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Multiplying the heterologous production of spinosad through tandem amplification of its biosynthetic gene cluster in *Streptomyces coelicolor*

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Summary

Heterologous expression of the biosynthetic gene cluster (BGC) is important for studying the microbial natural products (NPs), especially for those kept in silent or poorly expressed in their original strains. Here, we cloned the spinosad BGC through the Cas9-Assisted Targeting of Chromosome segments and amplified it to five copies through a ZouA-dependent DNA amplification system in *Streptomyces coelicolor* M1146. The resulting strain produced 1253.9 $\pm$ 78.2 μg l^{-1} of spinosad, which was about 224-fold compared with that of the parent strain carrying only one copy of the spinosad BGC. Moreover, we further increased spinosad to 1958.9 $\pm$ 73.5 μg l^{-1} by the dynamic regulation of intracellular triacylglycerol degradation. Our study indicates that tandem amplification of the targeted gene cluster is particularly suitable to enhance the heterologous production of valuable NPs with efficiency and simplicity.

Introduction

Microorganisms in a variety of habitats possess great potentials in mining natural products (NPs), and have been recognised as excellent sources for discovering and developing pharmaceutical drugs (Newman and Cragg, 2012; Blunt *et al*., 2015; Katz and Baltz, 2016). With stepping into the post-genomic era, microbial DNA sequencing has revealed a huge number of biosynthetic gene clusters (BGCs) for NP production (Luzhetskyy and Pelzer, 2007). However, most microbes are uncultivable or not amenable to genetic operability under the laboratory conditions (Rebets *et al*., 2014). To solve this problem, heterologous expression of the target BGCs in the genetically tractable host has been used (Yamanaka *et al*., 2014; Montiel *et al*., 2015; Huo *et al*., 2019). *Streptomyces* is the most important source of NPs and produces kinds of cellular intermediates of NPs with complex structures (Komatsu *et al*., 2013; Tan and Liu, 2017). Consequently, heterologous production of NPs is preferably performed in *Streptomyces* sp., especially in the strains with fully sequenced genome such as *Streptomyces coelicolor* (Jones *et al*., 2013; Zhang *et al*., 2013).

Many BGCs for NPs (Ripp, NRPS, PKS etc.) have been successfully expressed in *S. coelicolor* so far (Flinspach et al., 2010; Gomez-Escribano and Bibb, 2013; Ongley *et al*., 2013; Yamanaka *et al*., 2014). *S. coelicolor* M1146, which has been constructed by deleting four BGCs (*act*, *red*, *cpk* and *cda*) to remove the potential competition for acetyle-CoA, is considered as the super host for heterologous production of polyketides (Coze et al., 2013; Gomez-Escribano and Bibb, 2011, 2013; Nitta et al., 2021).

During fermentation process, *Streptomyces* undergoes a metabolic conversation from primary metabolism to secondary metabolism (Nieselt *et al*., 2010; Hwang *et al*., 2014). Since the intracellular triacylglycerol (TAG) metabolic process has been proven to direct carbon flux to polyketide biosynthesis at stationary phase, a strategy of TAG dynamic degradation (ddTAG) was used for mobilising the cellular TAG pool with selective control of time and strength to promote the precursor for the polyketide biosynthesis (Wang *et al*., 2019). Therefore, the ddTAG strategy used in *S. coelicolor* M1146 could further increase the polyketide production.

For heterologous production of NPs, direct cloning the target BGC is an imperative step. Thus, many kinds of methods have been developed such as RecE/T (Fu *et al*., 2012; Wang *et al*., 2016), TAR cloning (Larionov

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et al., 1996, 1997), PCR-based cloning and so on (Gibson *et al*., 2009; Li and Elledge, 2012). However, these techniques have limitations since they are dependent on the unique restriction sites flanking the targeted BGC. In 2015, a Cas9-Assisted Targeting of CHromosome segments (CATCH) technique was developed and used for one-step targeted cloning the near-arbitrary, large gene clusters up to 100 kb (Jiang *et al*., 2015; Jiang and Zhu, 2016). This technique facilitates BGC cloning and subsequent heterologous expression.

Since phylogenetic distance between the native producer and the heterologous host as well as the complex native regulation, the biosynthesis of NPs in heterologous host has extremely low efficiency or even no process (Martin *et al*., 2003; Komatsu *et al*., 2013; Galanie *et al*., 2015). This situation is detrimental to the heterologous expression of BGCs. Although the BGCs could be viewed as 'production lines' of NPs, it is usually hard to rationally and systematically identify the rate-limiting step in the biosynthetic pathway of NPs. A ZouA-dependent DNA amplification system (ZouA system) derived from *Streptomyces kanamyceticus* was applied for targeted amplifying the bacterial BGCs, and thus increasing the corresponding NP yield through multiplying the 'production lines' (Yanai and Murakami, 2006; Murakami *et al*., 2011; Zhou and Kim, 2014; Li *et al*., 2021). Consequently, this system could be used as a convenient strategy to enhance the heterologous production of NPs.

Spinosad, a novel insecticide with exceptional environmental safety, has been widely used in agriculture (Mertz and Yao, 1990; Sparks and Crouse, 2001). However, *Saccharopolyspora spinosa* ATCC49460 as the spinosad native producer is not amenable to genetic manipulation due to its genomic DNA with high-level methylation (Matsushima *et al*., 1994). Moreover, the spinosad biosynthesis including intramolecular cycloaddition, crossing-bridging, glycosylation and methylation is very complicated (Fig. S1). Of the biosynthetic genes, nineteen are clustered in a 74-kb region while the other four for the TDP-L-rhamnose biosynthesis are located in three different regions on the chromosome of *Sa. spinosa* (Madduri and Waldron, 2001; Waldron *et al*., 2001).

In this study, the heterologous production of spinosad in *S. coelicolor* was remarkably enhanced through amplifying its BGC by the ZouA system. With the ddTAG strategy, the heterologous production of spinosad in *S. coelicolor* was further increased to 1958.9 $\pm$ 73.5 μg l^{-1} (347-fold enhancement). This approach (Fig. 1), especially the ZouA system is likewise expected to become a generally tool for efficient heterologous production of the valuable NPs.

Result and discussion

Direct cloning the spinosad BGC on a plasmid carrying the ZouA system

To amplify the spinosad gene cluster under the control of the ZouA system, a plasmid pLHH001 carrying the elements including *zouA*-RsA, *kan*^{R} and RsB was constructed based on pSET152 (Kieser *et al*., 2000). Then, the TDP-L-rhamnose biosynthetic genes (*gtt*, *gdh*, *kre* and *epi*) with their native promoters were amplified respectively from *Sa. spinose* ATCC49460 and sequentially cloned into pLHH001 in the given order *gtt-gdh-kre-epi* (which was named as the TDP-L-rhamnose biosynthesis mini-cluster) to generate pLHH002 (Fig. 2). Two 500 bp DNA fragments from both sides of the spinosad BGC were amplified and each ended with 30 bp sequences overlapping with the target DNA. Both of them were cloned into pLHH002 to generate pLHH003, and a *Swa*I site was designed between these two fragments. Finally, the 74.6-kb spinosad BGC was directly cloned by CATCH and inserted into the *Swa*I site flanking the TDP-L-rhamnose biosynthesis mini-cluster in

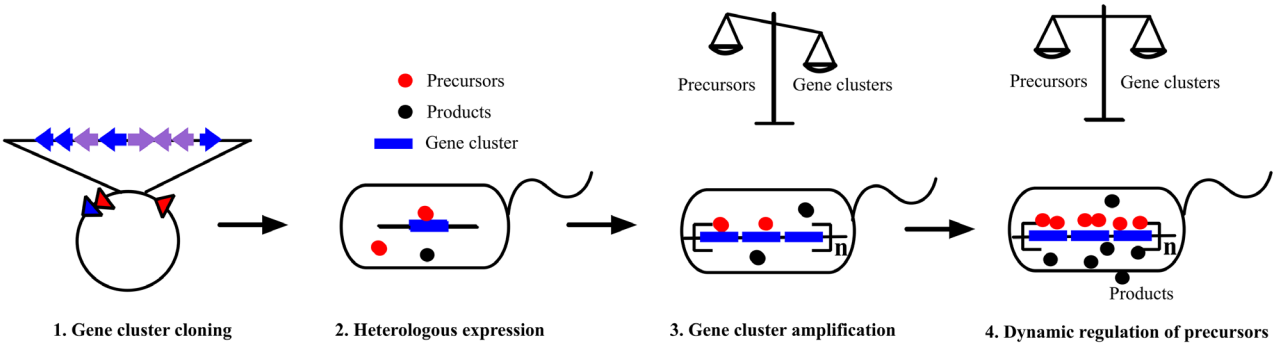

Fig. 1. The conceptual diagram of enhancing the heterologous production of spinosad through tandem amplification of the spinosad BGC and dynamic regulation of the precursors. The BGC of NPs was directly cloned into the plasmid carrying the ZouA system and then was transferred into the heterologous host. Subsequently, the recombinant strains with the amplified BGC were acquired by the ZouA system. Finally, engineering of the primary metabolism was performed to supply the precursors to adapt to the multiple BGCs in the heterologous host for the efficient heterologous production of the valuable NPs.

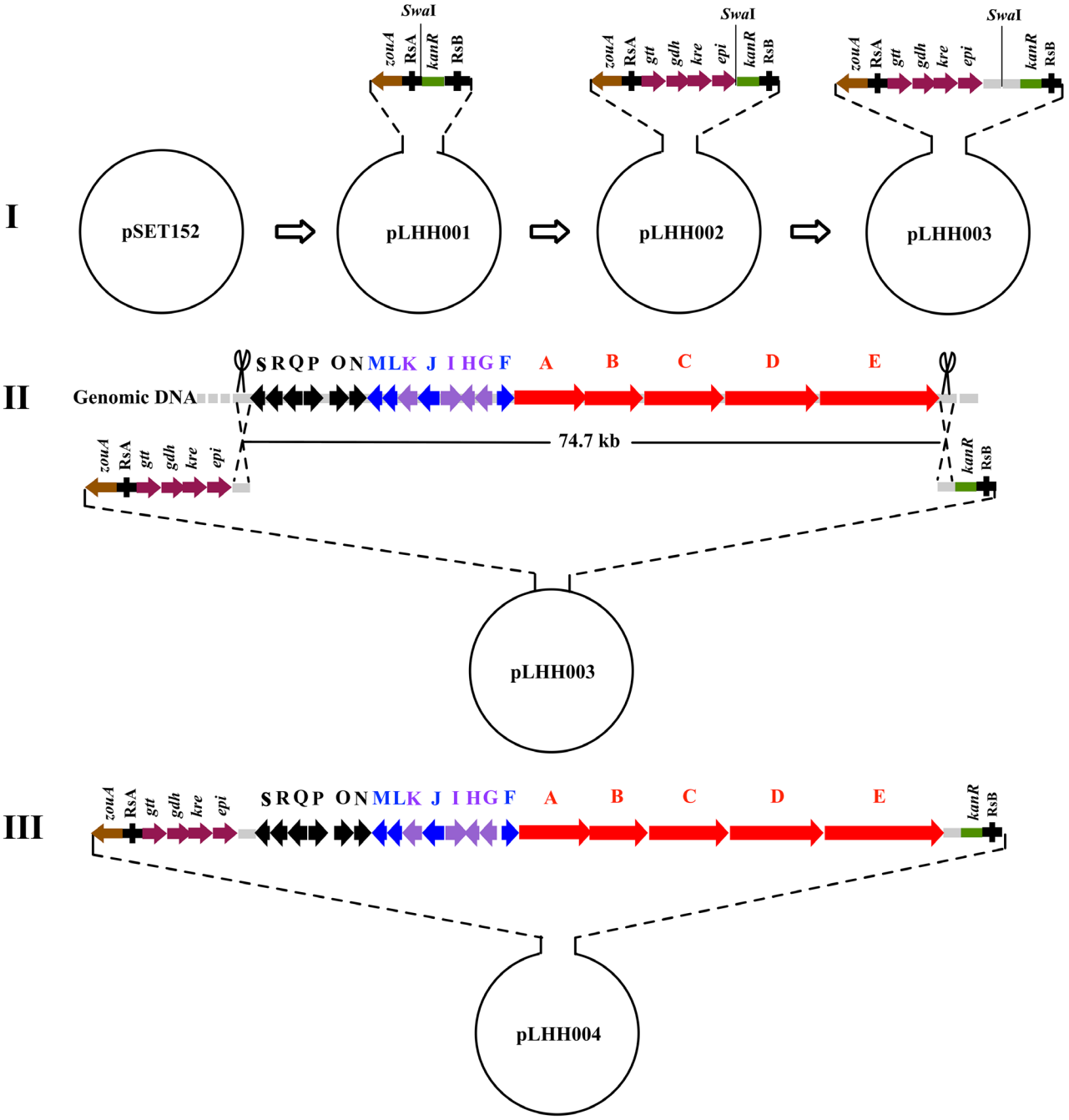

Fig. 2. Cloning and assembling the full set of spinosad biosynthetic genes in one cluster under the control of ZouA system. pLHH001, the plasmid carrying the ZouA system. pLHH002, the plasmid carrying the TDP-L-rhamnose biosynthetic genes under the control of the ZouA system. pLHH003, the plasmid containing the 30 bp overlapping sequence with the flanks of spinosad BGC was constructed based on pLHH002. pLHH004, the plasmid carrying the complete spinosad BGC under the control of the ZouA system.

pLHH003 to yield pLHH004 (Fig. 2). The colonies on the LB agar were selected and verified by amplifying the target genes flanking the spinosad BGC. After isolated, the recombinant plasmid pLHH004 was confirmed by restriction analysis (Fig. S2). The schematic diagram of plasmid construction is shown in Fig. 2.

Tandem amplification of the spinosad BGC in S. coelicolor

For heterologous production of spinosad, *S. coelicolor* M1146 was chosen as the host strain. Since it has been reported that the ddTAG strategy could increase

polyketide biosynthesis and *SCO6196* was responsible for degradation of TAG in *S. coelicolor* (Wang *et al.*, 2020), we transformed the plasmid pSET152-DT carrying the *SCO6169* gene under the control of a cumate-inducible promoter into *S. coelicolor* M1146 to yield the chassis strain M1146-DT. Then, pLHH004 carrying the complete spinosad BGC under the control of the ZouA system was transferred into M1146-DT to generate the recombinant strain IMSC001. M1146-DT and IMSC001 were verified by PCR respectively (Fig. S3).

To amplify the spinosad BGC through the ZouA system, the kanamycin selective enrichment was performed and subsequently the recombinant strain IMSC002, which carries multicopies of the spinosad BGCs was obtained through continuous passage. The schematic diagram of selective enrichment for generating the tandem repeats of spinosad BGC according to the mechanism of the ZouA system is shown in Fig. 3A. After kanamycin selective enrichment, the copy number of the spinosad BGC in IMSC001 and IMSC002 was determined by qPCR. Compared with IMSC001, which contains only one copy of the spinosad BGC, IMSC002 contains five copies of the spinosad BGCs (Fig. 3B).

Amplification of its BGC remarkably enhances the spinosad production in S. coelicolor

To test the spinosad production in *S. coelicolor*, fermentation of IMSC001 and IMSC002 was performed. The heterologous production of spinosad was analysed by high-resolution HPLC-MS. The production of spinosad was confirmed in the fermentation broth of IMSC001 and IMSC002 (Figs 4 and S4). As transcriptional level of the biosynthetic genes could indicate the biosynthetic level of spinosad, the transcriptions of all PKS genes (*spnA*–*spnE*), eight modification genes in the spinosad BGC and the TDP-L-rhamnose biosynthetic genes were analysed by RT-PCR after 8 days of fermentation (Fig. 5A and B). All detected genes were successfully transcribed in IMSC001 and IMSC002, indicating that the spinosad biosynthetic genes could be expressed in *S. coelicolor*. In consistence, IMSC001 produced a trace amount of spinosad consisting of only spinosyn D (5.6 ± 1.2 μg l^{-1}), while IMSC002 carrying five copies of the spinosad BGCs produced more spinosad consisting of spinosyn A and D up to 1253.9 ± 78.2 μg l^{-1} (Fig. 5C and D). Under the congruent PCR conditions, the intensity of RT-PCR products indicated the transcriptional level of corresponding genes. In this study, all detected genes in IMSC001 were transcribed at a lower level, especially for *spnC*, *spnE*, *spnH*, *spnI*, *spnM* and *gdh* (Fig. 5B), possibly resulting in the low spinosad production. The transcriptional level of the spinosad biosynthetic genes in IMSC002 was significantly enhanced and the spinosad production was increased by 224-fold compared with that of IMSC001 (Fig. 5B–D). These results demonstrated that tandem amplification of the spinosad BGC by the ZouA system remarkably enhanced the spinosad production in *S. coelicolor*.

Low or undetectable production of the target NPs in the heterologous host is generally resulted from inadequate expression of the BGC. To sufficiently produce the target NPs, improving the expression of target BGC is required. It has been reported that transcriptional factor was decoyed through amplifying the *cis*-acting element to release the repressors from the regulatory region and the silent BGC was activated (Wang *et al.*, 2019). Similarity, amplifying the spinosad BGC could de-repress the expression of the biosynthetic genes and thus significantly enhanced the spinosad production in IMSC002.

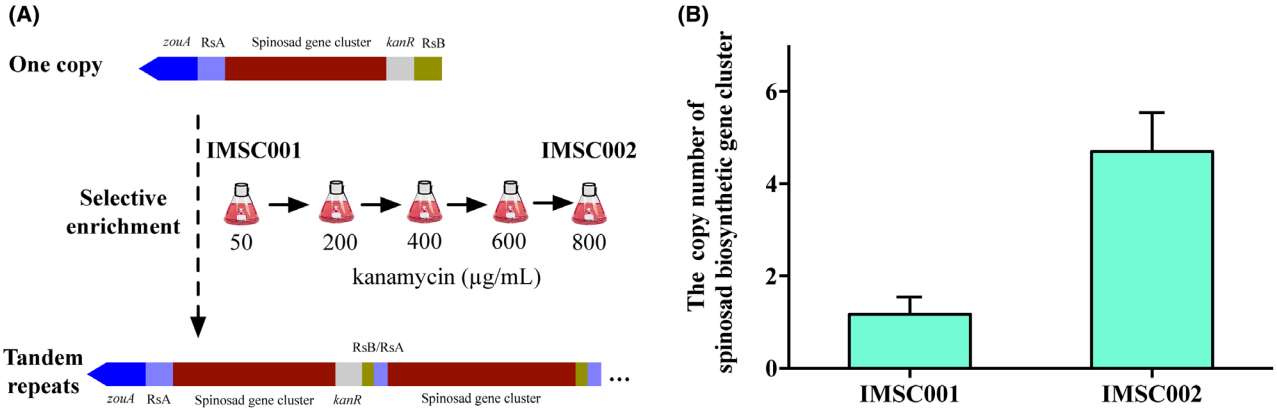

Fig. 3. Acquisition of the recombinant strain IMSC002 carrying the amplified spinosad BGC by selective enrichment.
A. The schematic diagram of acquisition of the IMSC002 strain carrying the amplified spinosad BGC through kanamycin selective enrichment and tandem amplification of the spinosad BGC by the ZouA system.
B. The copy number of spinosad BGC in IMSC002 was detected by qPCR. The primers used in qPCR were listed in Table S2.

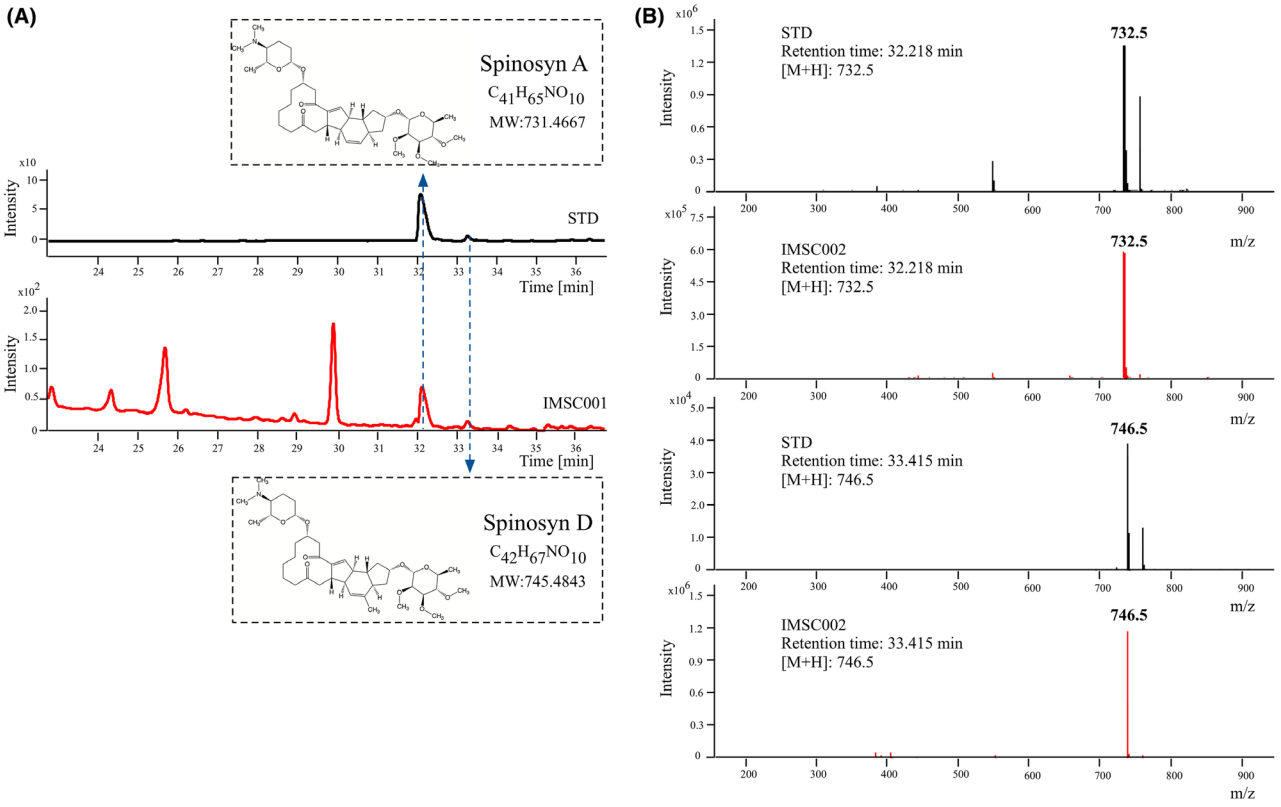

Fig. 4. Qualitative analysis of the spinosad production in IMSC002 by HPLC-MS.
A. HPLC-MS analysis of spinosyn A and spinosyn D in IMSC002. STD, the spinosad standard consisting of spinosyn A and spinosyn D.
B. The HPLC-MS analysis showed the characteristic fragment at *m/z* 732.5 $[M + H]^+$ (spinosyn A, top) and 746.5 $[M + H]^+$ (spinosyn D, down) in IMSC002 and the spinosad standard.

Although IMSC002 contains five copies of the BGCs, the transcriptional level of spinosad biosynthetic genes was not totally consistent with IMSC001, which contains single copy of spinosad BGC (Figs 3B and 5B). This result may be attributed to the complex regulation of spinosad biosynthesis and the variation of transcription of different genes in the spinosad BGC.

Generally, there are two methods for improving heterologous expression efficiency. One is to overexpress the genes for the rate-limiting steps based on analysis and identification of the rate-limiting steps of the NP biosynthesis pathway. This method is required for the complex analysis of multiomics and the rate-limiting steps in the biosynthetic pathway. Another is to replace the native promoters by the strong promoters and reconstruct the BGC. This method is time-consuming and labour-intensive. Compared with above two methods, enhancing the spinosad heterologous production with tandem amplification of the BGC is simplicity and efficiency. However, the amplification efficiency of the ZouA system is dependent on the selective enrichment. As we know, the resistance of the recombinant strain against the antibiotics is not completely dependent on the expression of the resistance genes. When the antibiotic is used for the selective enrichment, the copy number of the amplified cluster could not continue to rise with the increase of the antibiotic concentration. Moreover, the genetic stability of recombinant strains obtained by ZouA system is also heavily dependent on selection pressures, which generally maintained by the high concentration of the corresponding antibiotics. Since it is impractical to add high concentration of antibiotics during fermentation in industry, the ZouA system has not been widely used in strain improvement. For the industrial application, the antibiotic selection marker could be replaced by the nutrition selection marker in the recombinant strains.

Improvement of the spinosad production through dynamic regulation of TAG degradation in S. coelicolor

Our study demonstrated that the BGC could be viewed as the 'production line' of NPs. The spinosad production has been remarkably enhanced through amplifying the spinosad BGC to five copies by the ZouA system. Meanwhile, the amplified 'production lines' need appropriate amount of 'materials' to efficiently produce the target NP.

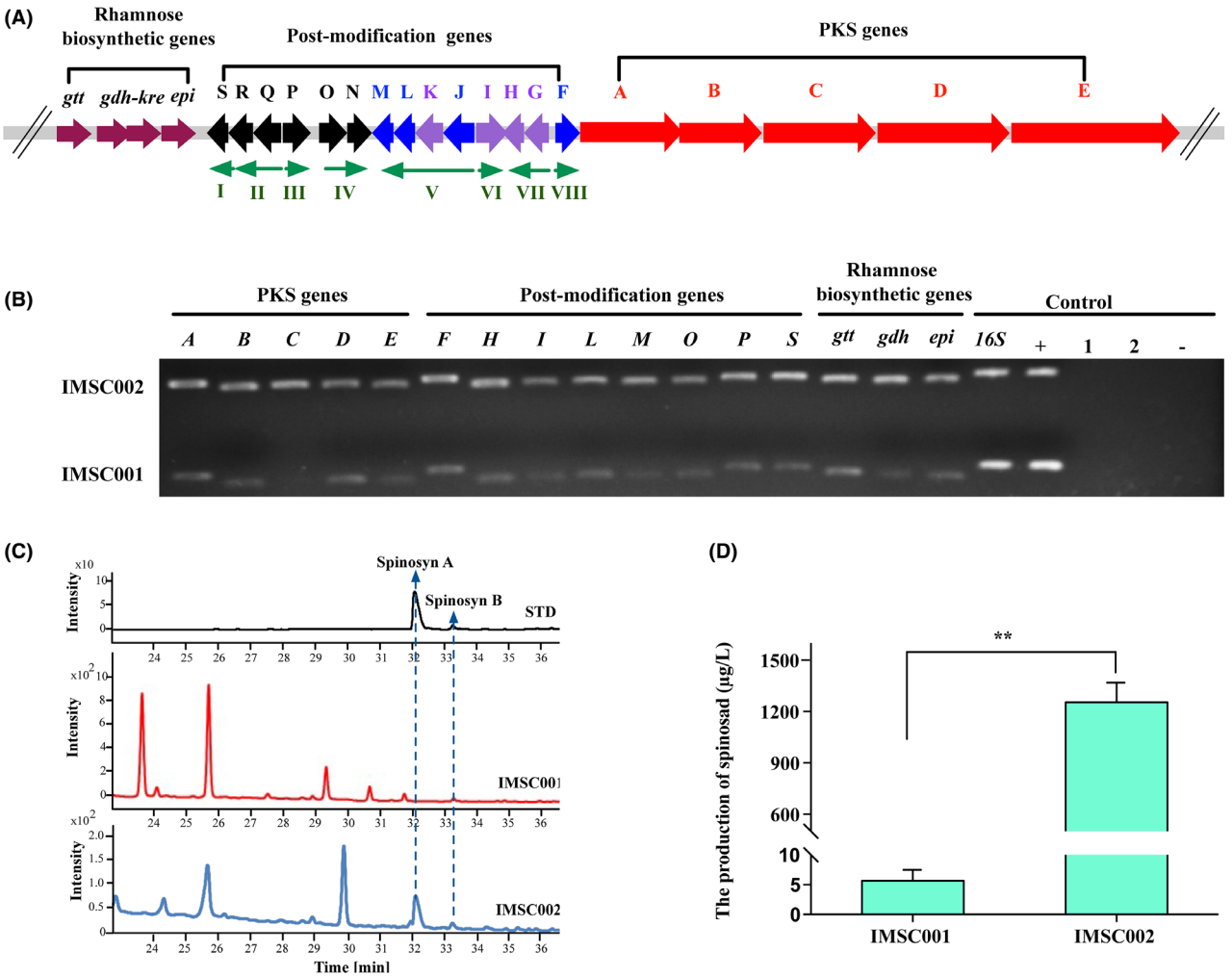

Fig. 5. The spinosad production and transcriptional analysis of its biosynthetic genes in IMSC001 and IMSC002.
A. The reconstructed spinosad BGC. The TDP-L-rhamnose biosynthetic genes are cloned and ligated into the spinosad BGC. Each green arrow indicates genes in the same operon.
B. Transcriptional analysis of the PKS genes, post-modification genes and TDP-L-rhamnose biosynthetic genes involved in the spinosad biosynthesis. These selected post-modification genes were located in different operons. The PKS genes *spnA*, *B*, *C*, *D* and *E* belong to the same operon. The cDNAs obtained by reverse transcription of RNA isolated from IMSC001 and IMSC002 were used as templates for PCR. As the negative control, the randomly selected *spnS* gene could not be detected by using the total RNA as template. The templates used in PCR reactions are the RNA samples from IMSC001 (1), IMSC002 (2), ddH_2O (−) and pLHH004 (+) respectively. The primer sequences are shown in Table S2.
C. The HPLC profiles of fermentation filtrates from IMSC001 and IMSC002. STD, HPLC profile of the spinosad standards.
D. The spinosad heterologous production in IMSC001 and IMSC002. $^{**}P < 0.01$.

It has been reported that the ddTAG strategy of mobilising the TAG pool with control of time and strength could supply appropriate precursors and increase the yield of polyketides such as actinorhodin, jadomycin B, oxytetracycline and avermectin significantly (Wang *et al.*, 2020). As spinosad is the polyketide compound, the ddTAG strategy could be used to regulate the supply of precursors in IMSC002 with selective control of time and strength. The ddTAG strategy and the brief illustration of mobilising TAGs to spinosad biosynthesis are illustrated in Fig. 6A. IMSC002 was chosen for fermentation to screen the optimal time and concentration of cumate which was used as inducer of the *SCO6169* expression (Fig. S5). In the optimal conditions (adding 50 μM of cumate in the fermentation culture at 96 h), the spinosad production was increased to 1958.9 ± 73.5 μg l^{-1} (Fig. 6B and C).

The production of NPs can be increased through overexpressing the rate-limiting biosynthetic genes. It has been reported that the heterologous production of spinosad was increased up to 1500 μg l^{-1} in *S. albus* 1074 after step by step overexpressing three rate-limiting biosynthetic genes (Tan *et al.*, 2017). Besides, the spinosad production in *S. albus* 1074 reached to 1100 μg l^{-1}

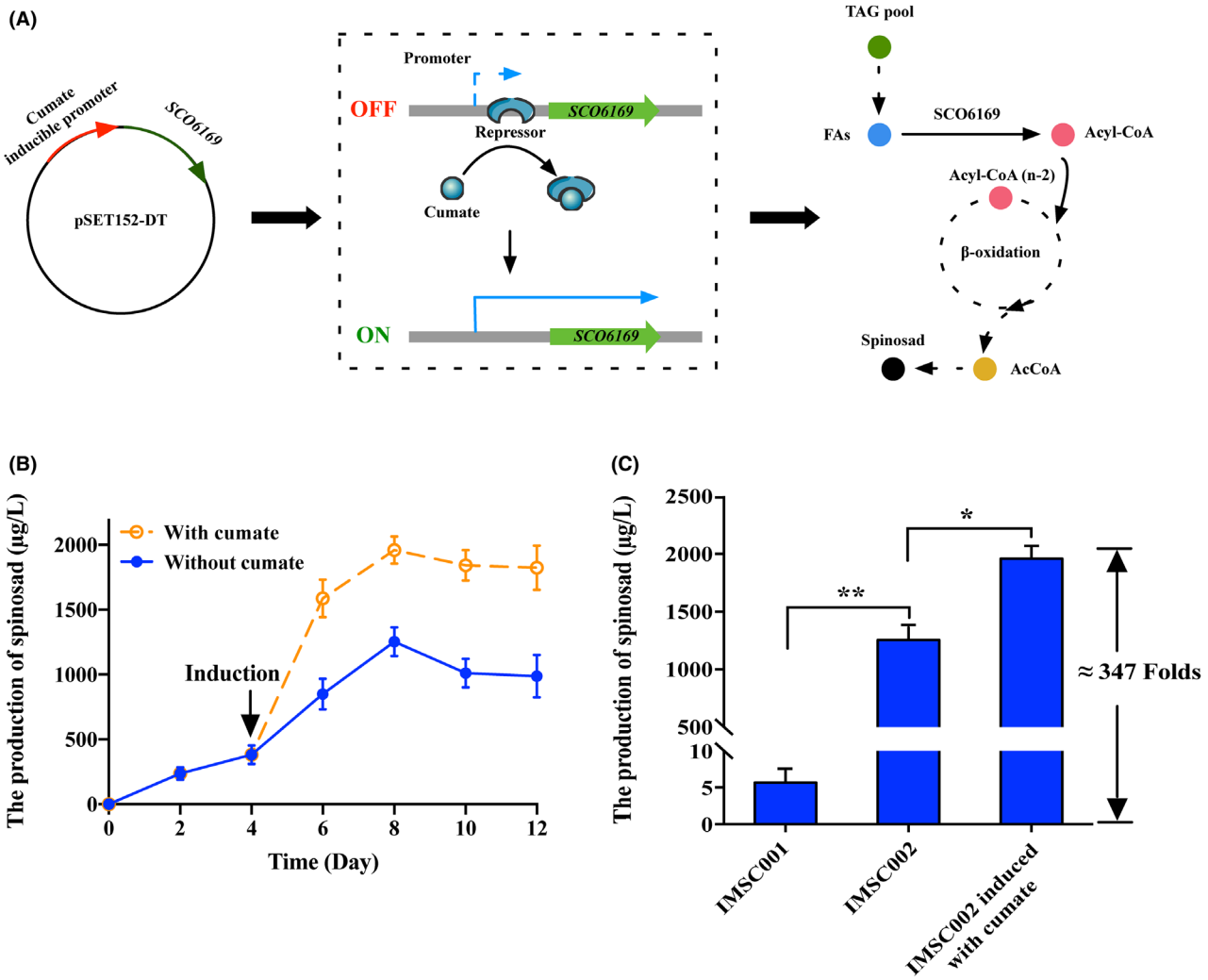

Fig. 6. The ddTAG strategy was used for improving the spinosad production in *S. coelicolor*.
A. Schematic of temporal control of the TAG mobilisation in *S. coelicolor* and brief illustration of the metabolic pathway from cellular TAG pool to spinosad.
B, C. Improving the spinosad production using the ddTAG strategy combined with the ZouA system. $^{**}P < 0.01$, $^{*}P < 0.5$.

when the whole spinosad gene cluster was reconstructed with the strong promoters (Song *et al*., 2019). However, both identification of the rate-limiting steps and reconstruction of the spinosad BGC are time consuming and tedious. In our study, tandem amplification of the BGC by the ZouA system in IMSC002 remarkably enhanced the spinosad production to 1253.9 ± 78.2 μg l^{-1}. Using the ddTAG strategy further enhanced its production up to 1958.9 ± 73.5 μg l^{-1}. Our study suggests that amplification of the BGC could be the simple and fast tool for heterologous production of the target NPs.

Concluding remarks

In summary, we applied a strategy of amplifying the spinosad BGC with the ZouA system in *S. coelicolor* and remarkably enhanced the heterologous production of spinosad. Based on our results, tandem amplification of the BGC by the ZouA system should be a simple and valuable tool for heterologous production of the target NPs in *Streptomyces*.

Experimental procedures

Bacteria, plasmids and culture conditions

The bacteria and plasmids used in this study are listed in Table S1. *Escherichia coli* and its derivatives were cultured at 37°C in the LB medium supplemented with 25 μg ml^{-1} of chloramphenicol, 100 μg ml^{-1} of kanamycin, 100 μg ml^{-1} of apramycin, or 100 μg ml^{-1} of ampicillin. *Streptomyces coelicolor*and its derivatives were cultured on the soybean flour-mannitol agar plates (2%

(W/V), soybean flour, 2% (W/V) mannitol and 2% (W/V) agar) for sporulation and conjugation or the tryptone soy broth (TSB) for *Streptomyces coelicolor* growth and the genomic DNA extraction.

Construction of the plasmid carrying the ZouA system

The genomic DNA was isolated from *Sa. spinose* ATCC49460 and *S. kanamyceticus* CGMCC 4.1441, respectively, by using the phenol-chloroform method (Pan *et al*., 2013). The integrative vector pSET152 was used for heterologous expression of the spinosad BGC in *Streptomyces* (Kieser *et al*., 2000). The elements of ZouA system including *zouA*-RsA and RsB were amplified by PCR using the genomic DNA of *S. kanamyceticus* CGMCC4.1441 as the template with the primers *zouA*-F/R and RsB-F/R respectively (Murakami *et al*., 2011). Then, a 963 bp fragment containing the kanamycin resistant gene (kan^R) from pUC119 was amplified with the primers kan^R-F/R. Subsequently, these fragments were inserted into pSET152 to generate the plasmid pLHH001 with a *Swa*I site (ATTTAAAT) upstream of kan^R by Gibson Assembly (Gibson *et al*., 2009). The schematic diagram of pLHH001 construction is shown in Fig. 2.

Construction of the plasmid carrying the spinosad BGC

The TDP-L-rhamnose biosynthetic genes *gtt*, *gdh-kre* and *epi* with their native promoters were amplified by PCR with the corresponding primers (Table S2). The amplified fragments were assembled in the specific *gtt-gdh-kre-epi* sequence (collinear with their biosynthetic sequence) and sequentially inserted into the *Swa*I site of pLHH001 to generate pLHH002 with the regenerative *Swa*I site (Fig. 2).

The Cas9-Assisted Targeting of CHromosome segments (CATCH) was used for one-step targeted cloning of the spinosad BGC according to previous reports (Jiang *et al*., 2015; Jiang and Zhu, 2016). Briefly, a pair of sgRNAs targeting *orfR1* and *orfR15* (flanking both sides of the spinosad BGC) were designed through searching for the PAM sequence 'NGG' near the target region. The templates of sgRNAs *in vitro* transcription were prepared by overlapping PCR with three primers including RX-sgP containing T7 promotor and target sequence, sgRNA-F and sgRNA-R containing crRNA-trcrRNA chimaera sequence of sgRNA (Table S2). The target sequences and the complete sequence of the sgRNA were shown in Fig. S2. The sgRNAs were transcribed *in vitro* by T7 RNA polymerase as referred in the instruction (New England Biolabs, Beijing). To obtain the recombinant Cas9, the gene encoding the *Streptococcus pyogenes* Cas9 fused to 6× His-tag at the C terminus was cloned into pET28a and expressed in *E. coli* Rossetta strain after cultured in LB medium at 28°C overnight and induced with 0.5 mM IPTG. The recombinant Cas9 was purified with Ni-NTA column. *Sa. spinose* mycelia were embedded in the agarose gel plugs at a maximal concentration (the agarose gel with extremely viscous). The well-washed gel plugs were stored in the 1× wash buffer at 4°C and used for digesting the genome DNA with the purified Cas9. Two well-washed plugs were transferred into Cas9 cleavage mixture which contained 0.1 mg ml^{-1} of Cas9 and 30 ng μl^{-1} of the corresponding sgRNA, and incubated at 37°C for 2 h. After incubation, the gel plugs were melted and digested with the β-agarase I (New England Biolabs, Beijing, China), and purified by ethanol. Finally,the purified DNA was stored at 4°C for ligation. To construct pLHH003, two 500 bp DNA fragments from both sides of the spinosad BGC were amplified and each ended with 30 bp sequences overlapping with the target DNA. These two fragments were cloned into pLHH002, and a *Swa*I site was designed between them. Then, pLHH003 was digested with *Swa*I, and 1 μl of the linearised pLHH003 and 4 μl of the above purified DNA containing the spinosad BGC were added into 5 μl of 2× Gibson Mix. The ligation was performed at 50°C for 1 h. After ligation, the mixture was transferred into *E. coli* S17-1 and the positive clones were screened with PCR and restriction analysis. The positive clone was named pLHH004. The schematic diagram of pLHH004 construction is shown in Fig. 2. In pLHH004, the kan^R as the marker gene in the ZouA system was used for IMSC001 verification. IMSC001 was continuously transmitted for five generations in the TSB medium containing progressively concentrations of kanamycin (50–800 μg ml^{-1}). The diagram of selective enrichment experiment is shown in Fig. 3.

Fermentation and analysis of spinosad

The conjugation was performed according to previous report (Kieser *et al*., 2000). *S. coelicolor* M1146 and its derivatives carrying pLHH004 were inoculated in 30 ml seed medium (TSB) of 250-ml flasks and cultured at 30°C with shaking (220 rpm) for 4 days; 500 μl of the seed culture was then transferred into 50 ml of the fermentation medium (40 g l^{-1} glucose, 10 g l^{-1} glycerol, 30 g l^{-1} soluble starch, 15 g l^{-1} soytone, 10 g l^{-1} beef extract, 6.5 g l^{-1} peptone, 0.5 g l^{-1} yeast extract, 1 g l^{-1} $MgSO_4$, 2 g l^{-1} NaCl, 2.4 g l^{-1} $CaCO_3$) in 250-ml flasks and incubated at 30°C with shaking (220 rpm) for 12 days as reported (Tan *et al*., 2017; Song *et al*., 2019). One millilitre of the adsorber resin Amberlite XAD-16 was added into the fermentation culture after 10 days fermentation (Song *et al*., 2019). Treatment of the fermentation samples were performed as previously reported for HPLC-MS analysis (Song *et al*., 2019). HypersilGold AQ, C18, 5 μm, 4.6 × 250 mm (Thermo

Scientific-CN, Pudong New Area, Shanghai, China) column was used and a calibrated curve was generated with the spinosad standard (CAS#131929-60-7; Shanghai yuanye Bio-technology, Songjiang District, Shanghai, China), analysis of the metabolites by HPLC-MS was performed as previously reported (Tan *et al*., 2017; Song *et al*., 2019).

RNA isolation and reverse transcription (RT)-PCR

Total RNA was extracted from IMSC001 and IMSC002 after 8 days of fermentation and RT-PCR were performed as previously reported (Tan *et al*., 2017). The primers used in RT-PCR were listed in Table S2.

Quantitative PCR

Absolutely quantifying the copy number of *metE* (GenBank accession no. AM397660.1) and *spnF* (GenBank accession no. AY007564.1) in the recombinant strains was performed by using Mastercycle® ep *realplex* equipment (Eppendorf, Germany) and SYBR® *Premix Ex Taq*™ II PCR kit (Takara, Dalian). As *spnF* in the spinosad BGC is located in the amplified region between the recombinant sites RsA and RsB while *metE* is a single copy gene, which is out of the region (Vockenhuber and Heueis, 2015), the ratio of *spnF* to *metE* based on the absolute quantification demonstrates the copy number of the spinosad BGC in the recombinant strains. The quantitative PCR was performed as described previously (Li *et al*., 2021).

Statistical analysis

All error bars displayed in this study are standard deviations based on the averages of three independent measurements. The data were analysed using the two-tailed Student's *t* test to demonstrate the differences in values.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

H. Li and G. Liu designed the experiments and conceived the study; H. Li, performed the experiments; H. Li, G. Liu and Y. Pan analysed data; H. Li and G. Liu wrote the manuscript. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The biosynthetic pathway of spinosad.

Fig. S2. Verification of the plasmid pLHH004 and the design of sgRNAs. (A) and (B) Amplification of the first gene *spnE* and the last gene *spnS* in the spinosad biosynthetic gene cluster by PCR. (C) Restriction analysis of pLHH004 by the *Hin*dIII digestion. (D) The plasmid map of pLHH004. The positive colonies are indicated with arrows. The PCR positive and restriction positive colonies (1, 4 and 7) were considered as the right recombinant strains. The primer sequences are listed in Table S2. (E) The sgRNAs were designed for targeting both sides of the spinosad biosynthetic gene cluster. The targeted sequences, cleavage sites and PAM sequences are highlighted.

Fig. S3. Verification of M1146-DT and IMSC001 by PCR. (A) Amplification of *aac(3)IV* from M1146-DT by PCR. (B) Amplification of *kanR* from IMSC001 by PCR. (C) Verification of the integrity of spinosad biosynthetic gene cluster in IMSC001 by PCR. Lanes 1-6 show the PCR products of *gtt, gdh-kre, epi, spnE, spnB and spnS* using the IMSC001 genomic DNA as template. The primers are listed in Table S2.

Fig. S4. Heterologous production of spinosad in IMSC001. (A) HPLC-MS analysis of the spinosad production in IMSC001. STD, the spinosad standard. (B) The MS fragmentation patterns of spinosyn A and spinosyn D produced in IMSC001. STD, the standard spinosyn A and spinosyn D are indicated. The fragment ion at *m*/*z* 732.5 and 746.5 are the characteristic spinosyn A and spinosyn D fragments respectively (Song *et al*., 2019).

Fig. S5. Determination of the optiomal conditions for the spinosad production in IMSC002-DT. (A) The optiomal time of induction with 30 μM cumate during spinosad production. (B) The optiomal dosage of cumate for inducing the spinosad production after 4 days of fermentation.

Table S1. Strains and plasmids used in this study.

Table S2. Primers used in this study.

