RsA* and *RsB* were PCR-amplified from genomic DNA of *S. kanamyceticus* CGMCC 4.1441 with primer pairs RsA F/R and RsB F/R, respectively. The two fragments were then assembled with the *neo* cassette, and then used to capture the *act* gene cluster *via* λ-Red-mediated recombination[5]. The resulting pIJ10500::*act* containing the *act* gene cluster flanked by *RsA* at the left border, and the *neo* cassette and *RsB* at the right border. For

the construction of pSET152::StrepT-switch-*zouA*, the coding region of *zouA* was PCR-amplified from genomic DNA of *S. kanamyceticus* CGMCC 4.1441 with primer pair ZouA F/R. The resulting amplicon was used to replace the *neo* cassette in pSET152::*TRS02-neo* to generate pSET152::StrepT-switch-*zouA*.

Overexpression and Purification of RheA

The coding region of *rheA* was PCR-amplified from genomic DNA of *S. albidoflavus* J1074 with primer pair rheA orfF/R. The PCR product was digested with *Nde*I and *Eco*RI, and then inserted into the corresponding sites of pET28a to generate pET28a::*rheA*. The recombinant plasmid was confirmed by DNA sequencing and subsequently introduced into *E. coli* BL21(DE3) for expression. RheA protein was overexpressed as a N-terminal His-tagged fusion protein, and purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography according to the manufacturer's instructions (GE Healthcare). The purified protein was quantified following the protocol of a bicinchoninic acid (BCA) protein assay kit (Sangon Biotech Co., Ltd.).

EMSAs.

The electrophoretic mobility shift assays (EMSAs) were performed essentially as described[6]. In brief, probes of *RS01* and *RS02* were PCR-amplified with primer pair rheO F/R from pSET152::*TRS01-neo* and pSET152::*TRS02-neo*, respectively. The probes were incubated with various concentrations of RheA in 20 μL of reaction buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin (BSA), and 5 % glycerol. The *hrdB* promoter was used to serve as negative control. After incubation at 30 °C for 25 min, protein-bound DNA and free DNA were separated by electrophoresis on nondenaturing

4.5 % (wt/vol) polyacrylamide gels with a running buffer containing 45 mM Tris-HCl (pH 8.0), 45 mM boric acid, and 1 mM EDTA. Gels were stained with the fluorescent dye ethidium bromide, and imaged by a Bio-Rad GelDoc 2000 system.

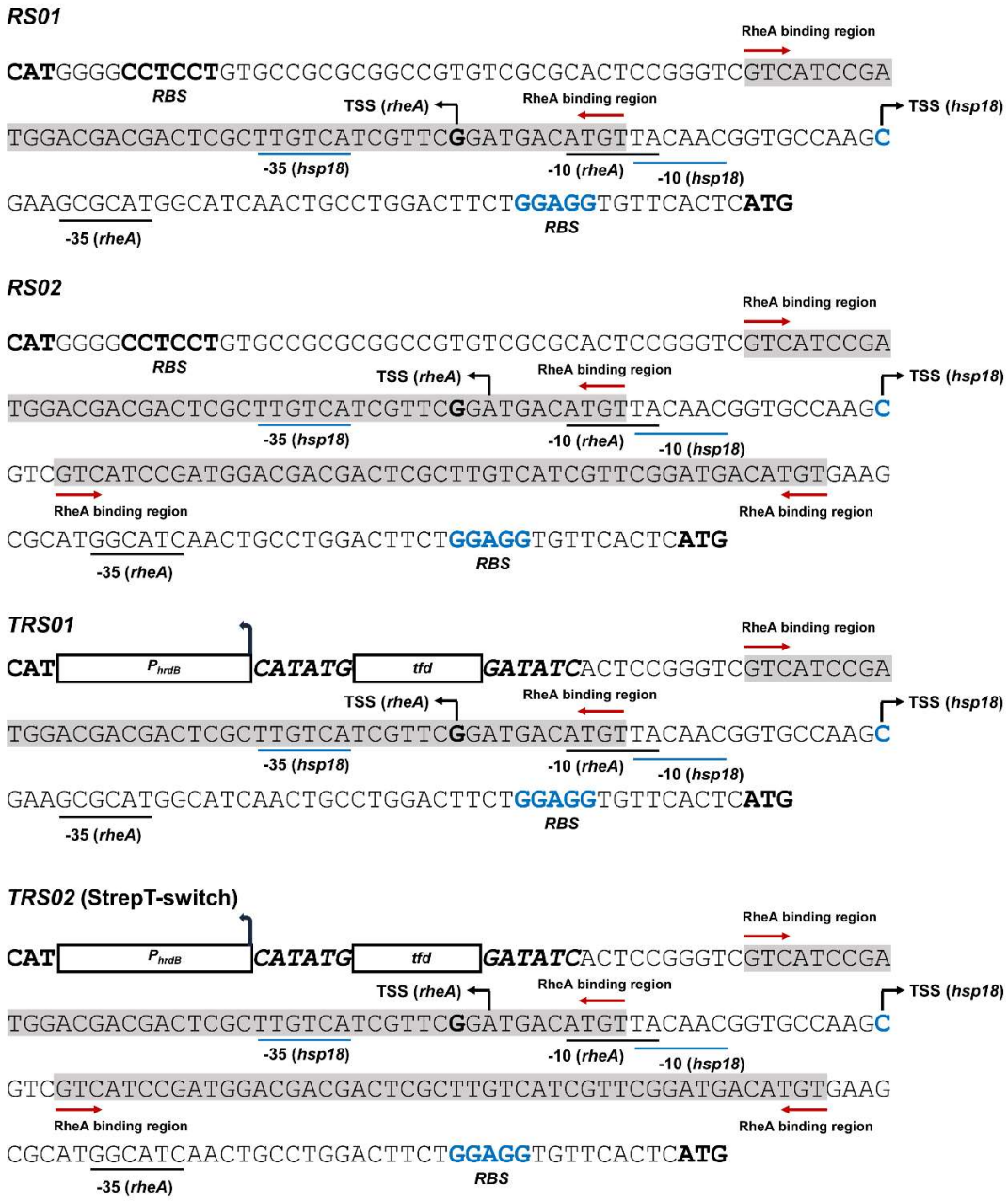

Fig. 1 Sequences of the thermal inducible modules. Promoter elements were as indicated. The RheA binding region was highlighted. The constitutive *hrdB* promoter was used to drive the expression of *rheA* in *TRS01* and *TRS02*. RBS, Ribosome Binding Site; TSS, Transcription Start Site; *tfd*, the *tfd* transcriptional terminator.

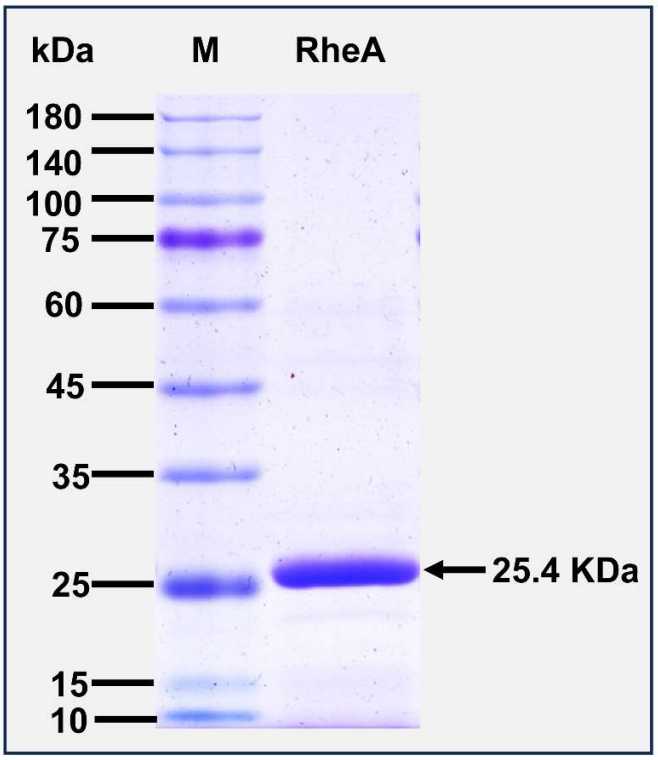

Fig. 2 SDS-PAGE analysis of purified RheA. M, molecular mass markers. Samples were separated by 12 % SDS-PAGE and stained with Coomassie brilliant blue R-250.

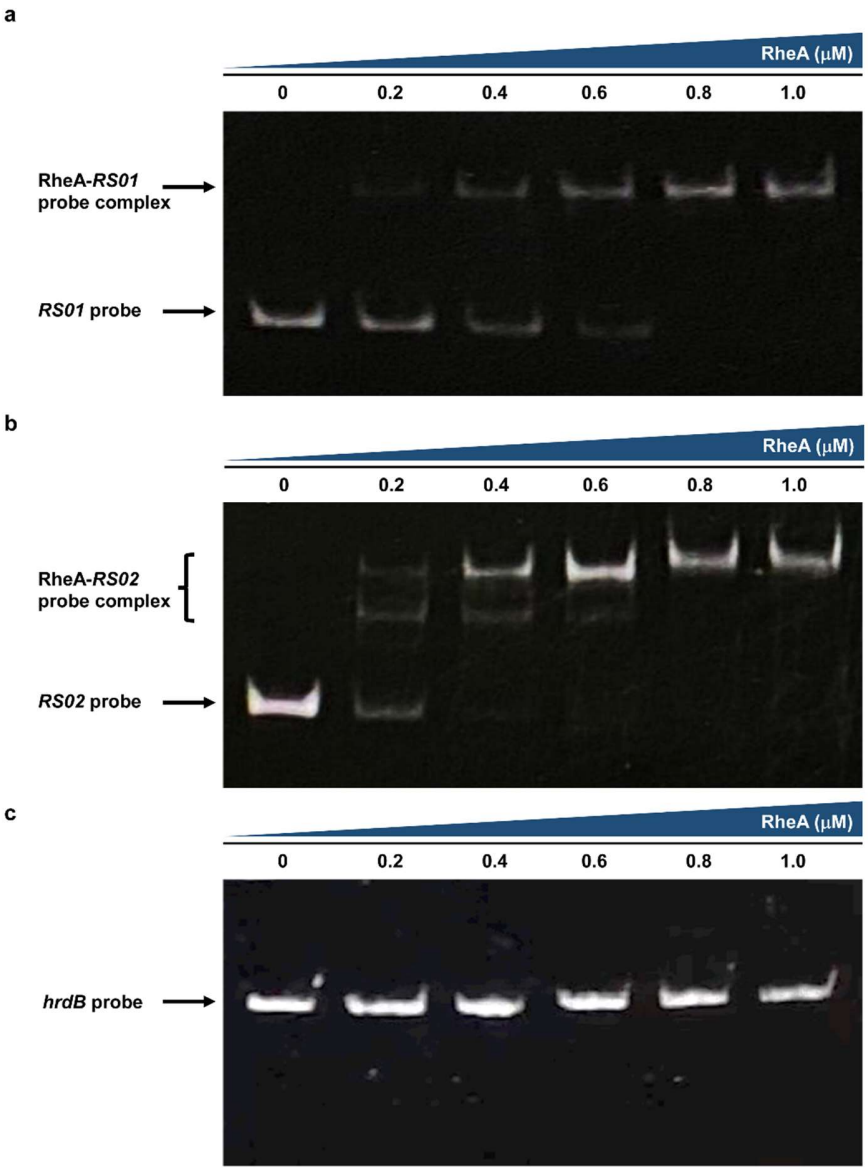

Fig. 3 Binding of purified RheA to *RS01* and *RS02* probes. Purified RheA protein was incubated with probes as indicated. The *hrdB* probe was included to serve as a negative control. DNA-protein complexes and free probes were indicated by brackets and arrows, respectively.

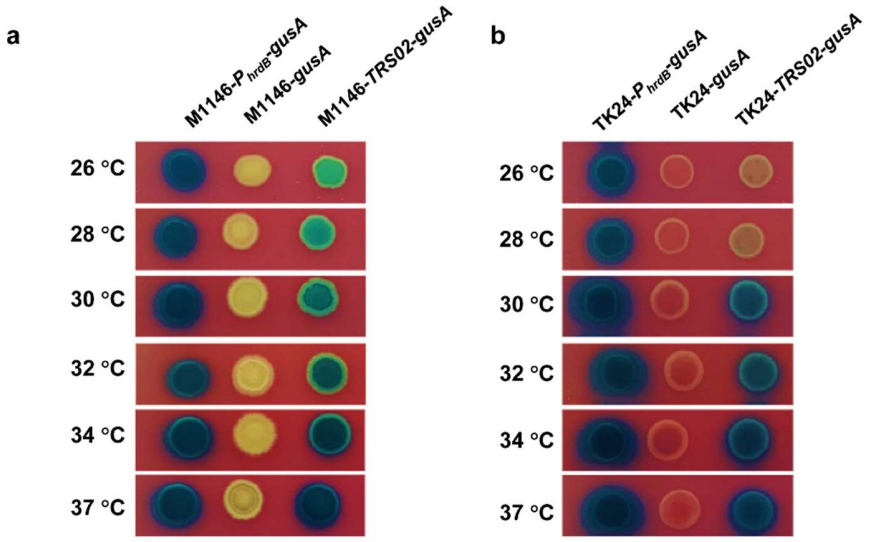

Fig. 4 GusA production in uninduced and induced states. The strains were cultivated on R2 agar plates at incubation temperature as indicated. The photograph was taken from the bottom of the plate after 5 days of cultivation. The representative image of three independent experiments with similar results was shown. J1074-P_{hrdB}-*gusA* was included to serve as a positive control, while M1146-*gusA* was used as a negative control.

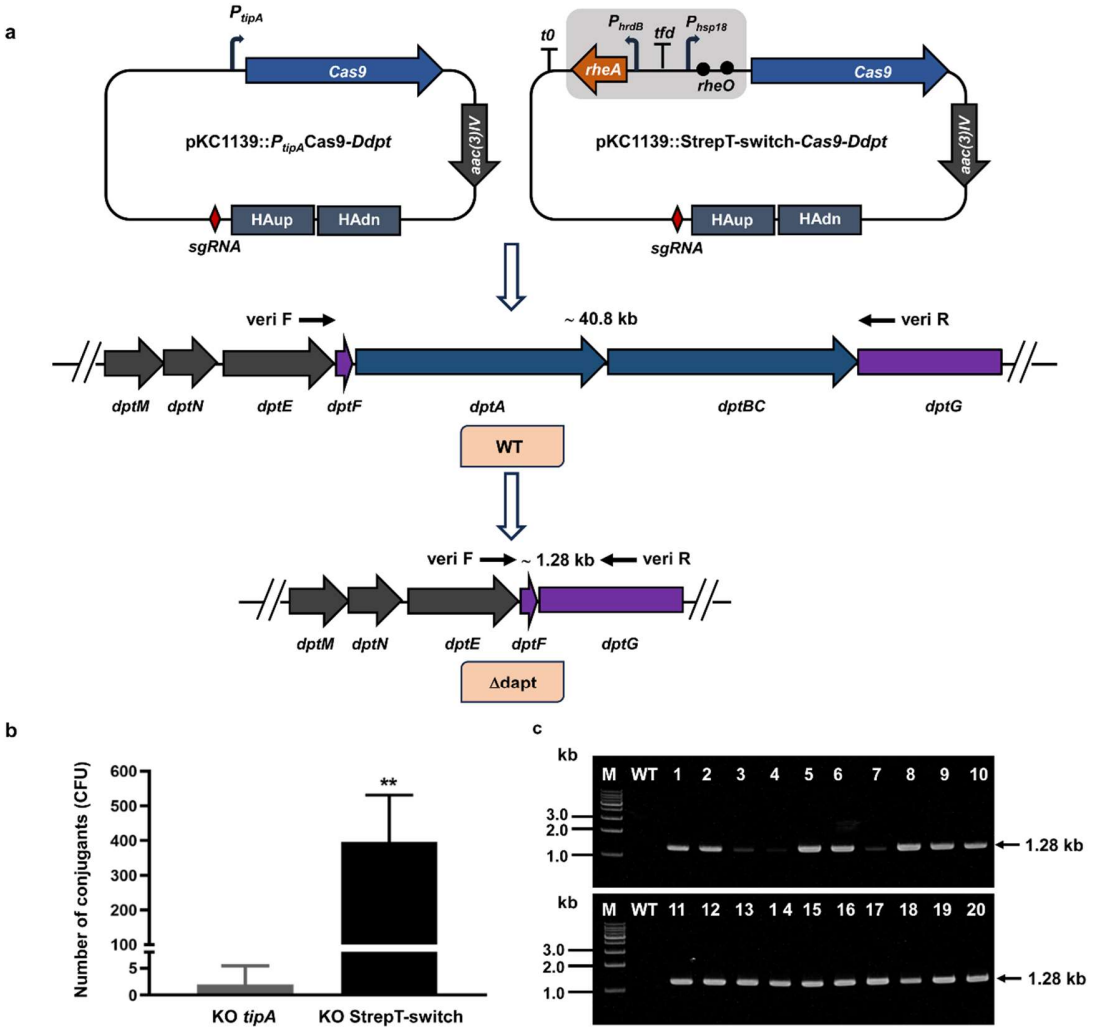

Fig. 5 Thermal inducible CRISPR/Cas9-mediated target knock-out. a CRISPR/Cas9-mediated knock-out of approximate 48 kb of the daptomycin gene cluster from the chromosome of *S. roseosporus*. The *tipA* promoter or StrepT-switch was used to drive the expression of Cas9. **b** Comparison of transformation efficiency between KO *tipA* and KO StrepT-switch. **c** Verification of transformants by PCR amplifications. The expected size of PCR amplicons was as indicated. Twenty transformants were randomly chosen for PCR amplifications.

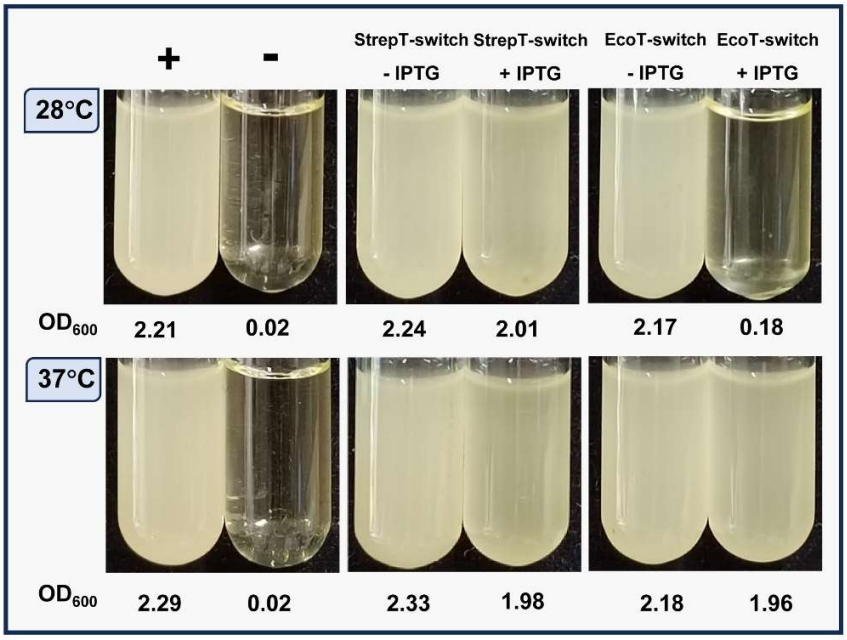

Fig. 6 Evaluation of the thermal bio-switch in *E. coli*. The StrepT-switch fails to switch off the expression of the *neo* cassette when the strain was cultivated at 28 °C. After codon optimization, the EcoT-switch is functional in *E. coli*. When cultivated at 28 °C, RheA repressor switched off expression of the *neo* cassette, and cells are unable to grow in the presence of kanamycin. When cultivated at 37 °C, the thermo-sensing repressor relieved its repression on the *neo* cassette, and cells grow normally in the presence of kanamycin.

Supplementary References

1. Sun, J., Kelemen, H. G., Fernández-Abalos, M. J. & Bibb, J. M. Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* **145**, 2221-2227 (1999).
2. Li, J. *et al*. NosP-regulated nosiheptide production responds to both peptidyl and small-molecule ligands derived from the precursor peptide. *Cell Chem. Biol.* **25**, 143-153 (2018).
3. Huang, H., Zheng, G., Jiang, W., Hu, H. & Lu, Y. One-step high-efficiency CRISPR/Cas9-mediated genome editing in *Streptomyces*. *Acta Biochim. Biophys. Sin.* **47**, 231-243 (2015).
4. Li, H., Gao, W., Cui, Y., Pan, Y. & Liu, G. Remarkable enhancement of bleomycin production through precise amplification of its biosynthetic gene cluster in *Streptomyces verticillus*. *Sci. China Life Sci.* **65**, 1248-1256 (2022).
5. Gust, B., Challis, L. G., Fowler, K., Kieser, T. & Chater, F. K. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl Acad. Sci. USA* **100**, 1541-1546 (2003).
6. Liu, M. et al. Comparative and functional analyses reveal conserved and variable regulatory systems that control lasalocid biosynthesis in different *Streptomyces* species. *Microbiol. Spectr.* **11**, e0385222 (2023).