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Original Research Article

Enhancing rufomycin production by CRISPR/Cas9-based genome editing and promoter engineering in *Streptomyces* sp. MJM3502

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ABSTRACT

Streptomyces sp. MJM3502 is a promising producer of rufomycins, which are a class of potent anti-tuberculosis lead compounds. Although the structure, activity, and mechanism of the main rufomycin 4/6 and its analogs have been extensively studied, a significant gap remains in our understanding of the genome sequence and biosynthetic pathway of *Streptomyces* sp. MJM3502, and its metabolic engineering has not yet been reported. This study established the genetic manipulation platform for the strain. Using CRISPR/Cas9-based technology to in-frame insert the strong *kasO*p* promoter upstream of the *rufB* and *rufS* genes of the rufomycin BGC, we increased rufomycin 4/6 production by 4.1-fold and 2.8-fold, respectively. Furthermore, designing recombinant strains by inserting the *kasO*p* promoter upstream of the biosynthetic genes encoding cytochrome P450 enzymes led to new rufomycin derivatives. These findings provide the basis for enhancing the production of valuable natural compounds in *Streptomyces* and offer insights into the generation of novel active natural products via synthetic biology and metabolic engineering.

1. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis,* which remains a global public health threat, with mortality rates higher than those caused by human immunodeficiency virus (HIV) [1]. The prevention and treatment of TB are further complicated by the emergence and rapid dissemination of multidrug-resistant and extensively drug-resistant strains [2]. Therefore, it is of great importance to develop novel natural products with anti-TB activities. More recently approved anti-TB agents include bedaquiline, sutezolid, levofloxacin, and clofazimine offer improved efficacy with reduced toxicity [3]. However, the long duration and possible serious side effects of TB treatment regimens necessitate research for alternative therapeutic targets and more selective inhibitors, particularly for drug-resistant strains [4].

One recently emerging anti-TB drug target is the caseinolytic protein C1 (ClpC1), which is essential for the survival of *M. tuberculosis* [5–9]. The rufomycins are a class of actinomycete natural products, also known as ilamycins [10–17], with prominent anti-TB as well as anti-cancer activities [18]. The rufomycins have become promising leads that target cellular proteostasis via ClpC1 [19–24]. The main congener, rufomycin 4/6, is an anomeric pair of cyclic peptides containing seven

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amino acids that is effective against both *M. tuberculosis* (0.02 μM of MIC90) and *M. abscessus* (0.4 μM of MIC90) [25–28]. Importantly, rufomycins decrease the proteolytic capabilities of the proteolytic complex without affecting the ATPase activity of ClpC1 [21]. Several naturally existing rufomycin analogs have been discovered [29–31]. Among these, the rufomycins 4/6 (same calcd for $C_{54}H_{76}N_9O_{12}^+$, 1042.5608) exhibit robust and selective anti-tuberculosis activity against *M. tuberculosis* H37Rv [19,30]. However, the initial fermentation conditions for the *Streptomyces* sp. MJM3502 producing rufomycins 4/6 only showed a low titer of 125 mg/L (data not shown), which makes translational drug development challenging. Therefore, increasing the titer and scaling up the production of rufomycin and its analogs is an important goal, and efforts have been made to enhance the titer of rufomycin through fermentation optimization [32–34]. Other means of improving the products involve metabolic engineering, including the deletion of negative regulator genes, such as the LysR family of transcriptional regulator genes [35], or the overexpression of positive regulators and the global regulator *bldD* [34,36]. Despite these efforts, rufomycin titers still have not reached levels conducive to industrial production.

Rufomycin biosynthesis involves a hybrid polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) assembly, which suggests that the efficient expression of these biosynthetic genes may hold the key to achieving higher yields [37]. Surprisingly, prior metabolic engineering studies have not comprehensively explored the efficient expression of biosynthetic rufomycin genes. Genome mining has led to the discovery of a wealth of cryptic biosynthetic gene clusters (BGCs), and promoter engineering has become a key tool in such studies aimed at improving the production or uncovering new natural products (NPs) from microorganisms [38,39]. As most metabolic gene clusters in *Streptomyces* remain un- or under-expressed under standard laboratory fermentation conditions [40,41], promoter engineering may enable the activation of silent BGCs through promoter refactoring and regulator manipulation, among other manipulations [42–44]. The high-efficiency promoter, *kasO*p*, can assist in gene overexpression and has been demonstrated to enhance transcription and protein expression in various *Streptomyces* host strains [45–47]. Promoter engineering has been successfully used to activate type I, II, and III PKSs; NRPS; hybrid PKS-NRPS; and phosphonate BGCs in multiple *Streptomyces* species [48–51]. Substituting the native with a well-characterized promoter is a widely employed promoter-engineering strategy to boost metabolite production [52–55].

Bacterial CRISPR/Cas9 genome-editing technology has revolutionized genetic manipulation in diverse organisms, including *Streptomyces* as well-known antibiotic producers [56–58]. This simple, cost-effective, and highly accurate approach has been used to edit DNA fragments, modify BGCs, and perform gene replacement and site-directed mutagenesis. Since its introduction in *Streptomyces* by Cobb et al. [59], CRISPR technology has been applied to a variety of model strains, such as *S. coelicolor*, *S. albus*, and *S. lividans*, as well as to many genetically recalcitrant, non-model organisms such as *Micromonospora chersina*, *S. formicae*, and *S. rimosus* [60–64]. However, no studies have yet been performed on the rufomycin producer, probably due to the low efficiency of genome editing.

To enhance rufomycin yields, this study established a genetic manipulation system in *Streptomyces* sp. MJM3502 that introduces the *kasO*p* cassette containing the strong constitutive promoter into six key promoter sites in the rufomycin BGC using CRISPR/Cas9 genome-editing technology. The developed strategies can potentially be adopted for other microbial strains of interest.

2. Materials and methods

2.1. Strains, plasmids, media, and culture conditions

The strains and plasmids used in the present study are listed in Table S1. Plasmids were propagated in *Escherichia coli* DH5α (TransGen Biotech, Beijing, China) cultured in Luria-Bertani (LB) broth with 50 μg/mL of apramycin at 37 °C. MS agar medium (2.0 % soy flour, 2.0 % mannitol, and 2.0 % agar with the addition of 10 mM of $MgCl_2$) was used for intergeneric conjugation between *E. coli* ET12567/pUZ8002 and the *Streptomyces* sp. MJM3502 strain [65]. *Streptomyces* sp. MJM3502 and their derivatives were cultivated and maintained on ISP4 plates for spore preparation. The ISP2 medium was used for the seed culture. Regarding the isolation and analysis of the metabolites, the *Streptomyces* sp. MJM3502 strain was incubated in a 3502-fermentation medium (2.0 % glucose, 7.0 % soluble starch, 1 % corn steep powder, 2.5 % soil bean flour, 0.25 % $(NH_4)_2SO_4$, 0.2 % $CaCO_3$, 0.1 % K_2HPO_4, 0.25 % $MgSO_4{\cdot}7H_2O$, 0.0005 % $ZnSO_4$, and 0.05 % NE-400) at 28 °C, 230 rpm, for 7.5 days.

2.2. Genome sequencing and bioinformatics analysis

The genomic DNA of *Streptomyces* sp. MJM3502 was extracted using a MiniBEST bacterial genomic DNA extraction kit (Takara, Tokyo, Japan). Sequencing was conducted by Bionics (Seoul, South Korea) using a combination of second-generation 454 and Illumina HiSeq 4000 sequencing technologies and third-generation PacBio sequencing technology. The secondary metabolites of *Streptomyces* sp. MJM3502 were analyzed using antiSMASH software [66]. Open reading frames (ORFs) in the rufomycin BGC were automatically predicted using Glimmer, which is available on the National Center for Biotechnology Information (NCBI) website. The gene cluster for rufomycin was deposited in GenBank CP121214. To investigate the function of rufomycin biosynthesis gene clusters, manual analysis using BLASTp was performed. To determine the genetic relatedness of *Streptomyces* sp. MJM3502 to closely related strains, an Average Nucleotide Identity (ANI) analysis was conducted using Orthologous Average Nucleotide Identity Tool (OAT) software [67]. To investigate the genomic organization and synteny among the genomes, collinearity analysis was conducted using the MAUVE version 2.4.0 alignment tool [68,69].

2.3. Construction of powerful promoter knock-in mutants using CRISPR/Cas9-based genome editing

The manipulations to construct the gene-inactivation and promoter-engineering plasmids were carried out in *Escherichia coli* DH5α. All primers for genetic manipulation are listed in Table S2. Restriction enzymes, PrimeSTAR Max DNA Polymerase, and In-Fusion HD Cloning kits were obtained from Takara Bio (Takara, Tokyo, Japan). For knock-in of the promoter in *Streptomyces* sp. MJM3502, the combined CRISPR/Cas9-CodA(sm) system was used according to a literature protocol [70]. Briefly, CasOT software was used as a genome-wide Cas9/sg-RNA off-target searching tool to select and identify the sg-RNA sequence [71]. Then, the protospacer (sg-RNA) of a target cluster was inserted in linearized pWHU2653 via B*ae*I-mediated Golden Gate Assembly. The promoter cassette containing the upstream homology arm, i.e., the *kasO*p* promoter, and the downstream homology arm was assembled via overlap PCR and subsequently inserted in a linearized construct containing the protospacer using the In-Fusion method to construct genome-editing plasmids for promoter engineering strategies.

The correct constructs were then introduced into *E. coli* ET12567/pUZ8002 for conjugation with *Streptomyces* sp. MJM3502. The conjugation processes were conducted in accordance with the procedure described in the literature [65]. To distinguish the genotypes of wild-type (WT) and successful genome-editing mutants, PCR and restriction enzyme analysis were conducted. Furthermore, positive samples were purified using a Universal DNA Purification Kit (Tiangen Biotech, Beijing, China) and confirmed by sequencing (Bionics, Seoul, South Korea).

2.4. Production, extraction, and UPLC-MS/MS analysis of metabolites

Seed cultures (25 mL ISP2 in 250 mL baffled flasks) of *Streptomyces* sp. MJM3502 were incubated at 28 °C with 230 rpm for 24–48 h until achieving high particle density. The seed cultures were inoculated into 100 mL of 3502 fermentation media in the 500 mL baffled flask and incubated at 28 °C for 7.5 days. The whole broth, including liquid broth and solid cell mass, was extracted with ethyl acetate. The extracts of the fermentation products were dried, resuspended in methanol, and filtered through a 0.22 μm syringe filter before analysis by HPLC. HPLC analysis was conducted using a YMC-Pack ODS-A column (5 μm, 4.6 × 250 mm) with UV detection at 215 and 285 nm. The mobile phases were 55 % aqueous acetonitrile solution buffered in 20 mM of sodium phosphate (pH 8.0). UPLC-MS/MS was performed in the positive auto MS(n) mode with a scan range of 150–1500 *m/z*.

2.5. RNA isolation, real-time quantitative PCR (RT-qPCR)

WT and engineered *Streptomyces* sp. MJM3502 strains were cultivated in ISP2 at 28 °C, 230 rpm, for 48 h. The total RNAs were isolated using a MiniBEST Universal RNA Extraction kit (TaKaRa, Tokyo, Japan). The RNA samples were treated with a gDNA Eraser (Takara, Tokyo, Japan) and checked with PCR to eliminate the possibility of chromosomal DNA contamination. The complementary DNA was synthesized with a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). RT-qPCR was performed on a Roche LightCycler 480 using SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan). The housekeeping gene *hrdB* was used as a constitutive reference to normalize the gene expression of each target gene. Gene-specific primers were designed and synthesized by TaKaRa bio (TaKaRa, Tokyo, Japan) and listed in Table S2.

2.6. Statistical analysis

To evaluate the enhancement, rufomycin production levels were tabulated and compared across all strains. Statistical significance was determined using Prism 8 software. The quantified rufomycin levels were compared to calibration standards based on peak area data from the HPLC results. The titer of rufomycin in engineered strains was then compared to that of the wild-type strain, using the same batch of culture. Significant differences are indicated as *P $<$ 0.05, **P $<$ 0.01, and ***P $<$ 0.001. Data are presented as mean ± s.e.m., with three biologically independent samples per condition unless otherwise specified in the figure legends. The relative transcription level of each gene was evaluated as the ratio of WT and engineered strain transcripts using the $2^{-\Delta\Delta Ct}$ method. All data represent the means ± SD of at least three biological repeats per condition. $p < 0.05$ was considered significant.

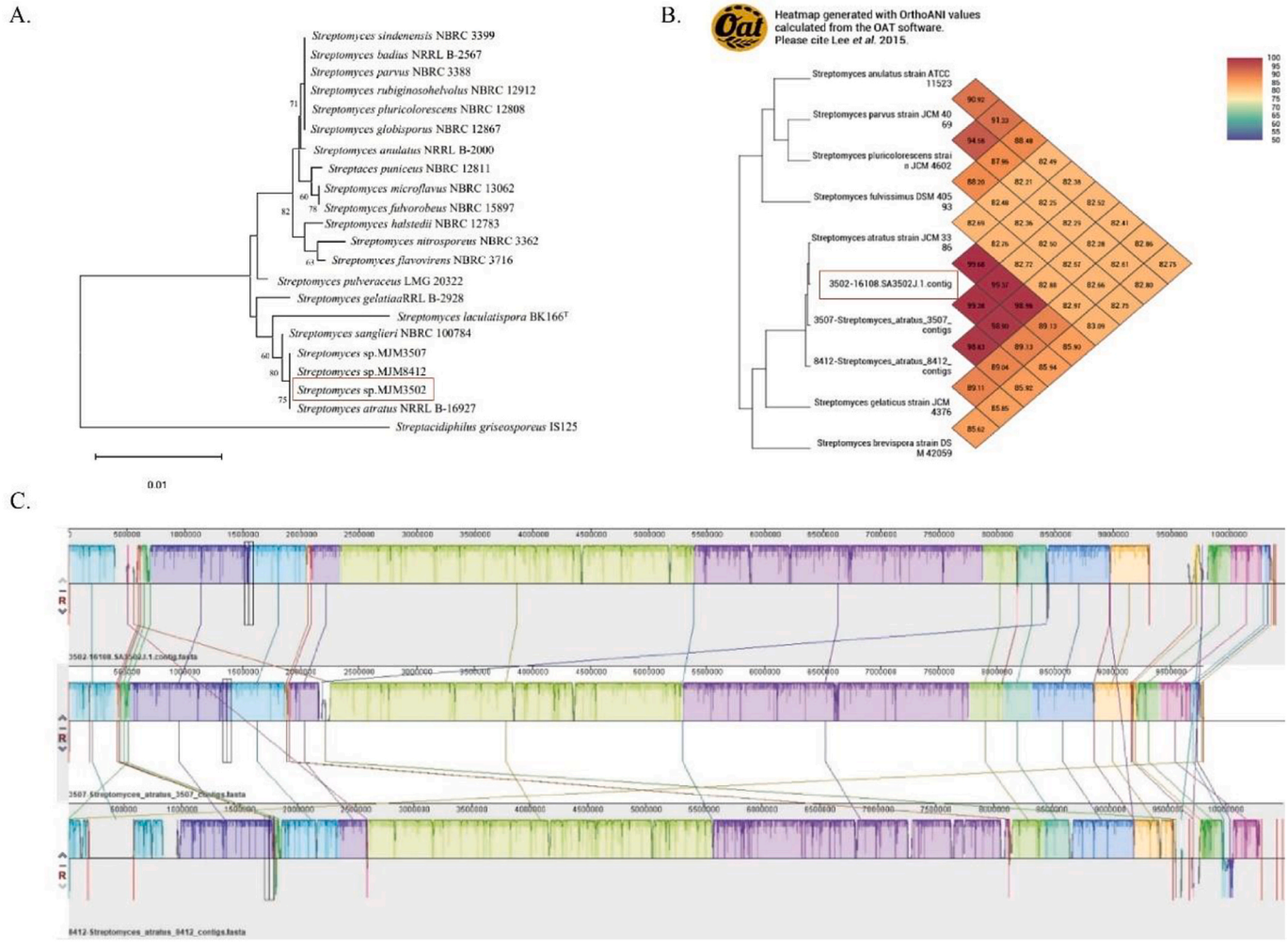


Fig. 1. Phylogenomic analysis of *Streptomyces* sp. MJM3502. (A) Phylogenetic analysis based on the 16s rRNA sequence. (B) A heatmap generated with OrthoANI values calculated using OAT software. (C) A collinearity diagram of *Streptomyces* sp. MJM3502 with *Streptomyces* sp. 3507 and *Streptomyces* sp. 8412. Each colored block (called locally collinear blocks) represents a region of a homologous sequence that aligns to part of another genome and is free from internal rearrangement. The colored lines indicate which blocks in each genome are homologous.

3. Results and discussion

3.1. Strain identification and classification based on genome sequencing

Genome sequencing is important for discovering new NPs and studying their biosynthesis. The *Streptomyces* sp. MJM3502 is a producer of the anti-Mtb rufomycins. To fully understand its biosynthetic potential, we sequenced its genome. The fully assembled genome consists of a linear chromosome with 9,314,038 bps and an average GC content of 69.60 %. The genome contains 8221 coding sequences, 18 rRNA genes, and 74 tRNA genes and has been deposited into Genbank (Accession number CP121214).

A phylogenetic analysis based on 16S rRNA gene sequences of the NJ tree revealed that *Streptomyces* sp. MJM3502 is most closely related to *Streptomyces atratus* NRRL B-16927 (DQ026638.1) (Fig. 1A). Additionally, *Streptomyces* sp. MJM3502 falls into the same clade as other known rufomycin-producing strains, *Streptomyces* sp. MJM3507 and *Streptomyces* sp. MJM8412 discovered in our laboratory [72]. Furthermore, a genome-scale phylogenetic tree was constructed using CVTree, and an OAT heat map was generated (Fig. 1B). Based on the whole-genome comparison, an ANI value score higher than 95 % indicates that the strains belonged to the same species [73]. *Streptomyces* sp. MJM3502 exhibited an ANI value of 99.68 % with *S. atratus* ATCC 14046 (also known as *S. atratus* JCM 3386 with NCBI Taxonomy ID: 1893). Therefore, it is phylogenetically closest to *S. atratus*. Importantly, rufomycin, whose chemical structure was first reported in the 1960s, was also isolated from *S. atratus* ATCC 14046 [11]. Another strain, SCSIO ZH16, was also phylogenetically classified as *S. atratus* [74]. Furthermore, collinearity analysis using the MAUVE alignment tool validated the genomic relationship of all three rufomycin-producing strains (Fig. 1C). *Streptomyces* sp. MJM3502 genome showed high collinearity with *Streptomyces* sp. 3507 and *Streptomyces* sp. 8412, indicating strong genomic similarities and further supporting their close phylogenetic relationship.

3.2. Identification of rufomycin BGC in Streptomyces sp. MJM3502

To identify the putative rufomycin BGC, we analyzed the genomic sequence of *Streptomyces* sp. MJM3502 and predicted the biosynthesis mechanism of rufomycin. The analysis revealed the possibility of 29 BGCs presented in the genome, including 7 non-ribosomal peptides, 5 polyketide synthases, 1 PKS-NRPS heterozygote, 5 lanthipeptides, 5 terpenes, and 6 other types of secondary metabolite BGCs (Fig. 2A, Table S3). Additionally, the predicted region 27 shows a match with all core genes, including the NRPS of bombyxamycin A (GenBank MK433001.1) [75,76]. The presence of these clusters suggests a far greater potential for producing specialized natural products than previously appreciated.

Previous studies have identified the BGCs of the rufomycins in two other *Streptomyces* strains: *S. atratus* ATCC 14046 [12] and SCSIO ZH16 [77]. A comparison of the rufomycin BGC encoded proteins in all three strains was conducted and the functions of the open reading frames (ORFs) were investigated (Fig. 2B–Table 1). The organization of the rufomycin BGC includes the multifunctional PKS and NRPS, and additional ORFs encoding modification enzymes are centralized between the PKS and NRPS. The comparative sequence analysis of the rufomycin BGC showed 97 % and 98 % identity at the amino acid level between *Streptomyces* sp. MJM3502 and the other two strains, respectively. While rufomycin biosynthesis in MJM3502 may be similar to that in *S. atratus* SCSIO ZH16, the two strains also produce different rufomycin analogs [77] (Fig. 2C). Initially, four rufomycin analogs similar to cyclopeptides initially reported as ilamycins, with two novel structure analogs (ruf 51 and 52) were isolated from the MJM3502 WT strain. The MIC values of 52 against *M. tuberculosis* is 0.58 μM [19,28]. Moreover, 20 naturally occurring rufomycin analogs [28,29] were identified from the MJM3502 strain out of the 44 structurally characterized rufomycins [35,78,79].

3.3. The spore conjugation and CRISPR/Cas9-based genome editing of Streptomyces sp. MJM3502

To facilitate the genetic manipulation of *Streptomyces* sp. MJM3502, we optimized spore production and conjugation methods. Previous attempts to use mycelia for conjugation with *E. coli* were hampered by low knockout efficiency and difficulty distinguishing exconjugants from dead mycelia. Spores of *Streptomyces* species are highly resistant to adverse conditions such as desiccation, UV radiation, and chemical stress [80]. These spores are known to have higher transformation efficiency compared to mycelial fragments or protoplasts in many *Streptomyces* species [81]. Therefore, we optimized the culture conditions to obtain abundant spores on modified ISP2 medium plates. *Streptomyces* sp. MJM3502 was maintained in the modified No. 2 medium with reduced concentrations of yeast extract, malt extract, and glucose and incubated for 14 days to induce sporulation. The pH was adjusted to 7.0 to further induce sporulation. Notably, it was important to maintain dry agar plates at 25 °C for 24 h before inoculation with fresh mycelia to promote spore formation. Using the collected spores, we observed a notable enhancement in conjugation efficiency compared to mycelium-based methods. Specifically, spore-based conjugation yielded an efficiency approximately 80 % higher than mycelium-based conjugation. Conjugation efficiency was calculated as the ratio of exconjugants to the total number of recipient cells plated, as determined by colony counts. We then successfully used CRISPR/Cas9-based genome editing to manipulate the *Streptomyces* sp. MJM3502 genome (Fig. 3). On the other hand, spores facilitate a cleaner background during genetic manipulation experiments, which improves the accuracy of selecting and screening for desired genetic modifications, reducing false positives and streamlining the downstream analysis. These results demonstrated the potential for genetic manipulation of this strain and the importance of optimizing spore production and conjugation methods for efficient genome editing in *Streptomyces* sp. MJM3502.

3.4. Promoter engineering for increasing rufomycin production

The structural composition of rufomycin 4/6 includes three non-proteinogenic amino acids: *N*-(1,1-dimethyl-1-allyl)-tryptophan (i), L-2-amino-4-hexenoic acid (L-AHA) (ii), and 3-nitrotyrosine (3NTyr) (iii) [77,82–84]. The biosynthetic pathway initiates with the individual synthesis of these amino acids. (i) *rufO* converts tryptophan to *N*-(1,1-dimethyl-1-allyl)-tryptophan. (ii) *rufD, rufE, rufF*, and *rufH*, likely via a putative operon, collaborate in the production of L-AHA. (iii) A cytochrome P450 enzyme, *rufN*, catalyzes the 3-nitration of tyrosine in conjunction with *rufM*, the nitric oxide synthase responsible for NO production, resulting in the formation of 3-nitrotyrosine. The genes *rufM* and *rufN*, also forming a putative operon, play a pivotal role in 3NTyr synthesis [34,77,82]. Subsequently, these non-proteinogenic amino acids are assembled by the NRPS, *rufS*.

To enhance rufomycin production in a native *Streptomyces* strain, promoter engineering was employed using two criteria for identifying potential promoter sites in the rufomycin BGC. Seven putative operons controlled by either bidirectional or unidirectional promoters were predicted to exist within the BGC. Six key proposal promoter sites located upstream of the genes *rufB*, *rufD*, *rufE*, *rufS*, *rufG*, and *rufM* were chosen for manipulation based on the gene function and biosynthesis mechanism (Fig. 4A). Since the PKS and NRPS genes are the main biosynthetic operon of rufomycin BGC, and the Streptomycin biosynthesis operon regulator is putative as positive regulator in rufomycin biosynthesis, we first inserted *kasO*p* upstream of the *rufE*, *rufS* and *rufE* genes.

To achieve this, the strong promoter *kasO*p* was introduced into the proposed promoter gene sequences in the *Streptomyces* sp. MJM3502 strain through a combined CRISPR/Cas9-CodA(sm) system (Fig. 4B). The gene-editing system resulted in the creation of genetic operations and high levels of knock-in efficiency (Fig. 4C). The mutants were

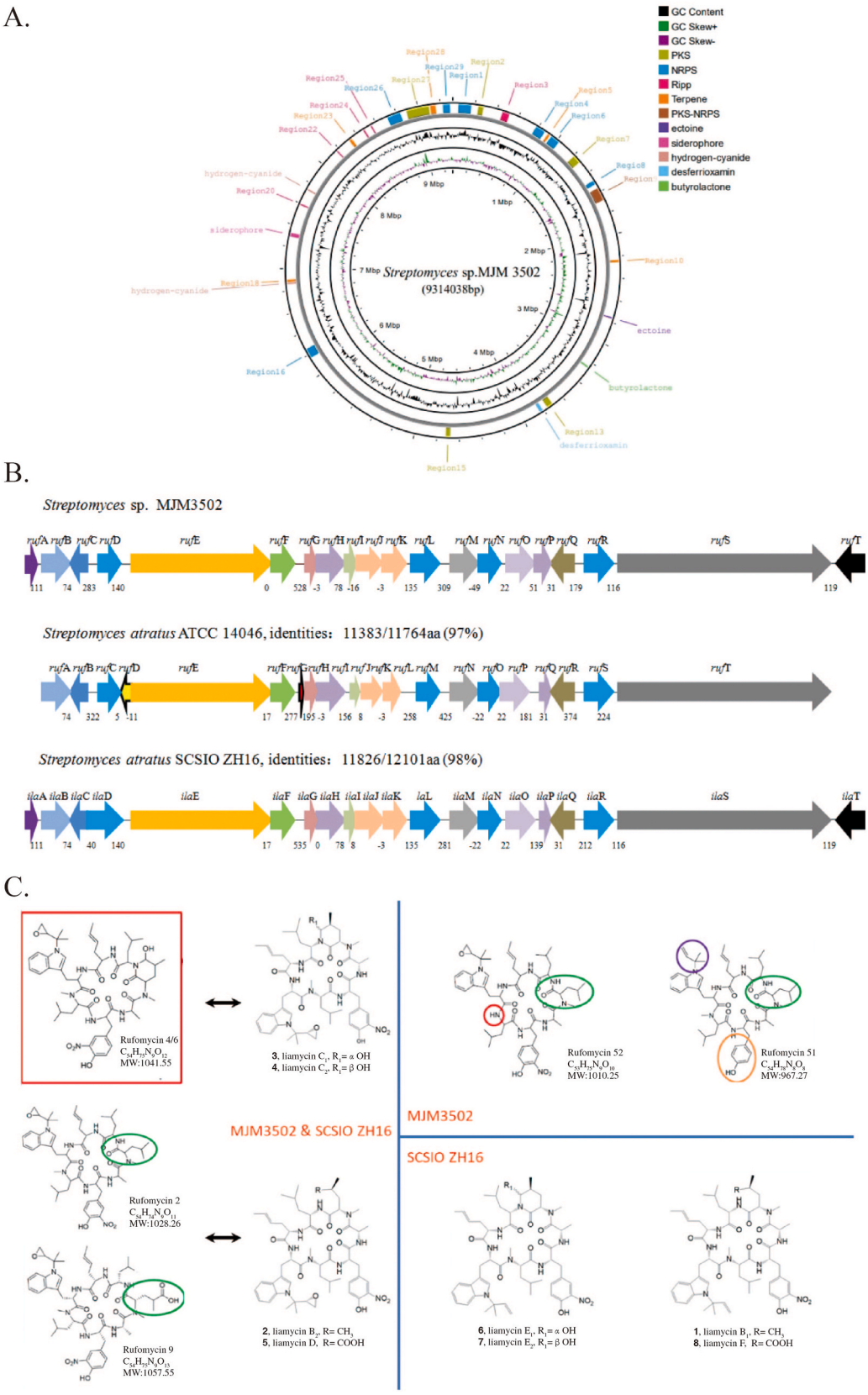


Fig. 2. Analysis of the biosynthetic potential and metabolites of *Streptomyces* sp. MJM3502. (A) Circular genome map of *Streptomyces* sp. MJM3502 (9,314,038 bp), highlighting various BGCs for secondary metabolites. The outermost circle denotes genomic DNA with color-coded BGCs, while the inner circles represent GC content and GC skew. (B) Comparative analysis of the rufomycin/ilamycin BGC in *Streptomyces* sp. MJM3502, *S. atratus* ATCC 14046, and *S. atratus* SCSIO ZH16. (C) The main chemical structures of rufomycin and ilamycin derivatives produced by *Streptomyces* sp. MJM3502 and *S. atratus* SCSIO ZH16. Structural differences, such as hydroxyl (OH), methyl (CH_3), and carboxyl (COOH) groups, are marked by colored circles.

Table 1
The deduced functions and homologs of the gene products from the rufomycin clusters. The relatedness of each *Streptomyces* sp. MJM3502 rufomycin BGC encoding the protein to its homolog in *S. atratus* ATCC 14046 and *S. atratus* SCSIO ZH16 is shown in the column comparing the rufomycin protein percentage sequence identity.

Protein	Comparison of rufomycin protein (aa/Identity)					Putative function
	Streptomyces sp. MJM3502	*Streptomyces atratus* ATCC 14046		*Streptomyces atratus* SCSIO ZH16		
RufA	105	0		104	101/105 (96 %)	LysR family transcriptional
RufB	353	255	254/255 (99 %)	353	352/353 (99 %)	Streptomycin biosynthesis operon regulator
RufC	267	254	253/254 (99 %)	267	265/267 (99 %)	Hydrolase
RufD	353	353	352/353 (99 %)	434	348/353 (99 %)	Cytochrome P450
RufE	4823	4823	4799/4825 (99 %)	4834	4760/4836 (98 %)	Type I polyketide synthase
RufF	246	238	13/33 (39 %)	247	10/29 (34 %)	Thioesterase
RufG	77	53	52/53 (98 %)	79	79/79 (100 %)	MbtH-like protein
RufH	381	381	380/381 (99 %)	380	374/380 (98 %)	Aminotransferase
RufI	161	135	134/135 (99 %)	161	158/161 (98 %)	DNA-binding protein
RufJ	359	351	349/351 (99 %)	351	349/351 (99 %)	ABC transporter
RufK	277	277	277/277 (100 %)	277	277/277 (100 %)	ABC-2 type transporter
RufL	402	361	360/361 (99 %)	402	401/402 (99 %)	Cytochrome P450
RufM	408	378	370/378 (98 %)	420	406/408 (99 %)	Nitric oxide synthase
RufN	403	394	392/394 (99 %)	394	390/394 (99 %)	Cytochrome P450
RufO	373	373	372/373 (99 %)	373	371/373 (99 %)	Aromatic prenyl transferase
RufP	167	124	124/124 (100 %)	137	136/137 (99 %)	Amidotransferase subunit A
RufQ	296	242	241/242 (99 %)	295	296/296 (100 %)	Tetrahydromethanopterin reductase
RufR	410	399	398/399 (99 %)	399	397/399 (99 %)	Cytochrome P450
RufS	8031	7991	7964/7995 (99 %)	8022	7865/8033 (98 %)	Nonribosomal peptide synthetase
RufT	416	0		408	405/408 (99 %)	Lactate dehydrogenase
BBA20954.1	0	33		0		Hypothetical protein
BBA20951.1	0	48		0		Hypothetical protein

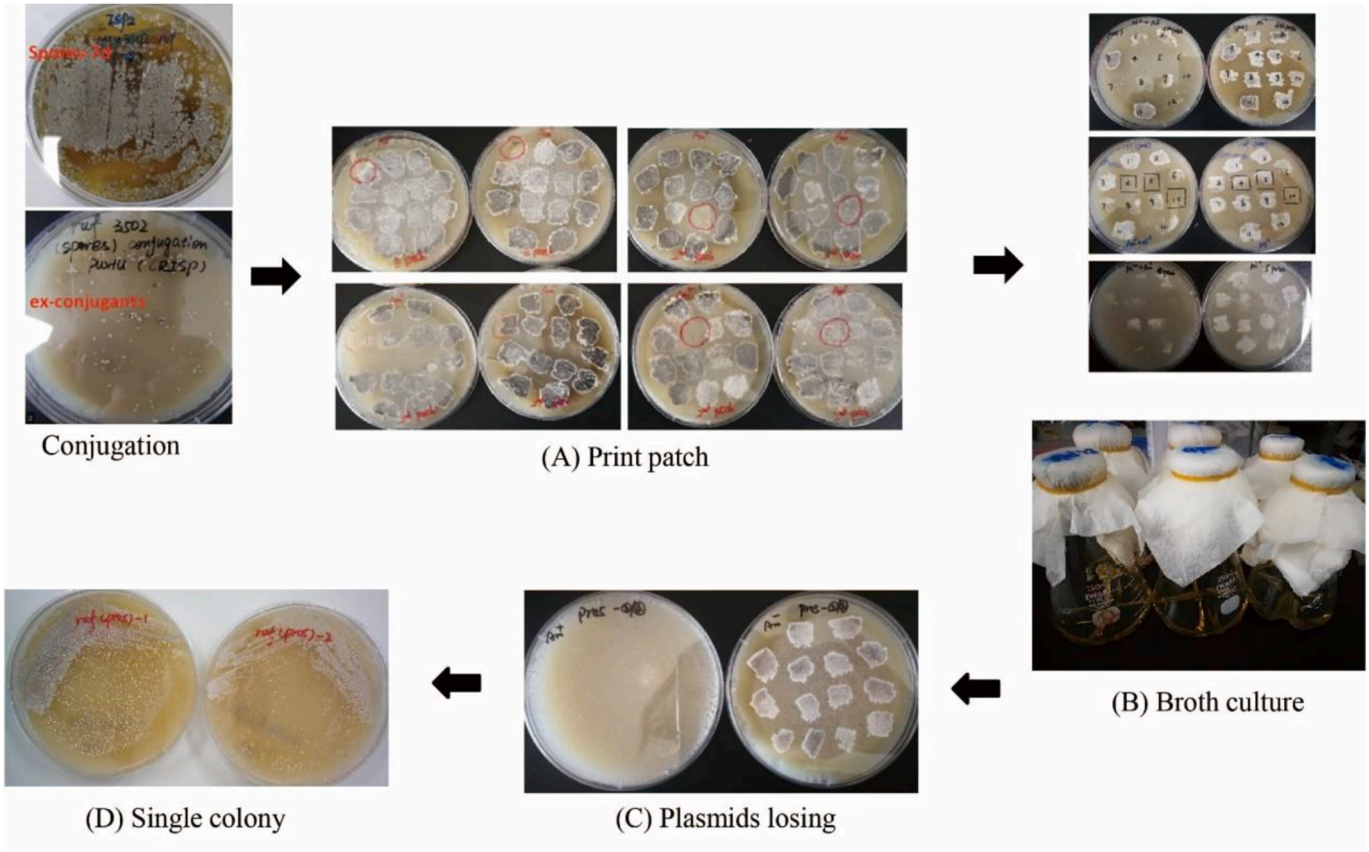


Fig. 3. Intergeneric conjugal transfer using spore conjugation and CRISPR/Cas9-based genome editing and screening for selected single-colony mutation. (A) Pick single exconjugants and streak on plates with and without apramycin, respectively, and grow at 28 °C for 3 or 4 days. (B) Colonies with reduced growth on the plate with apramycin were transferred in liquid media for losing the plasmid. (C) Replicate the grown colonies to plates with and without apramycin to confirm plasmid loss. (D) Extract genomic DNA of apramycin-sensitive single colonies from mycelium on the nonselective plate, followed by PCR amplification to check mutation.

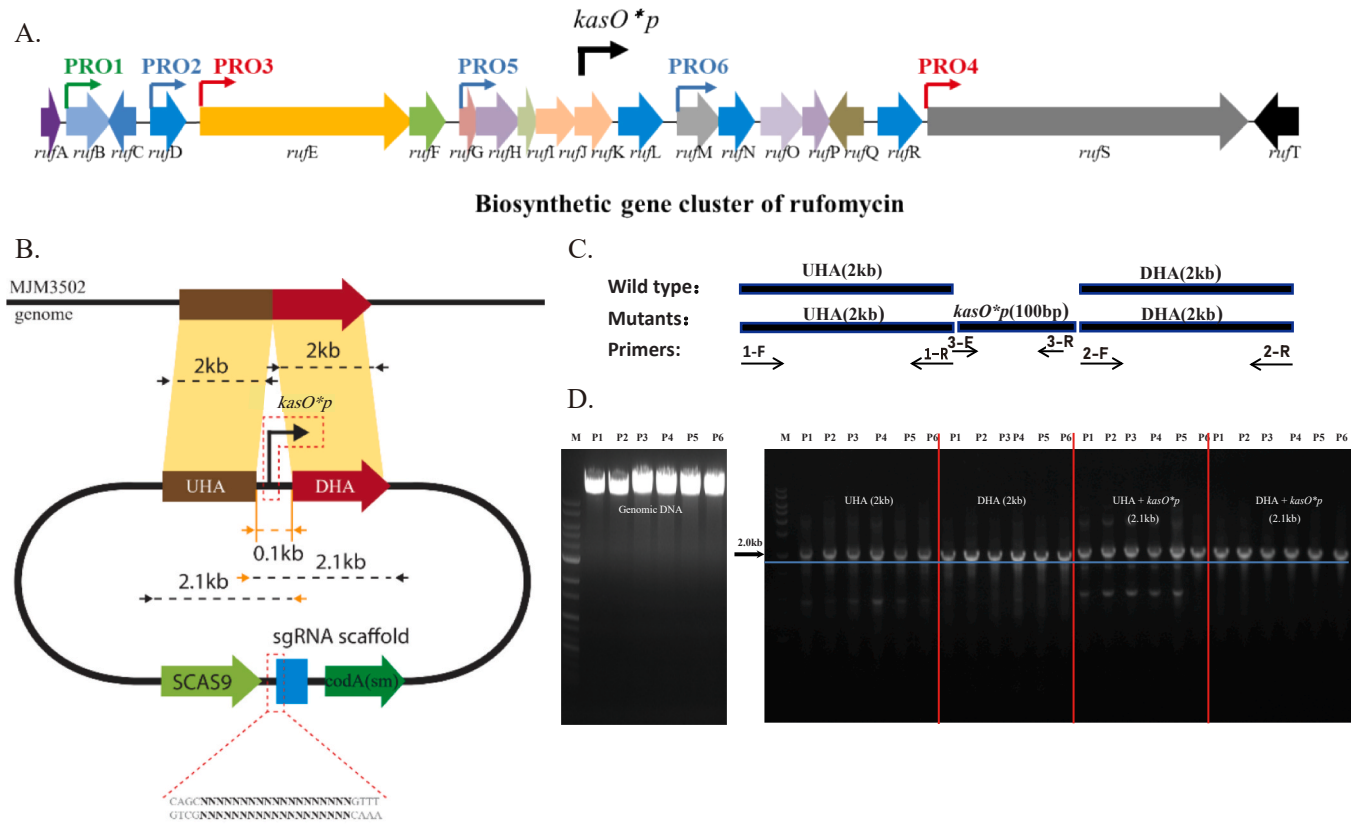


Fig. 4. The design of plasmids to introduce the powerful promoter *kasO*p* into MJM3502. (A) A schematic representation of the design positions where the promoter was inserted in the BGC of rufomycin. (B) The strategy of promoter engineering in six positions using CRISPR/Cas9. (C) A schematic representation of the primer design to verify *kasO*p* knock-in mutants. (D) PCR amplification products using templates of genomic DNA from the WT (W) and the engineered promoter knock-in strains at position PRO1-PRO6 (P1–P6). M (1 kb maker); UHA and DHA are both 2 kb; UHA + *kasO*p* and DHA + *kasO*p* bands are both 2.1 kb.

verified by their apramycin-resistant phenotype, PCR, and sequencing (Fig. 4D).

Subsequently, six promoter recombinant strains, PRO1-6, were engineered by inserting the *kasO*p* promoter in-frame upstream of the respective genes (*rufB*, *rufD*, *rufE*, *rufS*, *rufG*, and *rufM*, resp.). After fermentation, the culture broth of the mutants was extracted, and rufomycin 4/6 was quantified by HPLC (Fig. S1). The results showed a dramatic yield increase in PRO1, PRO3, and PRO4, emphasizing the significant role of *rufB*, *rufE*, and *rufS* in the rufomycin biosynthetic pathway (Fig. 5A and Fig. S2A). The notable yield enhancements observed in specific recombinant strains not only highlight the effectiveness of the engineering strategies employed, but also underscore the importance of these biosynthetic genes in the pathway. These findings provide valuable insights into the potential of metabolic engineering to improve secondary metabolite production. *rufB* is a streptomycin biosynthesis operon regulator, showing 99 % homology with *ilaB* and identified as a positive regulator [36]. In addition, overexpression of *rufB* by the *kasO*p* promoter increased rufomycin production. Notably, the titer of rufomycin 4/6 increased by 4.1-fold (Fig. 5B and Fig. S2B). This suggests that the promoter used in PRO1 effectively enhanced the biosynthesis of rufomycins 4/6. Considering total rufomycin yield, PRO1 shows the highest increase among the engineered strains. It not only boosts the production of rufomycins 4/6, but also enhances the overall yield across all rufomycin isoforms. And the overall rufomycin production via *rufB* overexpression shows a greater impact even more than the *rufE* or *rufS* overexpression, suggesting that the regulatory role of *rufB* exactly impacts the entire BGC and the *rufE* or *rufS* is specific enzymatic steps they mediate. The data provide evidence that promoter engineering can effectively enhance rufomycin production, whereas the level of enhancement varies based on the specific regulatory approach used.

Accordingly, *rufE* (PKS) and *rufS* (NRPS) are responsible for the biosynthesis of the main rufomycin scaffold. Rufomycin 4/6 production in PRO3 and PRO4 reached 2.0-fold and 2.8-fold relative to the WT strain, respectively (Fig. 5B). Accordingly, transcriptional analysis in mutant PRO4 showed higher gene expression levels owing to the *kasO*p* promoter (Fig. 5C and Fig. S2C). This suggests that, while the *rufE* gene responsible for synthesizing the C6 polyketide chain is essential for the release and cyclization of the heptapeptide from the NRPS assembly line, the role of *rufS* appears to be even more critical in rufomycin production. Additionally, the simultaneous knock-in of multiple promoters on the *rufE* and *rufS* genes substantially increased rufomycin production.

3.5. KasO*p promoter insertion enhances the gene expression of the rufomycin BGC

The *kasO*p* promoter was designed to be inserted upstream of the biosynthetic genes of cytochrome P450 enzymes (*rufD* and *rufN*), PKS (*rufE*), and NRPS (*rufS*) to increase the transcription level. Furthermore, the role of *kasO*p* in regulating rufomycin biosynthesis was investigated by analyzing its effect on the transcription of downstream genes in the PRO2, PRO3, and PRO4 strains. Quantitative real-time PCR (qRT-PCR) analysis used the total RNA extracted from WT and engineered strains after fermentation. The results showed that the transcription levels of *rufD, rufE,* and *rufS* genes were significantly upregulated in the PRO2, PRO3, and PRO4 strains, respectively, compared to the WT strain (Fig. 5C). Notably, the insertion of *kasO*p* before *rufS* led to a remarkable increase of about 29-fold in the transcription level of *rufS* in the PRO4 strain, the highest among the engineered strains. Moreover, we found that the insertion of *kasO*p* before *rufD* and *rufE* not only

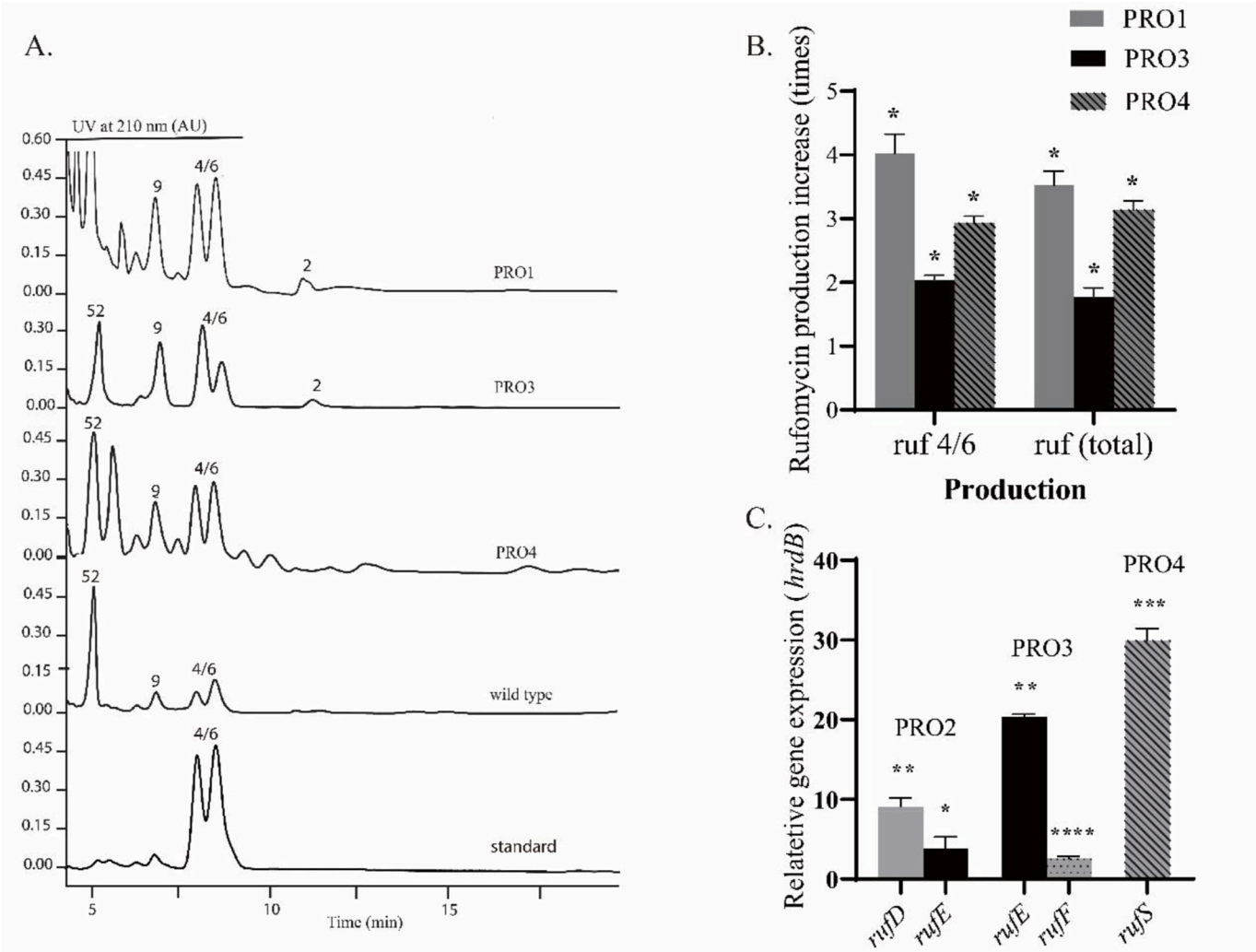


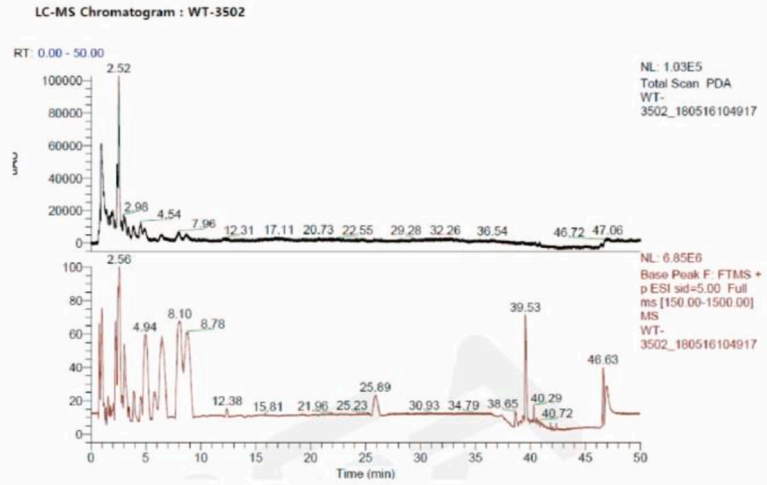
Fig. 5. CRISPR/Cas9-based knock-in of the *kasO*p* promoter to enhance the production of rufomycin in *Streptomyces* sp. MJM3502. (A) UPLC analyses of fermentation broth in the strains of *Streptomyces* sp. PRO1, PRO3, and PRO4. (B) The increasing ratio of rufomycin in the engineered strains *Streptomyces* sp. PRO1, PRO3, and PRO4. (C) qRT-PCR analysis of key gene expression after normalization to the housekeeping *hrdB* gene for both the WT and the engineered strains of *Streptomyces* sp. PRO2, PRO3, and PRO4. The error bars represent the standard deviation of biological triplicates.

increased their transcript levels by approximately 9 and 20 times, respectively, but also led to a significant upregulation of their downstream genes, *rufE* and *rufF*, by about 4 and 2 times, respectively. These findings indicate that *kasO*p* enhances gene expression at the transcriptional level for *rufD*, *rufE*, and *rufS* genes, as well as their downstream genes.

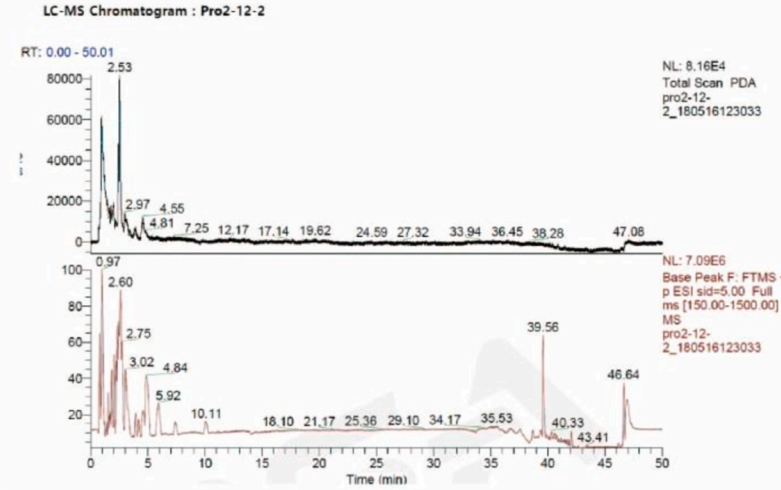
The recombinant strains, PRO2 and PRO6, led to the generation of potentially new rufomycin derivatives (Fig. 6A–S2A, S3, and S4). Despite the important role of *rufM* and *rufN* in supporting the basic precursor units [85], these proteins alone may be insufficient to activate the entire BGC and, thus, could not enhance rufomycin production. In Pro2, *kasO*p* promoter integration upstream of *rufD* likely enabled novel rufomycin analogs by enhancing *rufD* expression, leading to increased oxidative modifications, precursor availability, and broadened enzyme specificity. Further studies are needed to confirm how *kasO*p* alters the pathway to produce these analogs. Interestingly, inserting the *kasO*p* promoter of PRO2 primarily increased the transcription of *rufD* and increased *rufE* by 5-fold, although it did not enhance the overall production of rufomycin over that in PRO3. Therefore, *RufE*, serving as both the initiator and main contributor to L-AHA production, plays a crucial role in the production of rufomycin. Additionally, simultaneously enhancing the transcription of downstream genes of *rufE,* such as *rufF*, will play a more significant role than enhancing the transcription of *rufE* alone.

MbtH-like protein (MLP) plays a pivotal role in stimulating the integral amino acid adenylation components of NRPS [86,87]. The *rufG* gene-encoded MLP is an essential component in the production of rufomycin. However, inserting the *kasO*p* promoter before *rufG* in PRO5 completely abolished the ability of the strain to produce rufomycin. Further analysis revealed the presence of a transposase gene, *ruf**, which may be responsible for horizontal rufomycin BGC transfer to MJM3502, which overlaps with *rufG* (Fig. 6B and S5, Table S4). Collectively, this suggests that *ruf** and *rufG* form a single ORF. The insertion of a new promoter may have disrupted this integral ORF, leading to the failure of rufomycin synthesis. The identification of *ruf** as a critical gene in the rufomycin biosynthetic pathway is significant but not fully understood. Its disruption in PRO5 abolished rufomycin production, highlighting its essential role in pathway integrity. Potential functions of *ruf** include maintaining structural integrity, facilitating precursor biosynthesis, stabilizing protein complexes, or acting as a regulator of pathway flux. Its transposase-like elements suggest a possible role in BGC activation or stabilization. Furthermore, the importance of MLP was also observed in prior studies [88]. Inactivation of the *ila*G gene, which encodes an MLP, also led to a complete loss of ilamycin production [77]. Collectively, these results demonstrate the essential role of both NRPS modules and MLP activators in forming a complex that initiates the rufomycin

A.



NO.	RT (min)	[M+H]+	ruf (MW)
1-A	4.94	1019.5658	1014.24 (52)
1-B	6.10	1058.5682	1057.55 (9)
1-C	8.10	1042.5731	1041.55 (4/6)
1-D	8.78	1042.5613	1041.55 (4/6)
1-E	12.38	1028.5873	1028.26 (2)
1-F	25.89	1012.5921	1010.25 (1)



NO.	RT (min)	[M+H]+
2-A	3.87	1002.5362
2-B	4.21	1002.5374
2-C	4.84	1024.5202
2-D	5.92	1094.5638
2-E	7.38	1078.5678
2-F	10.11	1100.5484

B.

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Streptomyces sp. MJM3502(ruf*)          -GRTWTRWPWRPRQPSRCGQLRSGG-DGRSGRVFPPADSRLLAREMTYAMPMGLERRNCW
Streptomyces sp. UH6(WP_180220597.1)    MTTFNDQVAVEATIAKQVWRTAFGALMAEVADCFPRRDARLLARDMTEAMLMKLERRNCW
Streptomyces sp. CNS654(WP_078567041.1) MTTIKDQVAAEATMTEREWTNVIGAAMAAVANCFTRREPRLLAREMCEAMLMELDTRNCW
Streptomyces sp. TRM63209(WP_155058455.1) MKTIKNQVAVEATIAERVWTAAFGSLMAAVAVCFPRRDSRMLGRAMTQGMLMELERRNCW
Streptomyces sp. AVP053U2(WP_062190052.1) METIKNQVAVEATIAEQVWTAAFGSLMAAVAACFPRRDSRMLGRAMTQGMLMELERRNCW
                                              : . ..  ..:      *.  .  .  *.  :.*:*.* *  .* * *: ****

Streptomyces sp. MJM3502(ruf*)          TPAEALGHDGPHPVHLAPPSSAPIRRLPSALERRHRGSNHMTRPPISRLTTAVTERPCYA
Streptomyces sp. UH6(WP_180220597.1)    TLAEALGHDGPWRLQHFLSRGSWDHHLARDRLASWVSGELAEGDAVLVVDETGGRE----
Streptomyces sp. CNS654(WP_078567041.1) TLAEALGHCGPHRLQHFLARARIDHDLARDGLAAWITSELADGQAVLVVDETGDEKSSGD
Streptomyces sp. TRM63209(WP_155058455.1) TLAEALGHDGPHRLQHFLSRGAWNHDLARDRLAAWVAAELADNEAVLIVDETGDEKSSTD
Streptomyces sp. AVP053U2(WP_062190052.1) TLAEALGHDGPHRLQHFLSRGAWDHDLARDRLATWVAAELADDEAVLIVDETGDEKSSTD
                                         * ****** **  ::   . .   : *.        .:     .:  :  :  ..
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Fig. 6. The generation of potential novel rufomycin derivatives and a putative gene in the rufomycin BGC in different promoter engineering mutants. (A) The UPLC-MS/MS spectrum of rufomycin analogs in WT MJM3502 and PRO2. (B) The sequence alignments of *ruf** with other transposases in the *Streptomyces* strain.

biosynthetic assembly line.

Based on this study result, future efforts to enhance rufomycin production could integrate synthetic biology approaches, such as combinatorial overexpression of key genes like *rufB*, *rufE*, and *rufS* to synergistically amplify regulatory and enzymatic activities. Alternative strong promoters (e.g., *ermE*p* or inducible promoters) could optimize expression and balance pathway flux. Additionally, metabolic engineering to boost precursor biosynthesis, such as L-AHA and 3-nitrotyrosine, may expand the substrate pool and further improve yields. The methodology presented in this study can be applied to activate cryptic or silent biosynthetic gene clusters (BGCs) using CRISPR/Cas9 and promoter engineering. Integrating strong promoters, such as *kasO*p*, can unlock the production of otherwise inaccessible secondary metabolites by targeting pathway regulators, tailoring enzymes, or rate-limiting biosynthetic steps. This approach is particularly relevant for discovering novel natural products with therapeutic potential, such as antibiotics or anticancer agents, and could accelerate drug discovery pipelines to address challenges like antimicrobial resistance.

4. Conclusions

This study involved the generation of six recombinant *Streptomyces* sp. MJM3502 strains (PRO1-6) through the strategic insertion of the *kasO*p* promoter in-frame upstream of the key genes (*rufB*, *rufD*, *rufE*, *rufS*, *rufG*, and *rufM*). Alterations in the production ratio of rufomycin analogs were observed in PRO2 and PRO6, resulting in an augmented yield of previously unidentified rufomycin derivatives. PRO1, PRO3, and PRO4 exhibited enhanced production of rufomycin 4/6, indicating the potential of targeted promoter engineering. The introduction of robust promoter strains offers a strategy for achieving even higher rufomycin production when combined with PRO1, PRO3, and PRO4. By leveraging synthetic promoter engineering and combining our findings with other strategies to improve rufomycin analog production, a higher production titer for translational studies is within reach.

CRediT authorship contribution statement

Chun Su: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization. **Nguyen-Quang Tuan:** Writing – original draft, Software, Methodology. **Wen-Hua Li:** Software, Investigation. **Jin-Hua Cheng:** Writing – review & editing, Methodology, Conceptualization. **Ying-Yu Jin:** Writing – review & editing, Data curation, Conceptualization. **Soon-Kwang Hong:** Writing – review & editing, Data curation. **Hyun Lee:** Writing – review & editing, Formal analysis, Data curation. **Mallique Qader:** Writing – review & editing, Formal analysis, Data curation. **Larry Klein:** Formal analysis, Conceptualization. **Gauri Shetye:** Resources, Conceptualization. **Guido F. Pauli:** Writing – review & editing, Data curation. **Scott G. Flanzblau:** Writing – review & editing, Data curation. **Sang-Hyun Cho:** Writing – review & editing, Methodology, Data curation. **Xin-Qing Zhao:** Writing – review & editing, Writing – original draft, Project administration, Data curation, Conceptualization. **Joo-Won Suh:** Writing – review & editing, Validation, Project administration, Conceptualization.

Data availability statement

The genome sequences have been deposited on NCBI's GenBank Database at https://www.ncbi.nlm.nih.gov/nuccore/CP121214 and assigned Accession numbers in the range of CP121214. All other data generated or analyzed during this study are included in this published article [and its Supplementary Information Files].

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Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nguyen-QuangTuan is currently employed by R&D Center, Manbangbio Co. Ltd. Jin-HuaCheng is currently employed by Microbio Healthcare Co. Ltd. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2025.01.002.

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