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RESEARCH ARTICLE

Development of a group II intron-based genetic manipulation tool for *Streptomyces*

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Abstract
The availability of an alternative and efficient genetic editing technology is critical for fundamental research and strain improvement engineering of *Streptomyces* species, which are prolific producers of complex secondary metabolites with significant pharmaceutical activities. The mobile group II introns are retrotransposons that employ activities of catalytic intron RNAs and intron-encoded reverse transcriptase to precisely insert into DNA target sites through a mechanism known as retrohoming. We here developed a group II intron-based gene editing tool to achieve precise chromosomal gene insertion in *Streptomyces*. Moreover, by repressing the potential competition of RecA-dependent homologous recombination, we enhanced site-specific insertion efficiency of this tool to 2.38%. Subsequently, we demonstrated the application of this tool by screening and characterizing the secondary metabolite biosynthetic gene cluster (BGC) responsible for synthesizing the red pigment in *Streptomyces roseosporus*. Accompanied with identifying and inactivating this BGC, we observed that the impair of this cluster promoted cell growth and daptomycin production. Additionally, we applied this tool to activate silent jadomycin BGC in *Streptomyces venezuelae*. Overall, this work demonstrates the potential of this method as an alternative tool for genetic engineering and cryptic natural product mining in *Streptomyces* species.

INTRODUCTION

Streptomyces play a pivotal role in pharmaceutical industry due to their remarkable capacity to produce various secondary metabolites, including antibiotics, anticancer agents, immunosuppressants and so on (Barka et al., 2016; Hutchings et al., 2019; Liu et al., 2018). Genome mining (Blin et al., 2021) and genome sequencing analysis have unveiled numerous uncharacterized secondary metabolite biosynthetic gene clusters (BGCs) in *Streptomyces*, highlighting its immense potential for discovery and production of novel compounds (Baltz, 2017; Palazzotto et al., 2019; Sharma et al., 2021). Due to the high GC content of the genomes, the complex morphology, the slow growth rate, and the fastidious growth requirements, it is widely recognized that genetic manipulation poses a significant challenge in studying *Streptomyces* species (Lee et al., 2009). Therefore, it is imperative to develop convenient and efficient tools that facilitate fundamental and engineering research on these bioactive compounds encompassing their discovery, characterization, and titre improvement in *Streptomyces* strains (Baltz, 2016; Lee et al., 2019; Weber et al., 2015). However, traditional genetic manipulation strategies for *Streptomyces*, such as gene knock-out and knock-in, highly rely on plasmids-mediated homologous recombination, necessitating a laborious and time-consuming screening process. Several genome editing technologies have been developed for *Streptomyces* with a notable focus

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on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) system-based toolkits. These toolkits have demonstrated high efficiency in transcriptional regulation as well as genome editing capabilities (Alberti & Corre, 2019; Tao et al., 2018). Nevertheless, a common challenge arises from the toxicity exhibited by endonuclease proteins of CRISPR systems towards certain species of *Streptomyces*, thus leading to lower transformation and editing efficiency (Zhao et al., 2020). Henceforth expanding the repertoire of genetic manipulation tools is crucial to meet diverse experimental or host requirements, thereby unequivocally fueling exploration into the vast potential of *Streptomyces*.

Group II introns, consisting of a catalytically active intron RNA and an intron-encoded protein (IEP), offer promising prospects for genetic manipulation due to their ability to be stably inserted into any desired DNA target with high frequency and specificity (Lambowitz & Zimmerly, 2011). The IEP is a multifunctional reverse transcriptase that initially binds to excised intron RNA, forming a stable ribonucleoprotein (RNP). The RNP recognizes DNA target sites by base-pairing interactions between two loops of the intron RNA (the exon binding sites EBS1, EBS2, and δ site) and the target DNA. Afterward, the intron RNA cleaves and inserts into one strand of the DNA target site by a reverse splicing reaction, while the opposite strand is cleaved by the endonuclease activity of the IEP, leaving the 3′ end as a primer for reverse transcription of the inserted intron RNA. Finally, the intron RNA sequence is integrated into the DNA target site by the cellular recombination and repair mechanism (Mohr et al., 2000). The mobility process is termed “retrohoming”. The Ll.LtrB intron from *Lactococcus lactis* (Mills et al., 1996) represents one of the extensively investigated group II introns, and has been harnessed as a commercial gene knockout tool called TargeTron (Perutka et al., 2004). This tool has successfully facilitated gene disruptions in both Gram-negative and Gram-positive bacteria, such as *Escherichia coli* (Karberg et al., 2001; Yao & Lambowitz, 2007), *Clostridium acetobutylicum* (Shao et al., 2007), and *Staphylococcus aureus* (Yao et al., 2006), indicating the broad-host-range capability of prokaryotic group II introns. Moreover, this group II intron-based gene editing tool functions independently of RecA-mediated homologous recombination mechanisms, providing a considerable advantage over other techniques relying on homology-directed repair processes (Cousineau et al., 1998; Velazquez et al., 2019). Despite these advantages, such group II intron-based tools have not yet been applied in filamentous *Streptomyces* species, which are the largest genus of actinobacteria with over 900 described species and show great pharmaceutical and economic importance.

In this study, we presented a gene editing tool based on the group II intron Ll.LtrB for efficient genetic manipulation in *Streptomyces*. After improving the retrohoming efficiency of group II intron by repressing RecA-dependent homologous recombination, we utilized this tool to screen the secondary metabolite BGC responsible for red pigment synthesis in *S. roseosporus*, and further discovered that the biosynthesis of red pigment inhibited cell growth and daptomycin production. Additionally, through employing an intron carrying the constitutive promoter *ermE*p*, we readily activated the cryptic jadomycin BGC in *S. venezuelae*. This intron-based tool, as well as the derived screening strategy developed herein, offers a convenient approach to generate diverse mutants for desired phenotypes screening purposes, and activate targeted BGC to trigger the production of cryptic metabolites in *Streptomyces* species.

EXPERIMENTAL PROCEDURES

Strains, plasmids, primers, and culture conditions

All strains and plasmids used in this study are listed in Table S1, and primers are listed in Table S2. Culture conditions of *S. roseosporus* wild-type (WT) strain NRRL11379, *S. coelicolor* M145, *S. venezuelae* ISP5230 and their derivatives used for sporulation, mycelial growth, protoplast regeneration, and antibiotic production were employed as described previously (Li, Wang, et al., 2018; Liu et al., 2015; Zhang et al., 2015). Solid DA1 (Zhang et al., 2015) and RM14 (Kieser et al., 2000) were used for *S. roseosporus* phenotype observation. Solid MS (Kieser et al., 2000) and RM14 were used for *S. coelicolor* phenotype observation. Seed medium and fermentation medium A/B were used for daptomycin production. Seed medium A consisted of 2% Trypticase soy broth and 10 glass beads (Wang et al., 2014). Fermentation medium A contained 4% glucose, 0.5% tryptone, 0.3% yeast extract, and 0.3% malt extract (Wang et al., 2014). The primary and secondary seed medium B consisted of 3% Trypticase soy broth and 2.5% dextrin (Zhang et al., 2015). Fermentation medium B contained 1.1% yeast extract, 0.086% $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 1.07% glucose, 7.2% dextrin, and 0.72% cane molasses (Zhang et al., 2015).

E. coli strains JM109 and ET12567 were cultured in LB medium for, respectively, DNA cloning, and propagation of nonmethylated plasmids for transformation into *Streptomyces* strains.

Construction of the group II intron-based gene editing plasmids

Group II intron-based gene editing plasmids were constructed using the T5 exonuclease DNA assembly (TEDA) method (Xia et al., 2019). Using the plasmid pKCCpf1 (Li, Wei, et al., 2018) as the template, the strong constitutive

promoters *kasO*p* and *ermE*p* were obtained by PCR amplification with primers *kasO*p*-fw/rev and *ermE*p*-fw/rev. The two DNA fragments were inserted into *Xba*I-digested pKC1139 (Bierman et al., 1992) by the TEDA protocol to generate pKC1139ke. The *ltrA* gene, derived from *L. lactis subsp. Lactis* ML3, was codon optimized based on the bias of *S. coelicolor* M145 (the optimized sequence, named "*ltrAsco*," is available in Table S3) and was synthesized by Beijing Tsingke Biotechnology Co. Then, the *ltrAsco* gene was inserted into *Spe*I-digested pKC1139ke by the TEDA protocol to generate pKC1139ke-*ltrAsco*. Using the plasmid pSY6 (Shao et al., 2007) as the template, the intron Ll.LtrB was obtained by PCR amplification with primers intron-fw/rev. The DNA fragment was inserted into *Xba*I-digested pKC1139ke-*ltrAsco* by TEDA protocol to generate pZW28. On the basis of pZW28, the thiostrepton-inducible promoter *tipAp* was synthesized to replace *ermE*p* for expression of *ltrAsco*, generating plasmid pZW30.

Using the genomic DNA of *S. coelicolor* M145 as the template, the gene *recXsco* was obtained by PCR with primers *recXsco*-fw/rev. The strong promoter *SF14p* was synthesized by Beijing Tsingke Biotechnology Co. The two DNA fragments were inserted into *Eco*RI-digested pZW30 by the TEDA protocol to generate pSC30. On the basis of pSC30, the promoter *ermE*p* was amplified by PCR with primers Intron-*ermE*p*-fw/rev and inserted into the *Mlu*I site of pSC30 to generate pSC30E.

Group II intron-based gene disruption in *Streptomyces*

To target specific genes in *Streptomyces*, the ClosTron platform (www.clostron.com) was employed to identify potential Ll.LtrB intron insertion sites and design primers (IBS, EBS1d, EBS2, EBS universal) for modifying the intron RNA base pair optimally to those sites. Using pZW28, pZW30, pSC30, or pSC30E as the template, the DNA fragments 1 and 2 were obtained by PCR with primers IBS/EBS universal and EBS2/EBS1d. Using the resulting two PCR products as the template, the DNA fragment F was obtained by PCR with primers intron-fw/intron-universal. Then, the fragment F was inserted into *Nde*I/*Bsr*GI-digested pZW28, pZW30, pSC30 or pSC30E by the TEDA protocol to generate the mutagenesis plasmid which was introduced into *Streptomyces* by protoplast transformation.

Analysis of Ll.LtrB insertion by colony PCR

Ll.LtrB intron integration in the target site was detected by colony PCR, using primers flanking the insertion site in the target gene. The resulting amplicon was resolved on 1% w/v agarose gel at 150 V, and consequently, the DNA band was visualized through gel electrophoresis. The size of the DNA band from mutant strains was larger than that of the DNA band from the WT strain amplicon. Intron insertions were verified by DNA sequencing.

Phylogenetic analysis

The sequences used in this work were obtained from GenBank. The Neighbour-Joining method was adopted to construct the phylogenetic tree by MEGA software version 11 (Tamura et al., 2021). The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992). Tree files were viewed and embellished using online tool Interactive Tree Of Life (iTOL) v4 (Letunic & Bork, 2019).

Curing of the group II intron-based gene editing plasmid

The mutant strains were grown on solid medium MS or DA1 without apramycin at 37°C for two rounds of passage to remove the group II intron-based gene editing plasmids. The strains that had lost the donor plasmid were verified by colony PCR with primers *ltrAsco*-fw/rev.

Prediction of secondary metabolite BGCs

Prediction of secondary metabolite BGCs in *S. roseosporus* WT strain was performed using antiSMASH 6.0 (Blin et al., 2021). These BGCs are listed in Table S4.

Construction of the secondary metabolite BGCs mutant library

A total of 18 plasmids targeting core structural genes (listed in Table S5) of predicted secondary metabolite BGCs in *S. roseosporus* were individually constructed. Purified pooled plasmids were transformed into *S. roseosporus* WT strain. The resulting transformants were picked for DNA sequencing analysis of the transformed plasmids. Verification of the genome editing was conducted via PCR and sequencing analysis.

Complementation of *SSIG_RS15030* in SSIG_RS15030D

For complementation of SSIG_RS15030D, a 1293-bp DNA fragment carrying *SSIG_RS15030* coding region was amplified with primers CSSIG_RS15030-fw/rev. The promoter *ermE*p* was amplified with primers 152-*ermE*p*-fw/rev. The two PCR products were cloned into *Bam*HI site of integrative vector pSET152 (Bierman

et al., 1992) to create SSIG_RS15030D-complemented vector pCSSIG_RS15030, which was then introduced into SSIG_RS15030D to construct complemented strain CSSIG_RS15030.

Fermentation of *S. roseosporus* and HPLC analysis of daptomycin

S. roseosporus strains were cultured on DA1 solid medium for 7–10 days. For the fermentation process in which WT strain was capable of producing the red pigment, spores were collected and inoculated into flasks with 50 mL of seed medium A and shaken at 250 rpm, 28°C for 30 h. 2.5 mL of seed culture was inoculated into 50 mL of fermentation medium A and shaken at 250 rpm, 28°C for 10 days. After 36 h, sodium decanoate was added every 12 h at a final concentration of 0.2 g/L. For the fermentation process in which WT strain was not capable of producing red pigments, spores were collected and inoculated into flasks with 50 mL of primary seed medium B and shaken at 250 rpm, 28°C for 60 h. 2.5 mL of primary seed culture was inoculated into 50 mL of secondary seed medium B and shaken at 250 rpm, 28°C for 36 h. Then, 2.5 mL of secondary seed culture was inoculated into 50 mL of fermentation medium B and shaken at 250 rpm, 28°C for 10 days. After 48 h, sodium decanoate was added every 12 h at a final concentration of 0.2 g/L.

The fermentation cultures were collected and centrifuged. The supernatants were filtered through a Millipore membrane for high-performance liquid chromatography (HPLC) analysis (Shimadzu LC-20, Japan) on a reverse-phase column (ZORBAX SB-C18, 5 μm, 4.6 × 250 mm, Agilent, USA) at a flow rate of 1 mL/min. Solution A (H_2O containing 0.1% trifluoroacetic acid) and solution B (acetonitrile containing 0.1% trifluoroacetic acid) (A:B = 60:40, vol/vol) were used to separate daptomycin with UV detection set at 218 nm. Pure daptomycin (Beijing psaitong Biotechnology Co.) was used as a standard.

HPLC analysis of the red pigment

The HPLC conditions for analysis of the red pigment were employed as described above with some modifications. The percentage of solvent B changed linearly from 40% to 75% between 0 and 10 min, from 75 to 100% between 10 and 30 min, remained at 100% for 5 min, and then decreased to 40% between 35 and 40 min. The fermentation solution samples were recorded by full scanning in the wavelength range of 200–800 nm.

RNA preparation and qRT-PCR analysis

For RNA extraction, *S. roseosporus* strains were cultured in liquid fermentation medium. Samples were collected at various time points. Total RNAs were extracted with a RNAprep pure bacteria kit (TIANGEN Biotechnology Co.). Reverse transcription of total RNA and subsequent qRT-PCR analysis using the corresponding primers listed in Table S2 were performed as described previously (Zhang et al., 2015). Transcription values of test genes were normalized relative to internal control gene *hrdB* using the comparative Ct method. The WT value at the first time point for each gene was defined as 1. Experiments were performed in triplicate.

Fermentation of *S. venezaelue* and HPLC analysis of jadomycin B

The medium and culture conditions for jadomycin B production were employed as described previously (Doull et al., 1994). The extraction and HPLC analysis of jadomycin B were performed as described previously (Zheng et al., 2007).

RESULTS

Characterization of group II intron for gene disruption in *Streptomyces*

To develop a genome editing tool based on group II introns in *Streptomyces*, we initially constructed a plasmid harbouring the heterologous group II intron Ll.LtrB from *Lactococcus lactis* (Figure 1A). Due to the high GC content of *Streptomyces* genomes, we optimized the codon of gene *ltrA* (which encodes the intron-encoded protein in Ll.LtrB) based on the codon

FIGURE 1 Test of group II intron-based genetic manipulation tool for targeted gene inactivation in *Streptomyces*. (A) Group II intron-based gene editing plasmid. Plasmid pZW28 was a *pSG5* replicon-based, temperature-sensitive *E. coli*–*Streptomyces* shuttle plasmid, in which the Ll.ltrB intron system was codon optimized for *S. coelicolor*. (B) Secondary structure model of Ll.ltrB intron RNA. The EBS1, EBS2, and δ sites in red region could be modified to base pair with DNA target site. The *ltrA* gene (for intron-encoded protein) in domain IV was removed and expressed separately. The domain IV is the best location to insert heterologous sequences with minimal effect on intron mobility. (C) Insertion site 1017|1018a within *SCO5087* gene by group II intron. The exon binding sites EBS1, EBS2, and δ of the intron RNA were designed to base pair with corresponding complementary sequences of *SCO5087* gene. 'a' represented the antisense strand. The triangle represented the intron-insertion site. Critical bases recognized by LtrA were underlined. (D) Confirmation of *SCO5087* gene disruption in the genome of *S. coelicolor* by colony PCR with primers *SCO5087*-fw/rev. WT represented *SCO5087* gene in the genome of M145; Mut represented disrupted *SCO5087* gene in the genome of SCO5087D. (E) Phenotypes of WT strain and SCO5087D. The strains were grown on MS or RM14 plates at 28°C for 8 days.

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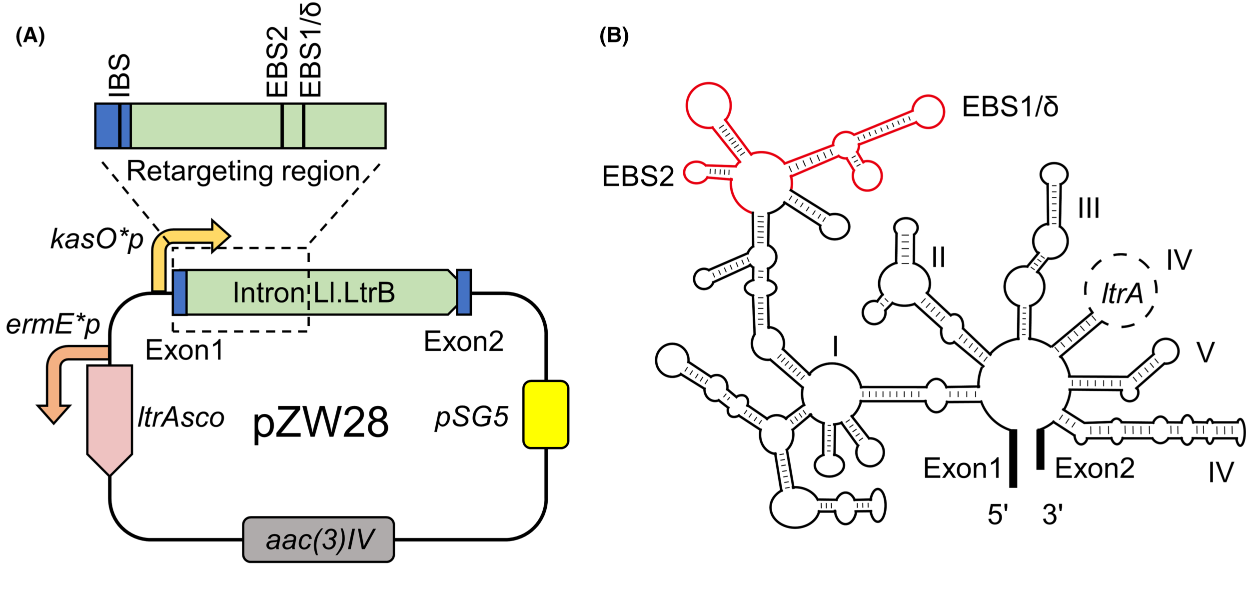
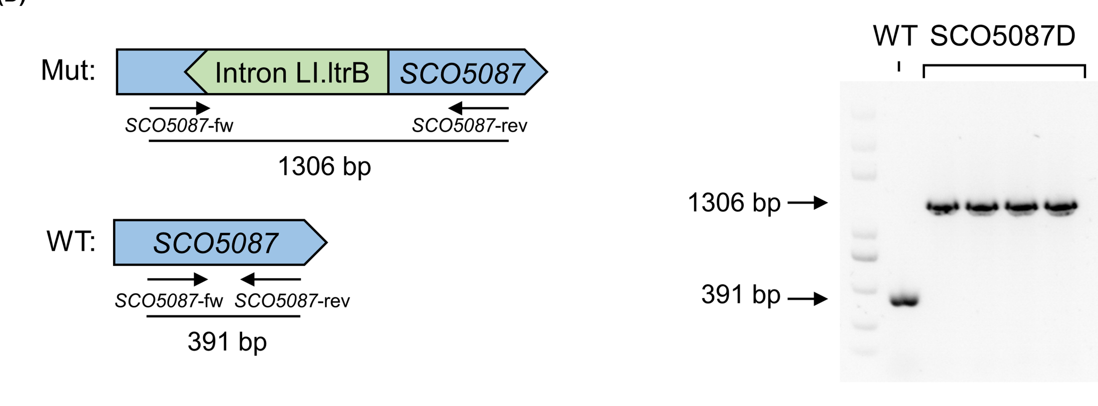
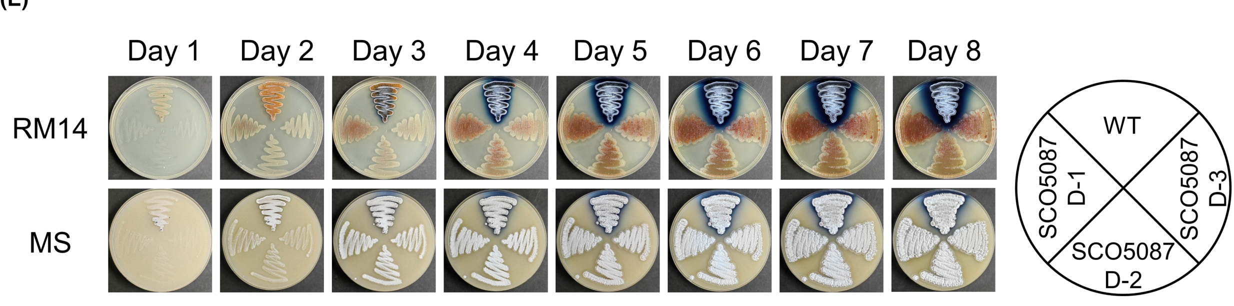






bias of *S. coelicolor*. Subsequently, we cloned the optimized *ltrA* (named *ltrAsco*) and its corresponding group II intron into the *E. coli-Streptomyces* shuttle vector pKC1139 to generate pZW28 (Figure 1A). In this plasmid, the expressions of *ltrAsco* and intron LI.LtrB were driven by strong constitutive promoters

*ermE*p* (Bibb et al., 1985) and *kasO*p* (stronger than *ermE*p*) (Wang et al., 2013), respectively. The recognition of DNA target sites by this group II intron largely depends on the base-pairing interactions between the intron RNA sequences and DNA target sequences (Figure 1B,C). Therefore, we could modify the target-site recognition sequences of the intron RNA (namely, the exon binding sites EBS1, EBS2, and δ site as shown in Figure 1C) to enable base pairing with desired retargeted DNA sites, and thus facilitate specific insertion of this intron into target sequences. To accelerate iterative targeted gene inactivation, we employed a temperature-sensitive replicon *pSG5* for convenient plasmid curing when cultivating edited strains at 37°C (Kieser et al., 2000).

To assess the functionality of our construct in *Streptomyces* species, we selected gene *SCO5087* as the target, which encodes beta-ketoacyl synthase from the biosynthetic pathway of actinorhodin (ACT), a blue-coloured antibiotic produced by the model strain *S. coelicolor* M145 (Tong et al., 2015). The insertion site for gene *SCO5087* was identified using the ClosTron online software (Heap et al., 2010), and site 1017|1018a received the highest score (score = 9.812) (Figure 1C). By employing specific primers provided by the software, we performed overlap PCR to amplify a DNA fragment containing modified EBS1, EBS2, and δ sequences that were designed to be complementary to site 1017|1018a in gene *SCO5087*. Subsequently, the fragment was cloned into plasmid pZW28 to replace the corresponding sequences to generate the plasmid specifically retargeting site 1017|1018a in gene *SCO5087*. The resulting plasmid pZW28-*SCO5087* was introduced into *S. coelicolor* by protoplast transformation.

Fifty initial transformant colonies were randomly selected for PCR screening using insertion-specific primers. We observed a lack of positive transformant with disrupted gene *SCO5087*, while plasmid pZW28-*SCO5087* was still present in all transformants. This result suggested that the retrohoming process may not have sufficient time to occur. To increase the likelihood of group II intron retrohoming, we passaged the primary transformants once on the MS agar plate prior to screening. Encouragingly, we successfully obtained mutant SCO5087D. As illustrated in Figure 1D, the DNA bands amplified with primers *SCO5087*-fw/rev from mutant strains were 915 bp larger than those from the WT strain, indicating that the intron had inserted into gene *SCO5087*. The insertion mutation at target site 1017|1018a of *SCO5087* was further confirmed through DNA sequencing. As expected, visual phenotype on RM14 and MS agar plates showed that SCO5087D lost its ability to biosynthesize blue pigment ACT (Figure 1E). Concurrently, we employed the tool to disrupt gene *SCO5087* by selecting a distinct insertion site at 975|976a (score = 9.182). We readily obtained the mutant SCO5087D2 (Figure S1A–C). These results suggested that the group II intron-based genome editing tool holds promise to be further developed as an alternative tool in *Streptomyces*.

Efficiency improvement of group II intron-based gene disruption

Although we demonstrated the applicability of a group II intron-based gene editing tool in *S. coelicolor*, the disruption rate of *SCO5087* was found to be only 0.49% in the absence of screening pressure. We noticed that the introduction of pZW28 harbouring Ll.LtrB into *S. coelicolor* resulted in a 92.8% decrease in transformant numbers compared to the control plasmid pKC1139 (Figure 2B), indicating potential toxicity or genetic burden caused by constitutive expression of group II intron Ll.ltrB for *Streptomyces*. This toxicity may arise from the presence of intron-encoded protein LtrAsco, which possesses both a C-terminal DNA-binding domain and DNA endonuclease domain (Enyeart et al., 2014). To mitigate these deleterious effects, we employed the thiostrepton-inducible promoter *tipAp* (Takano et al., 1995) as a replacement for the strong constitutive promoter *ermE*p* to drive the expression of *ltrAsco*, generating plasmid pZW30 (Figure 2A). The promoter *tipAp* requires the presence of the thiostrepton-responsive activator TipA, which is evolutionarily conserved among most *Streptomyces* (Yun et al., 2001). Introduction of *tipAp* led to the leaky expression of *ltrAsco* at a low level, however, such a level was sufficient for carrying out gene editing processes. Compared with pZW28, transformation efficiency significantly increased to 45% with pZW30 (Figure 2B), while enhancing the gene disruption efficiency to 0.83% (Figure 2C). These results highlighted that controlling the expression level of *ltrAsco* represents an effective approach for improving both transformation and gene disruption efficiencies of group II intron Ll.LtrB in *Streptomyces*.

Given that the retrohoming of group II intron Ll.LtrB occurred independently of RecA-dependent homologous recombination, and the retrohoming efficiency was somewhat more efficient in the absence of RecA in *E. coli* and *L. lactis* (Cousineau et al., 1998), we hypothesized that the two physiological events of retrohoming and RecA-dependent homologous recombination might compete to bind the cleaved substrates. However, deleting *recA* would introduce additional experimental procedures and prolong the time of mutant strain construction in some *Streptomyces* species. To enable versatile manipulation of various *Streptomyces*, a universal RecA repression system needs to be constructed without deletion of *recA* from the cognate genome. We therefore designed a strategy to inhibit the RecA-dependent homologous recombination for the improvement of retrohoming efficiency. The negative regulator of RecA, RecX, is present in genomes of a wide range

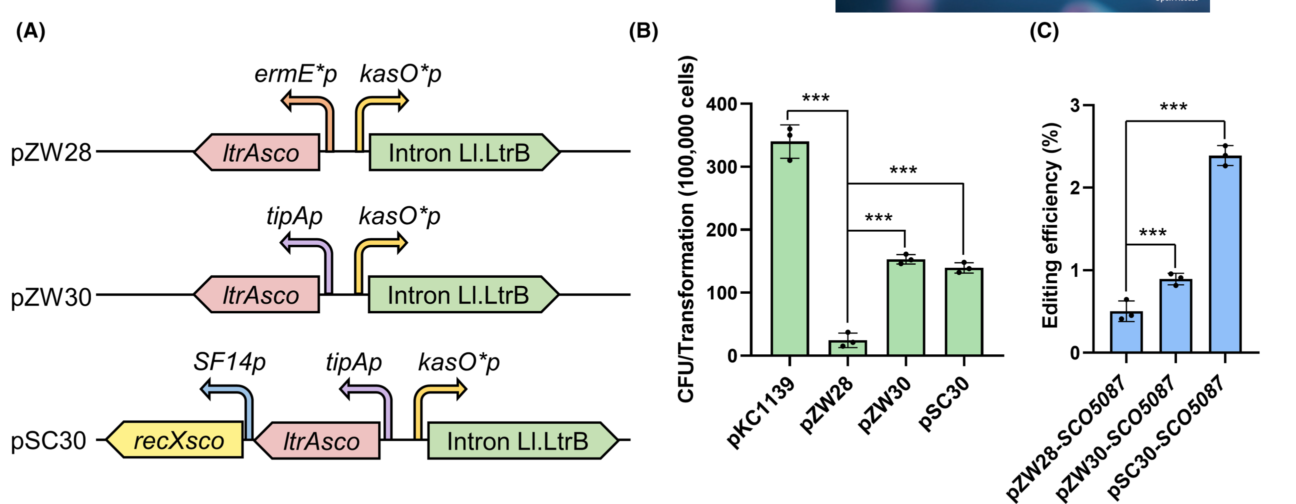


FIGURE 2 Improvement of the group II intron Ll.LtrB-based genetic manipulation tool for *Streptomyces*. (A) Schematic representation of the optimization of the group II intron Ll.LtrB-based genetic manipulation tool. In pZW28 and pZW30, the expression of *ltrAsco* was driven by *ermE*p* and *tipAp*, respectively. In pSC30, *recXsco* gene was overexpressed under the control of *SF14p*. (B) Transformation efficiency of the plasmids pKC1139, pZW28, pZW30, and pSC30 into *S. coelicolor*. The plasmid pKC1139 was used as the control. Transformation efficiency was indicated as the total number of CFU generated per transformation with 5 μg plasmid DNA. (C) Insertion disruption efficiency of the plasmids pZW28-*SCO5087*, pZW30-*SCO5087*, and pSC30-*SCO5087* in *S. coelicolor*. The efficiency was calculated as the ratio of the number of edited events to the total number of colonies analysed by PCR and sequencing. Error bars indicated the standard deviations from three independent experiments. Statistical significance is calculated based on two-tailed Student's *t* test (***$p < 0.001$).

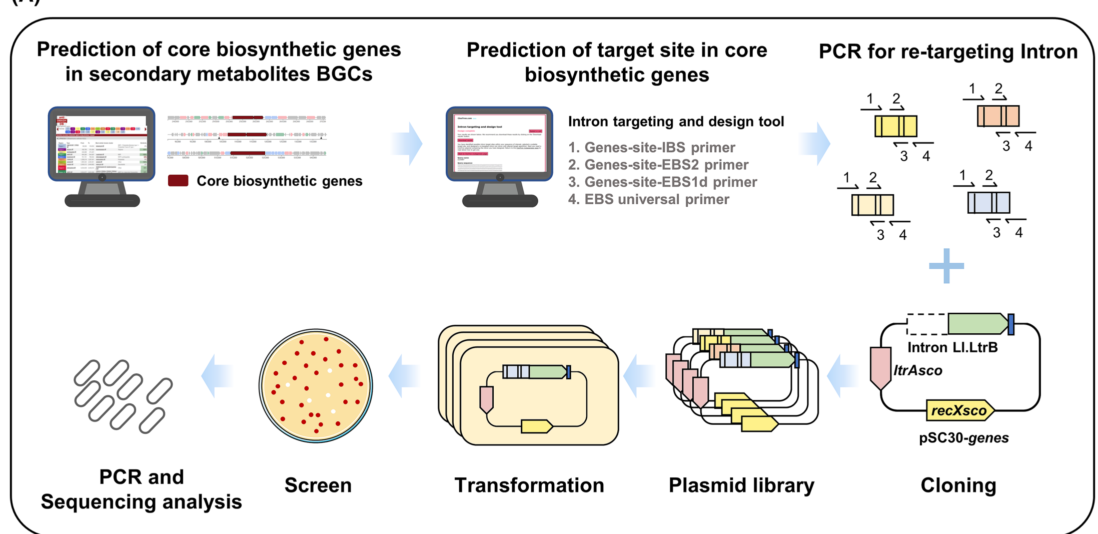
of bacteria and some plants (Lin et al., 2007). It inhibits recombinase, co-protease, ATPase, and strand exchange activities of RecA (Cox, 2007; Stohl et al., 2003). We investigated the distribution of RecA orthologs in the *Streptomyces* genus and observed that RecA is widely distributed and evolutionarily conserved in *Streptomyces* species (Figure S2). This information suggested that the co-expression of Ll.LtrB with RecX could suppress the RecA-dependent homologous recombination and avoid competition with the retrohoming process. Building on the pZW30 plasmid backbone, we constructed plasmid pSC30, wherein *S. coelicolor recXsco* was overexpressed under the control of promoter *SF14p* (Labes et al., 1997) (Figure 2A). Compared with pZW30, we observed that overexpression of *recXsco* in plasmid pSC30 did not decrease transformation efficiency, indicating that RecXsco showed no side-effect (Figure 2B). We then examined the gene disruption efficiency of pSC30 in *S. coelicolor*. As shown in Figure 2C, the proportion of strains with desired *SCO5087* disrupted mutant reached 2.38% of all the transformants, and this retrohoming efficiency was 2.87-fold higher than that of pZW30. Overall, via employing low expression of LtrAsco and co-expression of RecXsco, we improved the retrohoming efficiency of group II intron Ll.LtrB in *Streptomyces*.

Identification of BGC for red pigment biosynthesis in *S. roseosporus*

Having developed the group II intron-based genic manipulation tool for *Streptomyces*, we proceeded to demonstrate its application in other *Streptomyces* species. Due to its potent bactericidal activity and the low rate of drug-resistance, daptomycin is recognized as the first line antibiotic to treat serious infections caused by drug-resistant Gram-positive pathogens (Robbel & Marahiel, 2010). In addition to daptomycin production, *S. roseosporus* also synthesizes an unknown red pigment, which affects the separation of daptomycin from fermentation broth. As a proof-of-concept study, we sought to establish an efficient screening method for identifying BGC responsible for producing the red pigment in *S. roseosporus* (Figure 3A). Firstly, according to the prediction by antiSMASH, we identified 31 secondary metabolite BGCs in the genome of *S. roseosporus* (Table S4). Among these BGCs, 12 BGCs exhibited over 75% sequence identity with the known BGCs, and none of the cognate secondary metabolites were predicted to be in red based on the previously identified secondary metabolites in other *Streptomyces* (Table S4). Consequently, we selected the remaining 18 BGCs to determine which one was responsible for red pigment production. For each predicted core structural gene within these 18 BGCs, we constructed a mutant library by inserting an intron at specific insertion sites into plasmid pSC30 (Table S5). Subsequently, we transformed this library into *S. roseosporus* cells to screen mutants lacking the red pigment.

Approximately 4000 transformants were obtained upon introduction of the plasmid library into *S. roseosporus*, out of which four colonies exhibited no red pigment production. These four colonies were selected and subjected to DNA sequencing analysis

(A)



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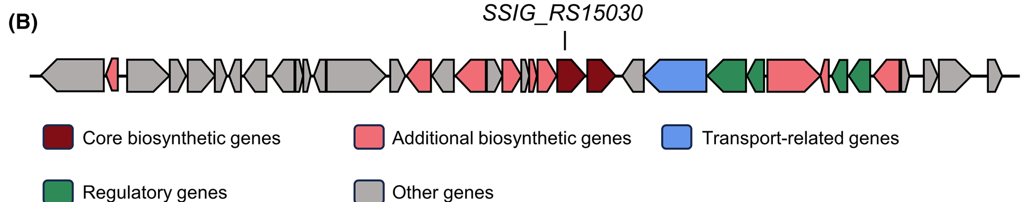

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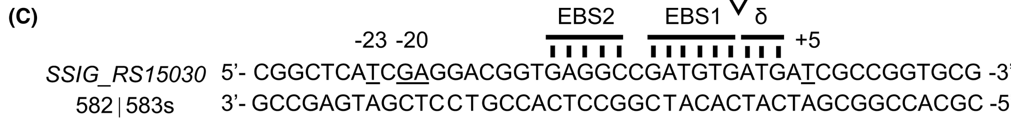

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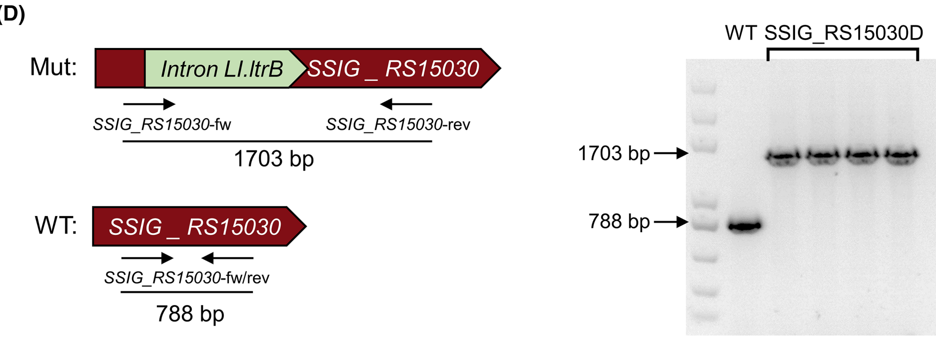
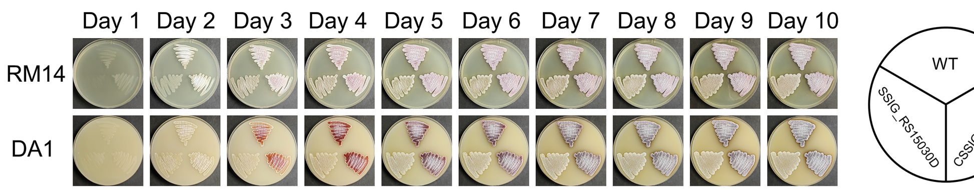


(E)



to identify the presence of intron RNA within them. Interestingly, we consistently found that introns in these colonies specifically targeted the core structural gene *SSIG_RS15030* (Figure 3B,C), which was predicted to be involved in Type II polyketide synthesis according to BGC analysis (Table S5). To confirm the disruption caused by insertion mutations in *SSIG_RS15030*, colony PCR and DNA sequencing using primers

FIGURE 3 Demonstration of the genetic manipulation tool by screening the BGC responsible for red pigment biosynthesis in *S. roseosporus*. (A) Schematic overview of the workflow for screening the BGC responsible for red pigment biosynthesis. (B) Genetic architecture of the complete BGC for red pigment biosynthesis in *S. roseosporus* genome. (C) Insertion site within *SSIG_RS15030* gene by group II intron. The exon binding sites EBS1, EBS2, and δ of the intron RNA were designed to base pair with corresponding complementary sequences of *SSIG_RS15030* gene. 's' represented the sense strand. The triangle represented the intron-insertion site. Critical bases recognized by the LtrA were underlined. (D) Confirmation of *SSIG_RS15030* gene disruption in the genome of *S. roseosporus* by colony PCR with primers *SSIG_RS15030*-fw/rev. WT represented *SSIG_RS15030* gene in the genome of *S. roseosporus* NRRL11379; Mut represented disrupted *SSIG_RS15030* gene in the genome of SSIG_RS15030D. (E) Phenotypes of WT, SSIG_RS15030D, and CSSIG_RS15030 strains grown on RM14 or DA1 plates at 28°C for 10 days.

SSIG_RS15030-fw/rev which flanked its insertion site were performed. The results confirmed that *SSIG_RS15030* was indeed disrupted by the intron insertion in these strains (named SSIG_RS15030D) (Figure 3D), and the gene disruption efficiency was about 1.78%. To confirm that the BGC involving *SSIG_RS15030* was responsible for biosynthesis of the red pigment, we constructed the complemented strain CSSIG_RS15030 based on SSIG_RS15030D. Furthermore, the phenotypes of SSIG_RS15030D and CSSIG_RS15030 were compared with those of WT on DA1 and RM14 plates. Complete impairment of red pigment biosynthesis was observed in the mutant SSIG_RS15030D, whereas CSSIG_RS15030 showed restored red pigment biosynthesis (Figure 3E). These results indicated that the *SSIG_RS15030*-containing BGC is responsible for biosynthesis of the red pigment. Notably, our BGC analysis revealed less than 14% similarity between this BGC and known ones, suggesting that the red pigment is likely a novel compound.

Improvement of daptomycin production by disruption of the red pigment biosynthesis

During the fermentation of daptomycin production by *S. roseosporus*, a considerable amount of undesired red pigment is generated, leading to a reddish coloration of the fermentation broth. Therefore, we investigated the impact of disrupting core structural gene *SSIG_RS15030*, responsible for the red pigment, on daptomycin production. We assessed both daptomycin titre and biomass (dry cell weight) of WT and SSIG_RS15030D strains cultured in fermentation medium A over a period of 1 to 10 days. In comparison with WT strain, SSIG_RS15030D exhibited increased daptomycin titers (Figure 4A). Moreover, during the stationary phase, SSIG_RS15030D displayed a slower decline in biomass compared to that observed in the WT strain. After 10-day culture, SSIG_RS15030D exhibited a 1.28-fold higher biomass than that of WT strain (Figure 4B). Concomitantly, the final daptomycin titre was 2.14-fold higher in SSIG_RS15030D than that in WT strain (Figure 4A). These findings indicated that the biosynthesis of the red pigment had a negative effect on both cell growth and daptomycin production in *S. roseosporus*. Furthermore, we explored an alternative fermentation medium B in which the red pigment synthesis was absent (Figure S3), while daptomycin titre was much higher. Under these conditions, we observed similar values for both daptomycin titers and biomass between SSIG_RS15030D and WT strains (Figure 4C,D). In addition, the relationship between the red pigment and daptomycin production was also investigated by qRT-PCR analysis of daptomycin biosynthetic genes (*dpt*) using RNAs prepared from WT and SSIG_RS15030D grown in fermentation medium A for 2 or 6 days (Figure S4). On day 2, the red pigment was not yet produced, and there were nonsignificant changes in the transcription levels of half of *dpt* genes between WT and SSIG_RS15030D, whereas most of the *dpt* genes were upregulated in SSIG_RS15030D on day 6 when the red pigment had been synthesized. These results indicated that the synthesis of the red pigment hampered cell growth, daptomycin production and transcription of *dpt* genes.

Activation of silent BGC using the developed tool

In addition to disrupting the target genes, the group II intron-based genetic manipulation tool could also be used as a carrier for integrating heterogenous DNA sequences into genome DNA (Velazquez et al., 2021). The majority of secondary metabolite BGCs in *Streptomyces* remain transcriptionally silent under conventional laboratory conditions. For example, jadomycin B, a type II polyketide antibiotic, was produced by *Streptomyces venezuelae* WT stain ISP5230 upon induction by heat shock, ethanol stress or phage infection (Yang et al., 1996). To investigate whether our developed group II intron-based gene editing tool could be used to precisely introduce heterologous promoters into *Streptomyces* genomes for BGC activation, we inserted the constitutive promoter *ermE**p* into the Ll.LtrB intron cargo vector pSC30 to generate pSC30E (Figure 5A), and utilized the ClosTron to identify the insertion sites of promoter region within the jadomycin BGC. The intron containing the promoter *ermE**p* was designed to insert at the position G|A in sense strand, located 85 nt upstream of the putative *jadJ* start codon (GTG) (Figure 5B). The resulting plasmid pSC30E-*jadJp* was transformed into *S. venezuelae* ISP5230. Colony PCR showed that the promoter *ermE**p* was efficiently inserted upstream of *jadJ* through retrohoming

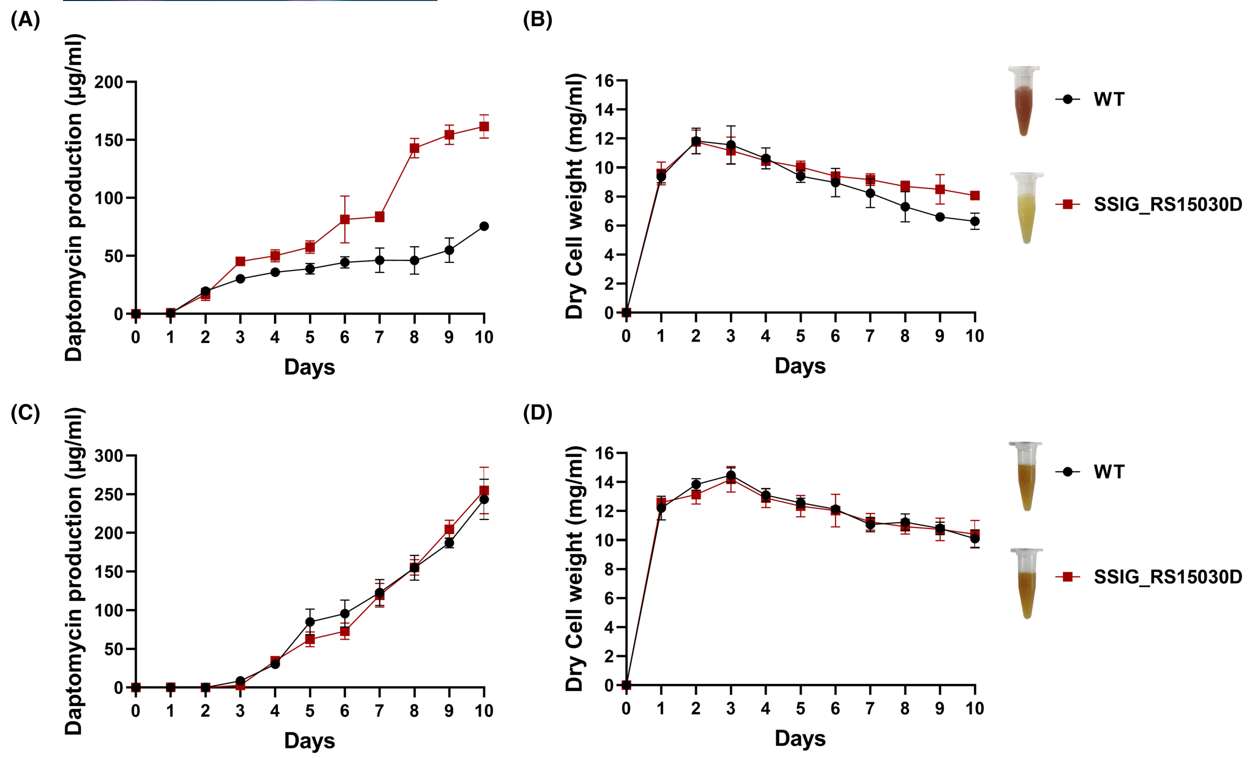


FIGURE 4 Effects of red pigment on daptomycin production and cell growth in *S. roseosporus*. (A) Curves of daptomycin titre. WT and SSIG_RS15030D strains were cultured in fermentation medium A for 10 days. (B) Growth curves of WT and SSIG_RS15030D cultured in fermentation medium A. (C) Curves of daptomycin titre. WT and SSIG_RS15030D strains were cultured in fermentation medium B for 10 days. (D) Growth curves of WT and SSIG_RS15030D cultured in fermentation medium B. Fermentation medium A: the medium in which WT strain could synthesize red pigment. Fermentation medium B: the medium in which WT strain could not synthesize red pigment. Error bars: SD for three biological replicates.

mediated by Ll.LtrB intron in the mutant strain EJAD23 (Figure 5C).The genome editing efficiency was 2.01%. To validate BGC activation, we tested the production of jadomycin B in *S. venezuelea* WT and mutant strain EJAD23. In flask fermentation, negligible jadomycin B was detected in the WT culture. However, the mutant strain EJAD23 accumulated approximately 29.74 µg/mL of jadomycin B (Figure 5D). These results demonstrated that the group II intron-based genetic manipulation tool could also serve as a vector for introduction of heterologous genetic elements into *Streptomyces* genomes, and such manipulation could conveniently activate cryptic BGCs.

DISCUSSION

Gene knock-in tools that do not rely on homologous recombination include sequence-specific Targetron and non-sequence specific transposon. The non-sequence specific transposon has been successfully applied to various prokaryotes, including *Streptomyces* species (Petzke & Luzhetskyy, 2009). Here, we established a sequence-specific genetic manipulation tool based on group II intron Ll.LtrB and implemented it for efficient gene disruption and insertion in three different *Streptomyces* species. Furthermore, to ensure the universality in *Streptomyces* genus and avoid potential toxicity, two improvements were introduced into this tool. Firstly, we employed the thiostrepton-inducible promoter *tipAp* to restrict the expression of intron-encoded protein, thereby reducing LtrAsco toxicity to cells and improving transformation efficiency. Secondly, we suppressed the RecA-dependent homologous recombination by overexpressing RecXsco to enhance the retrohoming efficiency of group II intron, enabling easy transferability of the tool without any genome modification in *Streptomyces* species. Empowered by these two improvements, we demonstrated the utility of our developed group II intron-based tool in two scenarios: gene disruption and promoter insertion. For gene disruption, we achieved efficient disruption in different *Streptomyces*, especially construction of secondary metabolite BGC mutant library and screening of red pigment BGC in *S. roseosporus*. Regarding promoter insertion, we activated cryptic jadomycin BGC and achieved successful production of jadomycin B in *S. venezuelae* ISP5230. These demonstrations evidenced the conveniency and universality of our developed group II intron-based tool.

This unique retrohoming mechanism of group II intron provides the system with several distinct traits.

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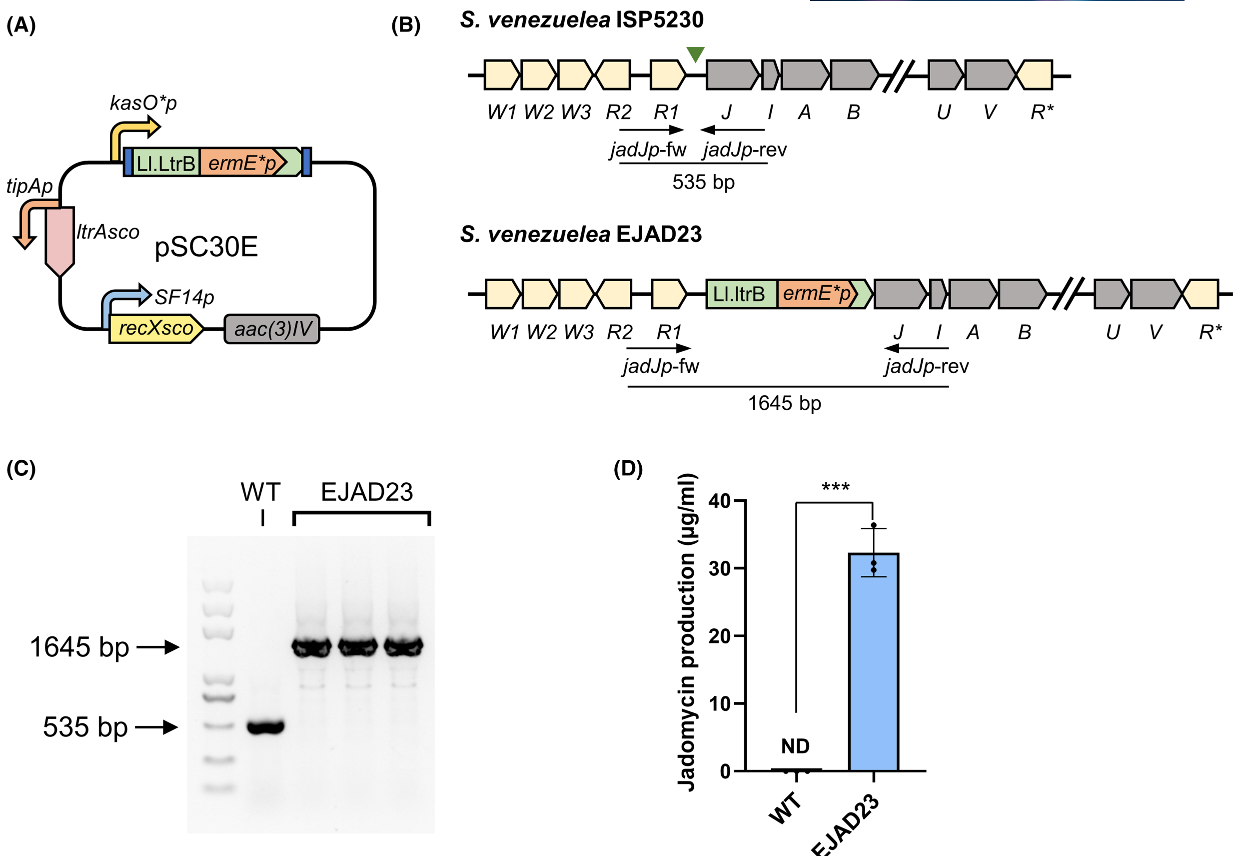

FIGURE 5 Demonstration of the genetic manipulation tool by activating silent jadomycin BGC in *S. veneluezae*. (A) Schematic representation of construction of pSC30E, in which the intron Ll.LtrB carrying the constitutive promoter *ermE*p*. (B) Genetic architecture of construction of *S. venezuelae* ISP5230 derivative EJAD23 with *ermE*p*-containing intron Ll.LtrB by retrohoming. The green triangle represented the intron-insertion site. (C) Confirmation of *ermE*p*-containing intron Ll.LtrB insertion in the genome of *S. venezuelae* ISP5230 (WT strain) by colony PCR. WT represented the DNA fragment amplified with primers *jadJp*-fw/rev in the genome of *S. venezuelae* ISP5230; EJAD23 represented the DNA fragment amplified with primers *jadJp*-fw/rev in the genome of *S. venezuelae* EJAD23. (D) Jadomycin B production in *S. venezuelae* WT and EJAD23 strains. ND means that jadomycin B was not detected. The values are means ± SD from three independent experiments. Statistical significance is calculated based on two-tailed Student's *t* test (***$p<0.001$).

Firstly, the sequence-specificity of the system could achieve precise gene disruption. This characteristic distinguishes it from the transposon system, making it an interesting tool for rational library design and high-throughput gene editing. Additionally, unlike CRISPR tools and traditional gene knock-in strategies that rely on large customized homologous arms and screening of recombination events, this system does not rely on homologous recombination. As a result, plasmid construction and gene disruption are simplified. Moreover, besides its application as a mutagenic tool, the tool could be further developed as a genetic delivery system in *Streptomyces*. By inserting foreign sequences into domain IV of group II introns, the tool can serve as a cargo to integrate these sequences at desired chromosomal locations. Consequently, heterologous promoters could be efficiently and precisely introduced into *Streptomyces* genomes to activate silent BGCs and produce unique metabolites. It was worth noting that the mobility efficiency of group II intron drastically decreased when the cargo sequences were longer than 1 kb (Plante & Cousineau, 2006). To overcome the limitation, the retrotransposition-activated selectable marker and the CRISPR/Cas9-facilitated counterselection strategy could be used to improve the screening of mutants (Velazquez et al., 2019; Zhong et al., 2003).

We found that the synthesis of the red pigment hindered cell growth, daptomycin production, and transcription of *dpt* genes in *S. roseosporus*. Biosynthesis of the red pigment probably competed with daptomycin production for energy and primary metabolism precursors, resulting in decreased daptomycin yields. Subsequently, when the strains ceased to produce red pigment, there was a noticeable increase in daptomycin production. Furthermore, the BGC responsible for synthesizing red pigment exhibits only 14% similarity to the known type II polyketide BGC associated with arixanthomycin (Table S5). The low similarity suggests that the red pigment likely distincts from arixanthomycin and may represent a novel compound. Arixanthomycin belongs to pentangular polyphenols (Kang & Brady, 2014) which are characterized by their large conjugated

systems and diverse colour range. Therefore, we hypothesized that the red pigment produced by *S. roseosporus* could potentially fall within the category of pentangular polyphenols which have a wide range of biological activities. Further experimental investigations are required to elucidate the chemical structure and properties of the red pigment.

In conclusion, the group II intron-based genetic manipulation tool described here enables specific disruption of the target genes and insertion of short heterologous DNA fragments in *Streptomyces* genome, providing a versatile gene editing tool for facile construction of *Streptomyces* mutants. To the best of our knowledge, this represents the first application of group II introns in *Streptomyces*. This tool complements other technologies, such as CRISPR and transposon, for facilitating fundamental research, identification of novel natural products, and development of cell factories in *Streptomyces*.

AUTHOR CONTRIBUTIONS

Ziwei Sang: Data curation; formal analysis; investigation; validation; visualization; writing – original draft. **Xingwang Li:** Data curation; formal analysis; investigation; validation; visualization. **Hao Yan:** Data curation; funding acquisition; investigation; validation; visualization. **Weishan Wang:** Conceptualization; funding acquisition; resources; supervision; writing – original draft; writing – review and editing. **Ying Wen:** Conceptualization; funding acquisition; resources; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interest.

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SUPPORTING INFORMATION

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

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