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RESEARCH ARTICLE

Editing *Streptomyces* genome using target AID system fused with UGI-degradation tag

Pamella Apriliana[1] | **Prihardi Kahar**[1] | **Norimasa Kashiwagi**[1] | **Akihiko Kondo**[1,2] | **Chiaki Ogino**[1]

[1]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Kobe, Japan

[2]Department of Graduate School of Science, Technology, and Innovation, Kobe University, Kobe, Japan

Correspondence
Chiaki Ogino, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan.
Email: ochiaki@port.kobe-u.ac.jp

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Abstract

The utilization of *Streptomyces* as a microbial chassis for developing innovative drugs and medicinal compounds showcases its capability to produce bioactive natural substances. Recent focus on the clustered regularly interspaced short palindromic repeat (CRISPR) technology highlights its potential in genome editing. However, applying CRISPR technology in certain microbial strains, particularly *Streptomyces*, encounters specific challenges. These challenges include achieving efficient gene expression and maintaining genetic stability, which are critical for successful genome editing. To overcome these obstacles, an innovative approach has been developed that combines several key elements: activation-induced cytidine deaminase (AID), nuclease-deficient cas9 variants (dCas9), and Petromyzon marinus cytidine deaminase 1 (PmCDA1). In this study, this novel strategy was employed to engineer a *Streptomyces coelicolor* strain. The target gene was actVA-ORF4 (SCO5079), which is involved in actinorhodin production. The engineering process involved introducing a specific construct [pGM1190-dcas9-pmCDA-UGI-AAV-actVA-ORF4 (SCO5079)] to create a CrA10 mutant strain. The resulting CrA10 mutant strain did not produce actinorhodin. This outcome highlights the potential of this combined approach in the genetic manipulation of *Streptomyces*. The failure of the CrA10 mutant to produce actinorhodin conclusively demonstrates the success of gene editing at the targeted site, affirming the effectiveness of this method for precise genetic modifications in *Streptomyces*.

KEYWORDS
actinorhodin, AID system, CRISPR dCas9, *Streptomyces*, UGI

Abbreviations: AID, activation-induced cytidine deaminase; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, dead variant Cas9; UGI, Uracil DNA glycosylase inhibitor.

1 | INTRODUCTION

The *Streptomyces* genus, a group of bacteria known for their remarkable capacity to produce a diverse array of bioactive compounds, holds significant importance in the field of biotechnology and pharmaceuticals. These compounds exhibit a range of biological activities, including antifungal, antimicrobial, antibiofilm, antitumor, anticancer, insecticidal, and anthelmintic properties. They are also sources of industrially relevant enzymes [1–11]. A major challenge in harnessing the full potential of these compounds lies in the fact that many of them are not fully expressed under standard laboratory conditions in their native *Streptomyces* hosts. This has led researchers to delve into the realm of genetic engineering to activate biosynthetic gene clusters and boost the production of these valuable compounds [12].

In recent years, the CRISPR-Cas system, particularly inspired by the adaptive immune system of *Streptococcus pyogenes*, has emerged as a powerful tool in genome editing. The system centers around the Cas9 protein, an RNA-guided endonuclease renowned for its precision in creating double-strand DNA breaks. This capability is essential for genomic alterations such as insertions, deletions, or substitutions, which can be achieved through mechanisms like nonhomologous end joining or homologous recombination [13, 14].

The adoption of the CRISPR-Cas9 system in *Streptomyces* research represents a significant leap forward. It allows for precise editing of the bacterial genome, thereby enabling the activation of previously silent biosynthetic pathways [15, 16]. This enhances the production of known bioactive compounds and paves the way for the discovery of novel compounds with potential therapeutic applications.

Nevertheless, while CRISPR-Cas9 is a widely used tool, it has certain limitations that depend on the host cell. In numerous actinomycetes, the heightened expression of Cas9 is often associated with significant toxicity and results in a substantial increase in undesirable off-target effects; the linear chromosomes demonstrate notable intrinsic instability and are capable of enduring extensive chromosomal deletions and rearrangements. The arm regions of these chromosomes are particularly prone to DNA double-strand breaks (DSBs), which are significant catalysts of this instability. These DSBs frequently accompany gene manipulation techniques that utilize DSBs, such as CRISPR-Cas9 [17–20]. In most bacteria, the absence of the nonhomologous end-joining (NHEJ) pathway means that chromosomal cleavages caused by nucleases typically result in cell death. In contrast, *Streptomyces* and other actinomycetes primarily rely on the homologous recombinant (HR) pathway to repair DNA double-strand breaks. This is unlike many eukaryotes that employ the NHEJ pathway for DNA repair. The preference of actinomycetes for HR is based on their evolutionary adaptability, which prioritizes the precision of this repair mechanism. Significantly, the genome often lacks essential NHEJ proteins, such as Ku and Ligase D, suggesting a possible absence of the NHEJ mechanism. This absence of NHEJ has streamlined genetic manipulations in research, ensuring precise DNA integrations through HR and eliminating the unpredictability associated with random insertions [21, 22]. This restriction can be overcome through the modification of a catalytically inactive Cas9 (dCas), which is achieved by introducing mutations in the HNH and RuvC domains (D10A and H840A). While lacking endonuclease activity, this modified system can efficiently bind to the target DNA by forming a complex with sgRNA.

Furthermore, it can be fused with various effectors for gene regulation and visualization of specific genomic loci [23, 24]. In recent development, a novel method has been introduced for the direct alteration of individual nucleotides at precise locations within DNA, all without the need for DNA cleavage. This innovative technique uses a modified vector that combines the deaminase PmCDA1 with dCas9 and incorporates the uracil DNA glycosylase inhibitor (UGI). Furthermore, in *E. coli*, this method employs a degradation tag known as LVA for enhanced efficacy [25]

Here, we propose a method for inducing mutations in *Streptomyces*, which does not necessitate a double-strand break (DSB). By specifically converting cytidine (C) to thymidine (T), this approach facilitates the introduction of stop codons. This study explores the fundamental characteristics of the CRISPR target-AID system, particularly its suitability for implementation in *Streptomyces* species, and the wide array of applications this adaptation enables. Choosing an expression host that is well-characterized and

Practical Application

The application of the Target AID system has shown remarkable success in engineering precise mutations in actinorhodin as a target *Streptomyces coelicolor*. In *S. coelicolor*, a mutation in actinorhodin has enabled researchers to knock out actinorhodin production with a single nucleotide mutation to create a stop codon. These successes affirm that the target AID system is an invaluable tool in the genetic engineering of *Streptomyces*.

TABLE 1 Strain and plasmids used in this study.

Strain & plasmid	Description
e.coli HST04	F^-, *ara*, Δ(*lacproAB*) [Φ80d*lacZ*ΔM15], *rpsL*(*str*), *thi*, Δ(*mrr-hsdRMS-mcrBC*), Δ*mcrA*, *dam*, *dcm*
e.coli JM109	*recA1, endA1, gyrA96, thi-1, hsdR17 (*rk^- mk^+*), e14*$^-$ *(*$mcrA^-$*), supE44, relA1*, Δ *(lac-proAB)/F' [traD36, proAB*$^+$*,* $lacI^q$*, lacZΔM15*]
pGM1190	OriT, thiostrepton-induced gene, Apr^r, Tip A
pMA-RQ_UGI_dCasPmCDA1	Codon-optimized dCas9, PmCDA1 gene
17ACQ2GP_ermEp-gRNA_pMA-RQ	*ermE*p and gRNA
pUB307	PΔTnA^c, IncPα replicon Km^r TC^r
pGM1190-dCas-linker-PmCDA1-UGI-AAV	OriT, thiostrepton-induced gene, Apr^r, Tip A and dCasPmCDA1 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 8	OriT, thiostrepton-induced gene, Aprr, Tip A and dCasPmCDA1-target 8 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV- target 9	OriT, thiostrepton-induced gene, Aprr, Tip A and dCasPmCDA1-target 9 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 10	OriT, thiostrepton-induced gene, Apr^r, Tip A and dCasPmCDA1-target 10 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 11	OriT, thiostrepton-induced gene, Apr^r, Tip A and dCasPmCDA1-target 11 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 12	OriT, thiostrepton-induced gene, Apr^r, Tip A and dCasPmCDA1-target 12 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV- target 13	OriT, thiostrepton-induced gene, Aprr, Tip A and dCasPmCDA1-target 13 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 14	OriT, thiostrepton-induced gene, Apr^r, Tip A and dCasPmCDA1-target 14 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 15	OriT, thiostrepton-induced gene, Aprr, Tip A and dCasPmCDA1-target 15 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 16	OriT, thiostrepton-induced gene, Apr^r, Tip A and dCasPmCDA1-target 16 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 17	OriT, thiostrepton-induced gene, Apr^r, Tip A and dCasPmCDA1-target 17 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 18	OriT, thiostrepton-induced gene, Aprr, Tip A and dCasPmCDA1-target 18 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 19	OriT, thiostrepton-induced gene, Aprr, Tip A and dCasPmCDA1-target 19 sequence
pGM1190-dCas-linker-PmCDA1-UGI-AAV-target 20	OriT, thiostrepton-induced gene, Aprr, Tip A and dCasPmCDA1-target 20 sequence

genetically flexible is essential for achieving optimal outcomes. To achieve this objective, we utilize *S. coelicolor* to express gene clusters derived from actinomycetes. The study further delves into the latest developments and case studies in the field of *Streptomyces* genome engineering, particularly focusing on the CRISPR-AID system. This system, incorporating elements such as dCas9, PmCDA, and a combined UGI design, is adept at specifically altering single nucleotides, changing cytosine to thymine in the actinorhodin genes of *S. coelicolor*, which serve as the target. In addition, we conducted an off-target assessment in *S. coelicolor* for the open reading frames (ORFs) associated with a crucial role in actinorhodin formation.

2 | MATERIALS AND METHODS

2.1 | Strains, plasmids, culture conditions, reagents, and enzymes

The bacterial strain used in this research is listed in Table 1. *Streptomyces coelicolor* NBRC 15146 were obtained from Biological Resource Center (NITE National Biological Resource Centre, Tokyo, Japan, https://www.nite.go.jp/en/) was used in this research, maintained in ISP2 medium (Yeast extract 4 gr/L, Malts extract 10 gr/L, Dextrose 4 gr/L, Agar 20 gr/L). Conjugated strains were grown into ISP4 medium (Soluble starch 10 gr/L, $MgSO_4 \cdot 7H_2O$, 1 gr/L

TABLE 2 Primers used in this study.

Primers	Sequence nucleotide	Comments
pGM1190-SnaBI-cassette-F2	ATGATTACGAATTGTACGTA-CGCGGTCGATCTTGACGGCTG	For construct ermE-target and compile with gRNA scafold (forward)
Target8 R8-SCO5085.actII-orf4.R (Code:BS)	GCTATTTCTAGCTCTAAAA-CCGGCGCGCCTGCCCATCTG–CCGCTGGATCCTACCAACCG	For construct ermE-target 8 (reverse)
Target9 R9-SCO5085.actII-orf4.R (Code:BT)	GCTATTTCTAGCTCTAAAA-CTATCGACGCGAATTGGCGGC–CCGCTGGATCCTACCAACCG	For construct ermE-target 9 (reverse)
Target10 R10-SCO5079.actVA-orf4.R (Code:BT)	GCTATTTCTAGCTCTAAAA–CGGCCGCGGAGCCGCCTTGCT-CCGCTGGATCCTACCAACCG	For construct ermE-target 10 (reverse)
Target11 R11-SCO5079.actVA-orf4.R (Code:BT)	GCTATTTCTAGCTCTAAAA-CGTGTCATCGGCGTTTGGATG–CCGCTGGATCCTACCAACCG	For construct ermE-target 11 (reverse)
Target12 R12-SCO5079.actVA4.R (Code:BW)	GCTATTTCTAGCTCTAAAA–CGCCGGTGTCATCGGCGTTTG-CCGCTGGATCCTACCAACCG	for construct ermE-target 12 (reverse)
Target13 R13-SCO5079.actVA4.R (Code:BX)	GCTATTTCTAGCTCTAAAA-CAGCCGCCAGTTCACGCCCTC–CCGCTGGATCCTACCAACCG	For construct ermE-target 13 (reverse)
Target14 R14-actVAORF5SCO5080.R (Code:CF)	GCTATTTCTAGCTCTAAAA-CTCAGGGACGGCCGCTCCTGG–CCGCTGGATCCTACCAACCG	For construct ermE-target 14 (reverse)
Target15 R15-actIIORF2(SCO5083) (Code:CG)	GCTATTTCTAGCTCTAAAA-CGCCGCGTTCGCGCCCCTGGA–CCGCTGGATCCTACCAACCG	For construct ermE-target 15 (reverse)
Target16 R16-actIIORF2(SCO5083) (Code:CH)	GCTATTTCTAGCTCTAAAA-CCCTACGCGCGCCGTTGG CCGCTGGATCCTACCAACCG	For construct ermE-target 16 (reverse)
Target-R17 actIORF2(SCO5088) (Code:CH.1)	GCTATTTCTAGCTCTAAAA-CGGTCCGCCTTCGCGTCCTGG–CCGCTGGATCCTACCAACCG	For construct ermE-target 17 (reverse)
Target18 R18-actIV(SCO5091) (Code:CI)	GCTATTTCTAGCTCTAAAA-CGGCCCTCACCGGGCTCTGGC–CCGCTGGATCCTACCAACCG	For construct ermE-target 18 (reverse)
TargetR19-actVII(SCO5090_17 bp) (Code:CJ)	GCTATTTCTAGCTCTAAAA-CAACGCTTCCGGATCTGGGCG–CCGCTGGATCCTACCAACCG	For construct ermE-target 19 (reverse)
TargetR20-actIII(SCO5086_17 bp) (Code:CK)	GCTATTTCTAGCTCTAAAA-CCCCGCCTTGAGGACCTGTTT–CCGCTGGATCCTACCAACCG	For construct ermE-target 20 (reverse)
sSCO5092.F	CGACCAGGGAATGCTCCG	For Sequencing target 6 & 7
sSCO5092.R	GGTACGCCCTTCTCCTCGAC	
sSCO5085_actIIORF4.F	AAAGCAATATCGCGCACCTG	For Sequencing target 8 & 9
sSCO5085_actIIORF4.R	TATCCGCCGGAGATTCCGAT	
target10-11-12 13_F_Cekseq2	GACTCGTGACGACGACCAAC	For Sequencing target 10-11-12-13
sSCO5079_actVAORF4.R	TTCTCCATGAAGTACGACGGC	
sSCO5083_actIIORF2.F	AAATGTTCCCGCCCAAGGAG	For Sequencing target 15
sSCO5083_actII-ORF2.R	ATCATGATGCCCATGCCGAG	

(Continues)

TABLE 2 (Continued)

Primers	Sequence nucleotide	Comments
sSCO5083_actII-ORF2_t16.F	TGAGTTCCGTTGAAGCCGAC	For Sequencing target 16
sSCO5083_actII-ORF2_t16.R	GAGCCCTTGCGGAATCATCA	
sSCO5088_actI-ORF2.F	AGATCGACGACTTCCACGC	For Sequencing target 17
sSCO5088_actI-ORF2.R	CCCATGTCGTAGTCGGTCAG	
sSCO5091_actIV.F	TCAACACCCACTTCCACGG	For Sequencing target 18
sSCO5091_actIV. R	TGGACCCAGCGCAGATAGTC	
sSCO5090_actIORF4.F	CTCTACGAGCTGGTCGCAC	For Sequencing target 19
sSCO5090_actIORF4.R	GTCCTCGAACGTGAAGACCA	
sSCO5086_actIII.F	TCGAGGCGATGTTGACGATT	For Sequencing target 20
sSCO5086_actIII.R	ACGAACTCTGGCTCGATGTC	

NaCl, $(NH_4)_2SO_4$ 2 gr/L, $CaCO_3$ 2 gr/L) supplemented with 1 mL trace salts solution ($FeSO_4{\cdot}7H_2O$ 0.1 gr/100 mL, $MnCl_2{\cdot}4H_2O$ 0.1 gr/100 mL, $ZnSO_4{\cdot}7H_2O$ 0.1 gr/100 mL) and over layer medium (Nutrient Broth 8 gr/L, Soft agar 5 gr/L, nalidixic acid 1 μg/mL, apramycin 50 μg/mL, aztreonam 50 μg/mL). Recombinant strains grown were transferred into a selective medium (ISP2 supplemented with antibiotic apramycin 10 μg/mL). *E. coli* JM109 (Takara-Bio, Shiga, Japan) and *E. coli* HST04 (Takara-Bio) were used as hosts for plasmid transformation and conjugation, respectively. These recombinant strains were grown in Luria–Bertani (LB) medium at 37°C, and agar was added at 20 g/L in the case of the solid medium used. Proper antibiotics were added to the medium using the concentration of apramycin 50 μg/mL; kanamycin 20 μg/mL; ampicillin 50 μg/mL and tetracycline 5 μg/mL.

All restriction enzymes used in this study were purchased from New England Biolabs Inc. (Germany), and In-Fusion HD for cloning fragment DNA was purchased from TaKaRa-Bio. The PCR technique was employed to verify the target sequence of *S. coelicolor* by amplifying nucleic acids using the high-precision polymerase KOD FX (Toyobo, Osaka, Japan).

2.2 | Plasmid constructions

All plasmids used in this research are listed in Table 1. Two different plasmids, pMA-RQ_UGI_dCasPmCDA1 was used as a source of dCas9 and PmCDA1, and 17ACQ2GP_ermEp-gRNA_pMA-RQ was used as sources of ermE and gRNA sequences, for generating ermE-target- gRNA construction. The primary temperature-sensitive pGM1190-dCas-PmCDA1-UGI plasmid was constructed from vector pGM1190 by inserting the genes for dCas9, PmCDa1, UGI, and protein degradation tag. Figure S1 illustrates the detailed sequence positioning of CasPmCDA-UGI along with the degradation tag. This plasmid was constructed by cutting pMA-RQ_UGI_dCasPmCDA1 (Geneart ThermoFisher) with *Eco*RI/*Bam*HI and *Nde*I enzymes, next cloned the genes into *Eco*RI/BamHI and *Nde*I sites by ligation after verified through the gel electroporation (Figures S2–S4).

To construct a completed vector, pGM1190-dCas-PmCDA1-target was made as follows. Firstly, by using PCR with KOD-FX (Toyobo), the generated ermE-target, 17ACQ2GP_ermEp-gRNA_pMA-RQ as a template, using forward primer (pGM1190-SnaBI-cassette-F2) ATGATTACGAATTGTACGTACGCGGTCGATCTTGACGGCTG and reverse primer depending on the target gene to be constructed, ermE-20 nt target will be formed, besides that forming gRNA was also using 17ACQ2GP_ermEp-gRNA_pMA-RQ as the template, forward primer (KnK9) GTTTTAGAGCTAGAAATAGCAAAG, reverse primer (pGM1190-SnaBI-cassette_R3) GTAGCTGACGCCTACGTAAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAG. All primers used in listed in Table 2. The PCR product is purified using the Fast Gen gel/PCR extraction kit (Nippon Genetics Co., Ltd., Tokyo, Japan). Secondly, the construction combining ermE promoter, target region, and gRNA amplified using overlap PCR generated the fragment ermE promoter-20 nt target-guide RNA and carries a *Sna*BI restriction site for easy cloning of new spacers. Next, the ermE promoter-20 nt target-gRNA was cloned into the basic vector between the *Sna*BI site using an Infusion HD cloning kit (Takara-Bio). The confirming the result of target infusion into plasmid is in Figure S5–S7. The workflow for constructing these vectors is described in Figure 1.

2.3 | Intrageneric conjugation

A helper plasmid PUB307 is required to assist the mobilization of pGM1190-dCasPmCDA-UGI-AAV plasmid into *Streptomyces* strains. After the PUB307 plasmid

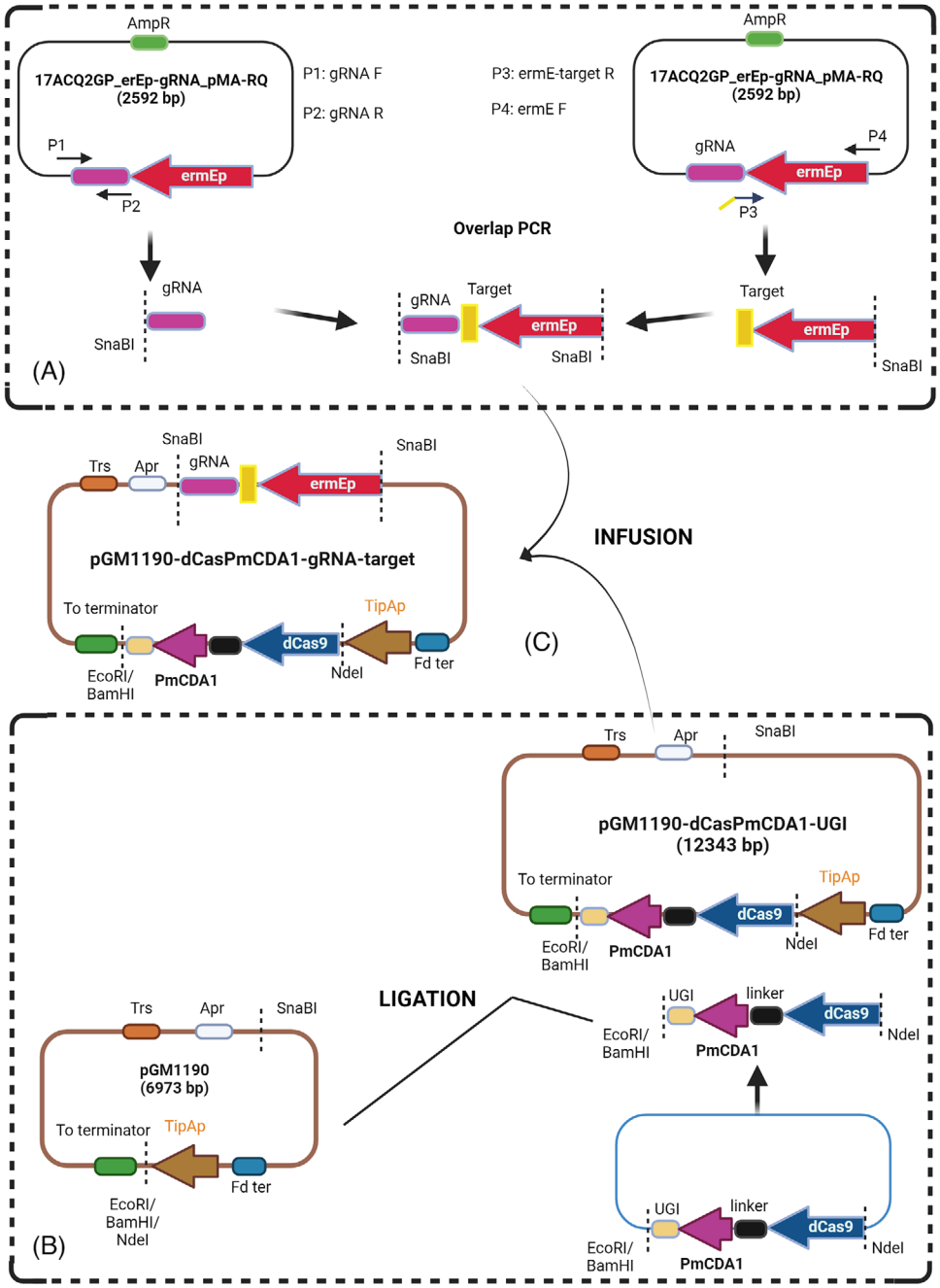


FIGURE 1 Plasmid construction. (A) Step to prepare ermE promoter and gRNA region. (B) The construction of vector backbones integrating the fused dCasPmCDA1-UGI. (C) Complete vector.

was transformed into *E. coli* JM109, it was necessarily transferred into *E. coli* HST04, which already contained pGM1190-dCasPmCDA-AAV/LVA_target, and we used the mating. The *E. coli* HST04 pGM1190-dCasPmCDA-AAV/LVA_target has an apramycin resistance gene from pGM1190 plasmid and a kanamycin resistance gene from PUB307 plasmid, used as selected markers. In the end, the positive transformant was selected based on the colony formation of *E. coli* HST04 strain containing these two different plasmids, which were generated with a Kanamycin-selected helper plasmid and an Apramycin-marked plasmid on the LB agar containing kanamycin and apramycin.

2.4 | Intergeneric conjugation for transferring plasmid from *E. coli* to *S. coelicolor*

Conjugation between *E. coli* HST04/ pGM1190-dCasPmCDA-AAV/LVA_target-PUB307 and *S. coelicolor* using a modified method [26]. Inoculation of a single

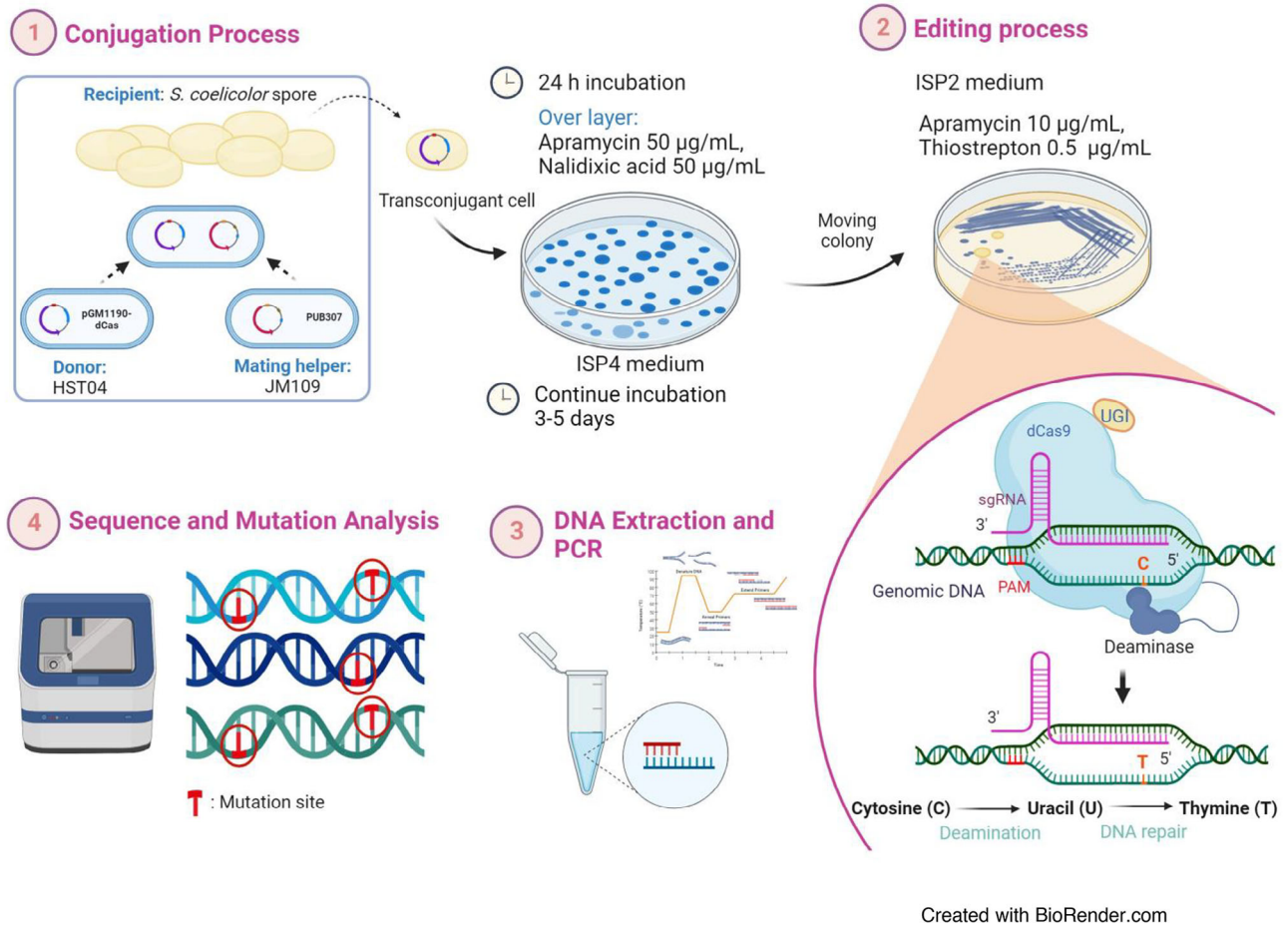


FIGURE 2 Overview of genome editing using CRISPR-dCas9-PmCDA-UGI in *S. coelicolor*.

colony of *E. coli* HST04 carrying pGM1190-dCasPmCDA-AAV/LVA_target and PUB307 was conducted in LB medium, supplemented with 50 µg/mL apramycin and 50 µg/mL kanamycin. After incubation at 37°C for 18 h with agitation at 200 rpm, the optical density at 600 nm was measured and then adjusted to 0.5 using LB medium. The cells were harvested and washed twice with LB medium to remove antibiotics by centrifugation at 5500 rpm for 4 min and resuspended in 1 mL LB medium. *S. coelicolor* spore suspension stored at −80°C was used as the recipient. The spores at 10^6 were washed using LB medium, resuspended in 200 µL 2 x TY medium, and incubated at 50°C for 10 min to activate the spore germination. The *E. coli* cultured were mixed with the heat-tread spores, incubated for 1 h at 28°C, then spread in the ISP4 medium plate medium. The plate was incubated for 16–24 h at 30°C, overlaid with 3 mL soft agar containing LB medium (0.8%), 50 µg/mL nalidixic acid to eliminate *E. coli* and 50 µg/mL apramycin. The incubation process continued at 30°C until colonies of exconjugants appeared. Each conjugant was transferred into ISP2 medium agar containing 20 µg/mL apramycine, 0.5 µg/mL thiostrepton. Generally, the experiment procedure is illustrated in Figure 2.

2.5 | Actinorhodin detection

To investigate extracellular actinorhodin using the modified method from [26–29] *S. coelicolor* at 10^9 spores were inoculated into 15 mL of ISP2 liquid medium and grown at 30°C for 3 days with shaking, used as a seed culture. For the fermentation process, 100 mL of basal medium containing K_2SO_4 (2 g), NaCl (1 g), K_2HPO_4 (15 mmol), and NH_4Cl or NO_3K (40 mmol) prepared in 500 mL Erlenmeyer flask was inoculated with 3 mL of the seed culture. Before adjusting the pH to 7, $MgSO_4 \cdot 7H_2O$ (80 mg), $ZnSO_4 \cdot 7H_2O$ (2 mg), and 0.1 mL of trace element solution were added. The trace element solution used was composed by $CuSO_4 \cdot 5H_2O$ 0.5 g/L, $MnSO_4 \cdot H_2O$ 5 g/L, H_3BO_3 4 g/L, $CoCl_2 \cdot 6H_2O$ 0.5 g/L, $NiCl_2 \cdot 6H_2O$ 2 g/L and $Na_2MoO_4 \cdot 2H_2O$ 3 g/L. Following autoclaving, 0.1 g/L of separately autoclaved $CaCl_2 \cdot 2H_2O$ and 5 g/L of glucose were added. The cultures were incubated at 30°C for 7 days in the dark with shaking after adding 50 mg/L of filter-sterilized yeast extract.

To extract actinorhodin, 30 mL sample was taken from each culture, centrifuged at 8000×g for 10 min at room temperature, transferred the supernatant to a 50 mL tube,

adjust the pH until 2 with 1 N HCl, and added chloroform to the supernatant at a ratio of 1:4. The mixed solution was shaken vigorously and then centrifuged at 8000×*g* for 5 min at room temperature. The chloroform phase was collected and evaporated. After drying, 2 mL solvent (methanol: chloroform = 1:1) was added. A UV spectrophotometer was used to analyze the solutions at various absorptions.

2.6 | Off-target confirmation

Nonspecific mutations occurring at unintended places, known as "off-targets," are a significant problem in the CRISPR system. This study employed Next Generation Sequencing (NGS) to assess the unintended impacts following the editing process. The complete genomic sequence of *S. coelicolor* (wild type) and genetically modified strains were compared and analyzed using CRISPR tools provided by Geneious prime (Biomatters, Inc., Auckland, New Zealand). A search was conducted for the gRNA CRISPR sites within the targeted gene, as well as for off-target binding sites within the genome of interest. This study focused only on conducting off-target evaluation specifically for the actinorhodin gene.

3 | RESULTS AND DISCUSSION

3.1 | Basic vector construction and AID system

In this study, we used a high copy number temperature-sensitive vector pGM1190 with apramycin as a selection marker as a backbone for a host-vector CRISPR complex. Constitutive ermE promoter and fd terminator were used for sgRNA expression, and thiostrepton inducible tipA promoter was used for dCas9 expression. The presence of oriT in the vector was necessary for vector mobility. The enzyme dead variant Cas9 (dcas9) lacks endonuclease activity caused by inactivating RuvC and HNH catalytic domain [23, 30], fused to a transcriptional activator, repressor, and effector domain to enact specific changes at the targeted site. To tightly control the levels of this protein in the cell, the tag variants LVA and AAV were employed. Besides that, to improve the efficiency of the base conversion by inhibiting the cell's own repair machinery, Uracil Glycosylase Inhibitor (UGI) was fused. Practically, after a cytidine deaminase converts a target cytosine to uracil, UGI prevents the cell from repairing this change, thus making the edit become permanent [31]. The basic construction vector was obtained after the transformation of *E. coli* JM109. The ermE promoter with target region and gRNA was amplified by PCR. In this preparation, gRNA (128 bp) and ermE promoter-target region (178 bp) were fused by using an overlap PCR, and the total length of the gRNA-ermE-target cassette was 306 bp (Figure 1). The fragment gRNA-ermE-target was introduced into the basic plasmid already digested with SnaBI, using In-Fusion HD Cloning Kit.

3.2 | Evaluation of the CRISPR-dCas9-AID-UGI-AAV/LVA fusion system in *Streptomyces*

The Target-AID system was evaluated in *S. coelicolor*, using a combination of dCas9 from *Streptococcus pyogenes*, activation-induced cytidine deaminase (AID), PmCDA from sea lamprey (*Petromyzon marinus*), and the uracil DNA glycosylase inhibitor (UGI). *S. coelicolor* has the ability to produce Actinorhodin (ACT), a blue pigment [32]. Actinorhodin is a type of polyketide that is synthesized through a polyketide synthase (PKS) pathway. This pathway encompasses a series of enzymatic reactions that ultimately lead to actinorhodin synthesis. Initially, precursor molecules such as acetyl-CoA and malonyl-CoA are produced. These precursors serve as building blocks for the synthesis of polyketide. Polyketide synthase (PKS) catalyzes the elongation of the polyketide chain, resulting in the production of an expanding polyketide chain. This chain undergoes cyclization, chemical modification, and the creation of an aromatic system [32].

Here, the base editing with the dCas9-PmCDA-UGI-AAV was feasible in the *Streptomyces*. Previous studies have reported the successful application of Target AID in *E. coli* through the fusion of PmCDA1 with an LVA tag and UGI. As to the report, Target-AID causes the substitution of cytosine (C) with thymine (T), resulting in the introduction of a stop codon [25]. To assess the suitability of deaminase-mediated targeting in *Streptomyces*, we constructed a Target activation-induced cytidine deaminase (Target-AID)-dCas-PmCDA. This fusion protein was designed to increase the efficiency of base mutation by incorporating the UGI (Uracil-DNA glycosylase inhibitor) and degradation tag fused at the carboxyterminus of dCas-CDA [25]. UGI is bacteriophage protein derived from *B. subtilis* that protects its genome from the repair enzyme Urasil-DNA glycosylase (UDG) by permanently inhibiting the action of urasil [33]. On the other hand, the degradation tag is a short peptide sequence that directs the protein toward degradation through the protein recycling machinery. Additional degradation tags, such as AAV ASV, LVA, and LAA, have been engineered with variations in the last three amino acids [34]. Banno et al. [25] highlighted the role of the LVA tag in genome editing in *E. coli*. This

FIGURE 3 Essential genes for actinorhodin production. (A) Actinorhodin gene cluster. (B) Mutated target gene actVA-ORF4. The positions of PAM sequence (GGC) and target 10 are given. (C) The position of SCO5079 protein (actVA-ORF4) in actinorhodin biosynthesis.

tag directs the degradation of the dCas9-CDA-UGI fusion protein, effectively reducing its potential toxicity. By controlling the concentration of this fusion protein, the LVA tag enhances genome editing efficiency while minimizing the risks of off-target effects and unintended mutations. In applying the LVA tag to *Streptomyces* strains, the model strains continued to produce actinorhodin, as evidenced in Figure S8. In several studies, the AAV tag is utilized for targeted protein degradation, which is beneficial in synthetic circuits that require a sustained presence of proteins [35]. We persist in utilizing the AAV degrading tag for further investigation due to its consistent generation of positive outcomes during the studies.

As previously mentioned, *S. coelicolor* was employed to target crucial genes involved in the biosynthetic route of actinorhodin production (Figure 3). actVA-ORF4 (SCO5079), actVA-ORF5 (SCO5080), actII-ORF2 (SCO5083), actI-ORF2 (SCO5088), actIV (SCO5091), actI-ORF4 (SCO5090), actIII (SCO5086) genes from these strains were selected as a target. The actII-ORF4 (SCO5085) is 768 nucleotides long. It contains two specific regions of interest: target 8, which is located in position 628–646 within the actII-ORF4 (SCO5085) sequence, and target 9, which is located in position 510–529. The actVA-ORF4 (SCO5079) is a gene that spans 885 bp long and has four specific targets as follows: target 10 is located

TABLE 3 Target sequence position in *S. coelicolor* NBRC15146 genome.

Name	Sequence nucleotide	Pam	Position
Target 8 (5′–3′)	CAGATGGGCAGGCGCGCCG	AGG	5.528.721-5.528.739
Target 9 (5′–3′)	TATCGACGCGAATTGGCGGC	TGG	5.528.603-5.528.622
Target 10 (5′–3′)	AGCAAGGCGGCTCCGCGGCC	CGG	5.520.903-5.520.922
Target 11 (5′–3′)	GCATCCAAACGCCGATGACAC	CGG	5.521.086-5.521.106
Target 12 (5′–3′)	CAAACGCCGATGACACCGGC	CGG	5.521.091-5.521.110
Target 13 (3′–5′)	CTCCCGCACTTGACCGCCGA	CGG	5.521.217-5.521.236
Target 14 (5′–3′)	CCAGGAGCGGCCGTCCCTGA	CGG	5.521.758-5.521.777
Target 15 (5′–3′)	TCCAGGGGCGCGAACGCGGC	TGG	5.524.827-5.524.846
Target 16 (3′–5′)	GGGATGCGCGCGGCAACC	CGG	5.524.178-5.524.195
Target 17 (5′–3′)	CCAGGACGCGAAGGCGGACC	CGG	5.531.449-5.531.468
Target 18 (3′–5′)	CCGGGAGTGGCCCGAGACCG	AGG	5.533.961-5.533.980
Target 19 (3′–5′)	TTGCGAAGGCCTAGACCCGC	CGG	5.532.860-5.532.879
Target 20 (3′–5′)	GGGCGGAACTCCTGGACAAA	CGG	5.529.335-5.529.354

at position 46–66, target 11 is located at position 230–250, target 12 is located at position 235–254, and target 13 is located at position 361–380. The actII-ORF2 (SCO5083) is 1737 bp long and contains two specific targets: target 15 at position 755–774 and target 16 at position 106–123. The actI-ORF2 (SCO5088) is 1224 bp long and has only one target (target 17) located at position 249–268. The actIV (SCO5091) is 894 bp long and has only one target (target 18) located at position 309–328. The actI-ORF4 (SCO5090) is 951 bp long and has only one target (target 19) located at position 155–174 bp. All target positions are listed in Table 3.

We have designed 13 sgRNAs toward various gene targets, with the position being approximately—17–20 nucleotides upstream from the PAM sequence. The pGM1190 vector, which carries our CRISPR system with the target, is introduced into the *S. coelicolor* strain using conjugation. The empty vector pGM1190-dCas9-PmCDA-UGI-AAV/LVA was used as a control, without any target inserted, following the same approach. The tipA promoter strictly regulates this mechanism. Thiostrepton functions as an inducer to facilitate the operation of this system. As a result, we transferred the exconjugant from the ISP4 medium to the ISP2 medium, which contains thiostrepton. Subsequently, we examined the editing efficiency using target region PCR and DNA sequencing in colonies that did not generate a blue color. Only one of the created targets underwent a successful mutation in a single nucleotide with stop-codon creation (Figure 4B,C). The mutant strain CrA10, which has already inserted plasmid pGM1190-dcas9-pmCDA-UGI-AAV-target 10 [actVA-ORF4 (SCO5079)], demonstrated a lack of actinorhodin production. The strains corresponding to the other 12 sgRNAs have mutations in a single nucleotide but did not create a stop codon that could knock out the expression of respective genes. Therefore, these mutants were still available to produce ACT (data not shown).

The actVA ORF4 gene, which is a part of the actVA-actVB system found in *S. coelicolor*, plays a crucial role in the two-component flavin-dependent monooxygenase system that is responsible for the production of actinorhodin (ACT). More precisely, it promotes the dimerization of a benzoisochromanequinone (BIQ) intermediate in ACT synthesis. The disruption or knockout of the actVA ORF4 gene leads to a cessation of ACT production, underscoring its crucial role in ACT biosynthesis and its association with cellular energy regulation. When the actVA-ORF4 is absent, a genetic variant generates a modified form of hydrokalafungin called 8-hydroxy-DHK, which shares structural similarities with the individual unit of ACT. This indicates that the gene is implicated in the intermediate dimerization of BIQ through C-C bond [36, 37]

The streak results on the ISP2 medium demonstrated that the designed system successfully suppressed actinorhodin production in the CrA10 mutant, as indicated by the observed phenotype. Conversely, strains harboring control vectors (pGM1190-dCas-PmCDA-UGI-AAV/LVA without the target) continued to produce actinorhodin, as depicted in Figure 4A. To validate our initial findings from the agar plate, we cultivated the clones in a liquid basal medium and used UV-visible spectroscopy to quantify the amount of actinorhodin produced. Notably, actinorhodin exhibits a color shift from blue to red when exposed to acidic conditions (pH of 2), as depicted in Figure 5A. We performed absorbance measurements at various wavelengths, and the results are depicted in Figure 5B. These results showed that the absorbance peaks at 500 nm were prominent in both the wild-type and empty vector samples. However, the mutant CrA10 (target 10) displayed markedly reduced absorbance values compared to the wild-type

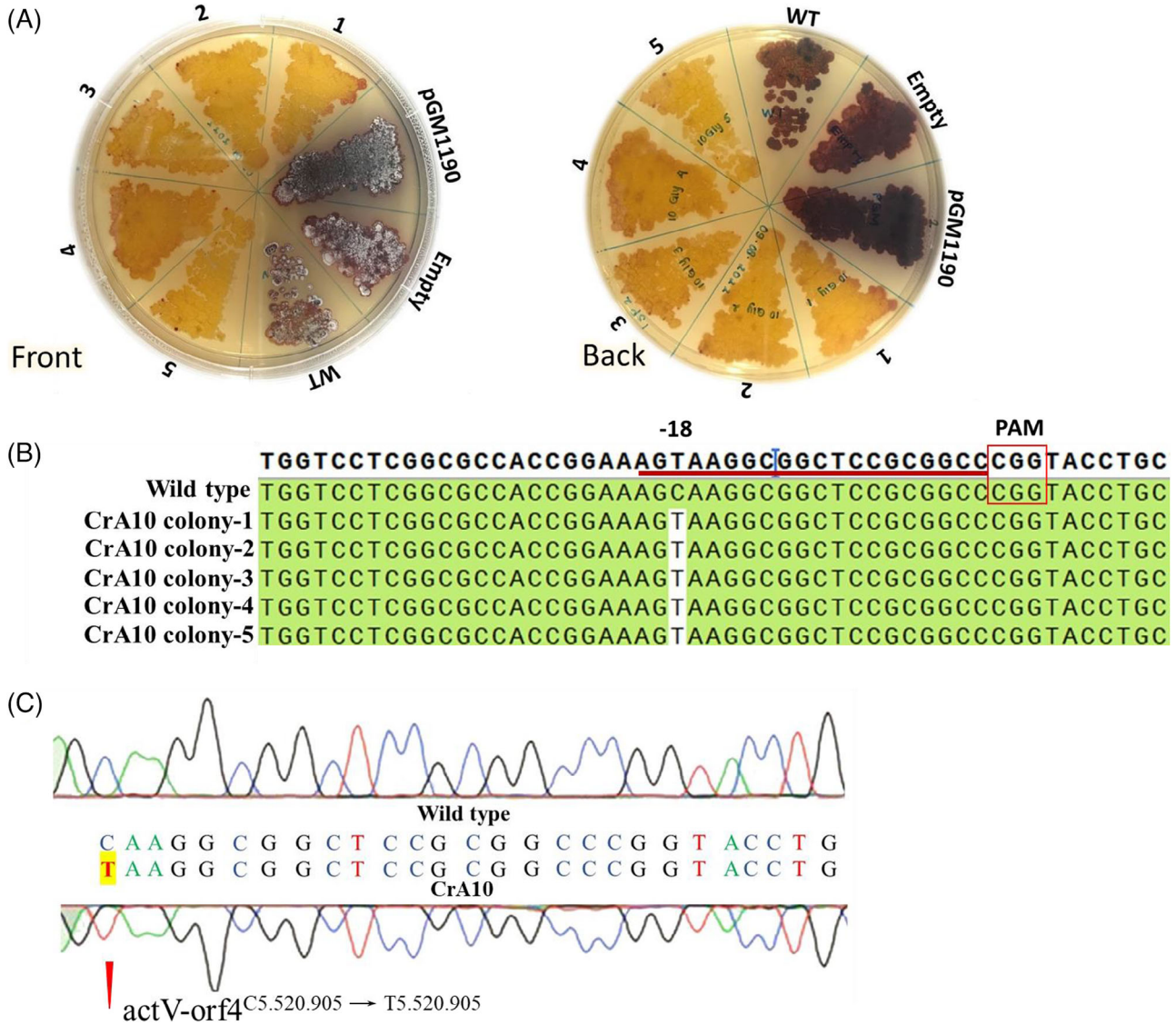


FIGURE 4 Genome editing using dCas9-PmCDA-UGI-AAV system. (A) Phenotypical analysis of five mutants selected and grew on ISP2 medium agar, the mutant with targeted actVA-ORF4. Wild-type *S. coelicolor*, pGM1190, and Empty vector (dcas9-PmCDA-UGI-AAV without target) were used as control experiments. (B) Sequence alignment between Wild type and target 10 (actVA-ORF4) edited by dCas9-PmCDA-UGI-AAV. The PAM sequence is shown in the red box, and the site targeted by sgRNA is indicated by a brown underline. Uncolored bases are seen in single mutation results. (C) A typical sequencing chromatogram showing the mutation from C to T in the target location. *S. coelicolor* wild-type strain was used as a control.

and empty vector samples. The substantial reduction in absorbance indicates that this approach successfully targeted the actVA-ORF4 (SCO5079) genes and inactivated its expression, leading to the inhibition of actinorhodin biosynthesis.

3.3 | Characterization of mutation and off-targets

To facilitate the conjugation from *E. coli* to *Streptomyces*, pUB307 is required. This is achieved through the mating process, where pGM1190, which contains the AID-system vector, is mobilized into *E. coli* HST04. The plasmid pUB307 enables the AID-system vector to translocate into *Streptomyces* spore. To initiate spore germination, the *S. coelicolor* spore was subjected to incubation at 50°C for 15 min, mixed with *E. coli* HST04/pUB307, pGM1190, and then spread on the ISP4 agar plates. After 18 h, overlayed each plate with 3 mL upper layer NB medium (consisting of NB 0.8% and soft agar 0.5%) containing Aztreonam 50 μg/mL, Nalidixic acid 5 μg/mL, and Apramycin 50 μg/mL.

Genome editing using the CRISPR system is one of the revolutionary techniques in metabolic engineering.

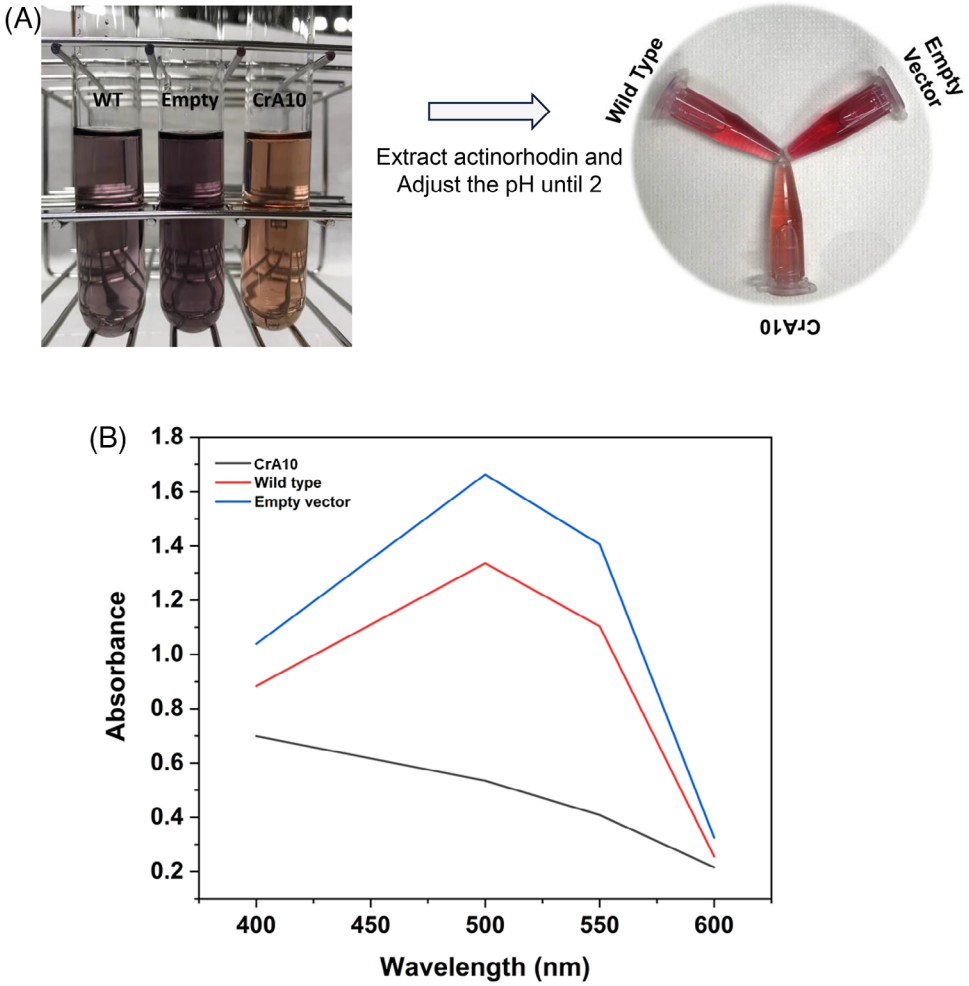


FIGURE 5 The actinorhodin color differences from the cultures of WT (Wild type), Empty (plasmid CRISPR system with no expected target), Target 10 (plasmid CRISPR system with 20 nucleotides from gen actVA-ORF4 as targets) At a pH of 2, actinorhodin undergoes a color change, shifting from its initial blue to a distinct red are depicted in the photograph (A) and observed at various absorbances (B).

In our study, we developed the CRISPR system by combining the dead cas9 protein (dCas9), cytidine deaminase (PmCDA1) from *Petromyzon marinus*, the uracil glycosylation inhibitor (UGI), and protein degradation tag AAV. The dCas9 gene, which has been optimized for codon usage, is controlled by the promoter from the tipA gen. The activation of this gene occurs after being induced by thiostrepton [38, 39]. The presence of the ermE promoter is necessary for expressing gRNA [29, 40]. Besides that, the apolipoprotein B mRNA-editing catalytic family includes another member called Activation-induced Cytidine Deaminase (AID). AID is an RNA editor that can convert the nucleotide C to U in DNA, leading to mutations in both DNA and RNA. It is also capable of converting deoxycytidine to deoxyuridine. Increasing the number of UGI copies in the C terminus does not enhance editing efficiency. However, including a single copy of UGI at the C terminus can enhance the editing level by three to ten-fold, as demonstrated in the absence of UGI [41].

Understanding and reducing off-target activity in genome editing is especially crucial because these impacts cannot just be momentary occurrences but also impact the longevity of modified cells, necessitating the need for off-target detection [42]. Formerly, the T7 endonuclease assay was the method of choice for detecting off-targets, but it has limited sensitivity and cannot confirm the existence of less than 1% [43, 44]. Several software tools have been developed in recent years to anticipate and identify CRISPR off-target regions, using methods such as Digenome-seq [45], CIRCLE-seq [46], GUIDE-seq [47], and others. The initial off-target detection algorithm identified potential off-target sites based on the sgRNA sequence.

We closely examined the complete genome sequence of CrA10 laboratory strains and compared it with the reference genome to investigate off-target potential caused by the target-AID system. We prioritize the study of pivotal genes implicated in the synthesis of actinorhodin.

(A)

ActVA-ORF4 SCO5079 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	0	0	0
T	1	0	0	0
A	0	0	0	1
G	0	0	0	0

ActVA-ORF5 SCO5080 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	2	2	4
T	2	0	1	1
A	1	0	0	2
G	7	2	0	0

ActII-ORF2 SCO5083 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	5	11	16
T	5	0	2	3
A	7	2	0	5
G	18	3	6	0

ActII-ORF4 SCO5085 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	0	0	3
T	0	0	0	0
A	0	0	0	0
G	0	0	0	0

ActIII SCO5086 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	3	2	7
T	2	0	0	1
A	2	0	0	1
G	5	1	2	0

ActI-ORF2 SCO5088 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	0	0	0
T	0	0	0	0
A	0	0	0	0
G	0	0	0	0

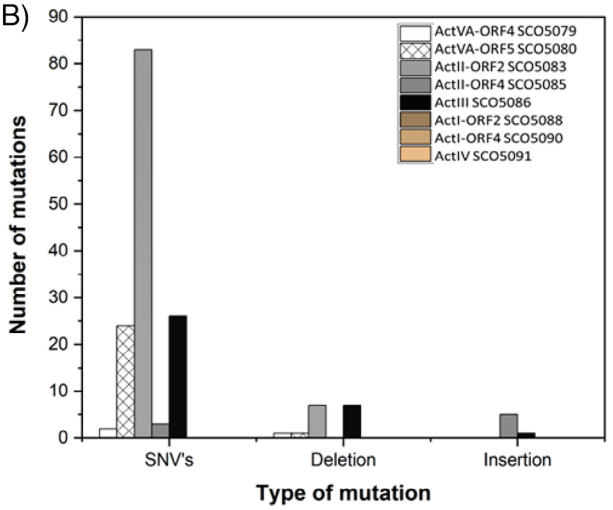
ActI-ORF4 SCO5090 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	0	0	0
T	0	0	0	0
A	0	0	0	0
G	0	0	0	0

ActIV SCO5091 Mutant vs WT

TO \ FROM	C	T	A	G
C	0	0	0	0
T	0	0	0	0
A	0	0	0	0
G	0	0	0	0

(B)



(C)

Gene	Mutation from	Mutation to	PAM	Position
ActVA-ORF4 SCO5079	C	T	CGG	5573921
	G	A	GGG	5573952
ActII-ORF2 SCO5083	A	C	CGG	5577904
	C	G	AGG	5578657
	C	G	GGG	5578749
ActIII SCO5086	G	C	CGG	5582313
	C	G	CGG	5582377

FIGURE 6 The outcome of assessing mutations in the open reading frame (ORF) genome plays a crucial role in producing actinorhodin. (A) The mutated gene critical for actinorhodin production was analyzed and compared to the reference sequence from the *S. coelicolor* wild type. The study recorded the total count of single nucleotide variants (SNVs) in this analysis. (B) The number of mutations based on the mutation types, including SNVs, deletions, and insertions, were observed in the critical genes. (C) The single nucleotide variations (SNPs) are observed in multiple ORFs, these mutations do not result in the creation of a stop codon.

Our detailed analysis of single nucleotide variants (SNVs) revealed various gene mutations. Specifically, we identified two mutations in astVA-ORF4 (SCO5079), 24 mutations in actVA5 (SCO5080), 83 mutations in actII-ORF2 (SCO5083), 3 mutations in ActII-ORF4 (SCO5085), and 26 mutations in actIII (SCO5086). Interestingly, three genes, actI-ORF2 (SCO5088), actI-ORF4 (SCO5090), and actIV (SCO5091), exhibited no alterations. We also noted the occurrence of deletions and insertions in several other genes, as outlined in Figure 6B. The significant finding was the prevalent substitution of C residue with T residue, as depicted in Figure 6A. This pattern strongly indicates that the functional activity of the Target-AID system caused these SNVs. Significantly, the Geneious program failed to anticipate these unintended consequences and did not correspond to any of the spacers we used. To confirm these findings, we re-examined the mutation position relative to the PAM sequence. However, the validation revealed a limited number of alterations, none of which resulted in the insertion of a stop codon, as depicted in Figure 6C.

4 | CONCLUDING REMARKS

This study investigates the utilization of a CRISPR-dCas9-AID-UGI-AAV/LVA fusion system in *Streptomyces* strains. The system is implemented using a high copy number vector, pGM1190, which contains unique genetic control elements. The approach efficiently targeted certain genes in *S. coelicolor*, resulting in the inhibition of actinorhodin synthesis. The procedure entailed meticulous genome editing followed by analysis to evaluate off-target impact, unveiling unforeseen changes in many genes. The findings illustrate the effectiveness of the system in

specifically disrupting genes, while simultaneously underscoring the significance of comprehensive examination for unintended genetic alterations. This underscores both the possibilities and difficulties associated with advanced genome editing methods in bacteria.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ORCID

Pamella Apriliana https://orcid.org/0000-0002-5455-5365
Prihardi Kahar https://orcid.org/0000-0003-1006-2283
Chiaki Ogino https://orcid.org/0000-0002-8906-7724

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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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