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Improvement of rimocidin production in *Streptomyces rimosus* M527 by reporter-guided mutation selection

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Abstract: In this study, we employed a reporter-guided mutation selection (RGMS) strategy to improve the rimocidin production of *Streptomyces rimosus* M527, which is based on a single-reporter plasmid pAN and atmospheric and room temperature plasma (ARTP). In plasmid pAN, P_{rimA}, a native promoter of the loading module of rimocidin biosynthesis (RimA) was chosen as a target, and the kanamycin resistance gene (*neo*) under the control of P_{rimA} was chosen as the reporter gene. The integrative plasmid pAN was introduced into the chromosome of *S. rimosus* M527 by conjugation to yield the initial strain *S. rimosus* M527-pAN. Subsequently, mutants of M527-pAN were generated by ARTP. 79 mutants were obtained in total, of which 67 mutants showed a higher level of kanamycin resistance (Kan^r) than that of the initial strain M527-pAN. The majority of mutants exhibited a slight increase in rimocidin production compared with M527-pAN. Notably, 3 mutants, M527-pAN-S34, S38, and S52, which exhibited highest kanamycin resistance among all Kan^r mutants, showed 34%, 52%, and 45% increase in rimocidin production compared with M527-pAN, respectively. Quantitative RT-PCR analysis revealed that the transcriptional levels of *neo* and *rim* genes were increased in mutants M527-pAN-S34, S38, and S52 compared with M527-pAN. These results confirmed that the RGMS approach was successful in improving the rimocidin production in *S. rimosus* M527.

Keywords: Reporter-guided mutation selection, Atmospheric and room temperature plasma, Single-reporter plasmid, Rimocidin, RimA

Introduction

The *Streptomyces* genus has become an excellent source of microorganisms for the development of biologically active compounds with agricultural and medical applications, including antibiotics, anti-infectious agents, insecticides, and therapeutic molecules (Kemung et al., 2018; Liu et al., 2018; Rey & Dumas, 2017). A variety of agricultural antibiotics such as validamycin (García & Argüelles, 2021), natamycin (Zong et al., 2022), kasugamycin (Kasuga et al., 2017), and nystatin (Ren et al., 2014) have been proved to exert good control effects on crop diseases. The polyene macrolide rimocidin is a glycosylated polyketide (Sowiński et al., 1995), synthesized by the action of so-called type I modular polyketide synthases (PKSs) in *Streptomyces* sp. (Seco et al., 2004). Due to its broad range of antifungal activity (Jeon et al., 2016), rimocidin became a potential fungicide for controlling plant diseases. However, native or wild-type (WT) bacteria for the production of agricultural antibiotics cannot meet the needs of the industry due to the limitations of low yield and long physiological cycle; the increasing requirement for the microbial preparation of antibiotics demands high-yielding microorganism strains applicable to industrial production. Thus far, microbial breeding has been fulfilled by traditional random mutagenesis and screening techniques (Huang et al., 2022; Jung et al., 2011; Yin et al., 2021; Yu et al., 2019a). Although the performance of such strategies seems fruitful, they are labor and time intensive.

Recently, the reporter-guided mutation-selection (RGMS) method has been devised to facilitate the identification of secondary metabolite-overproducing strains (Qin et al., 2017; Wang et al., 2016). The RGMS strategy includes two effective procedures: random mutagenesis to create various mutagenized populations, and a promoter-reporter system to facilitate the selection of mutants in which the expression level of the targeted promoter is enhanced. This method can be used to unlock unknown complex regulatory circuits that often regulate the biosynthesis of secondary metabolites, and to directly select mutants harboring the desired phenotype based on reporter genes (Guo et al., 2015; Li et al., 2018; Shi et al., 2021; Yin et al., 2021). For example, Wang et al. (2012) successfully applied reporter-guided mutant selection and ribosome engineering to generate an A21978C overproducing strain of *S. roseosporus*. Shin et al. (2021) increased the production of clavulanic acid of *S. clavuligerus* by improved glycerol utilization, which was achieved by iterative random mutagenesis and using the *neo* gene as a reporter for the enhanced expression of the *glp* operon.

In 2004, Seco et al. (2004) identified the gene cluster involved in rimocidin biosynthesis in *S. diastaticus* var. 108, and revealed that the gene *rimA* located in the gene cluster that encoded a 181 kDa PKS was the loading module of rimocidin biosynthesis. Thus, it was established that the *rimA* gene plays a crucial role in the final biosynthesis of rimocidin. *Streptomyces rimosus* M527, a rimocidin producer, was originally isolated by Lu et al. (2016)

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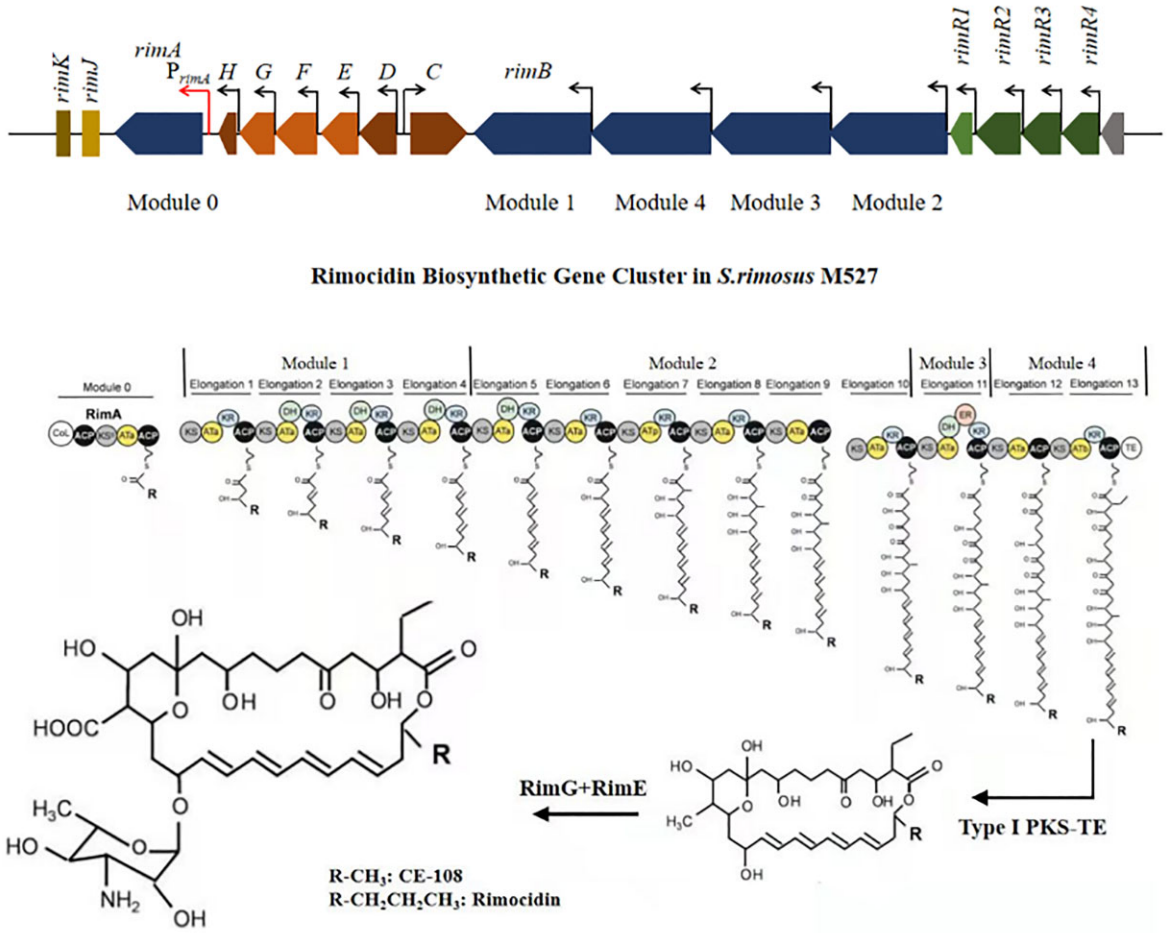

Fig. 1. Gene organization of *rim* gene cluster in the genome of *Streptomyces rimosus* M527 and rimocidin biosynthetic pathway. Module 0 (*rimA*, a loading module), Module 1 (*rimB*), Module 2, Module 3, Module 4, type I polyketide synthase; *rimK*, acetyltransferase; *rimJ*, crotony-CoA reductase; *rimH*, ferredoxin; *rimG*, cytochrome P450 monooxygenase; *rimF*, aminotransferase; *rimE*, glycosyl transferase; *rimD*, cholesterol oxidase; *rimC*, tyrosine phosphatase; *rimR* l, PAS-LuxR family transcriptional regulator; *rimR2*, *rimR3*, *rimR4*, LAL family transcriptional regulator. Proposed model for rimocidin and CE-108 biosynthesis in *S. diastaticus* var. 108 (Seco et al., 2004).

and deposited in the China Center for Type Culture Collection (CCTCC), under the name M2013270. Recently, the whole genome of *S. rimosus* M527 was sequenced (GenBank Accession No: NZ_SADA00000000.1) and the rimocidin biosynthetic gene cluster was predicted (GenBank Accession No.MK300953) (Fig. 1). Several strategies have been successfully employed to promote the rimocidin production of *S. rimosus* M527, such as ribosome engineering (Zhao et al., 2019), the addition of elicitors (Song et al., 2020) and enhanced precursor supply (Liao et al., 2022). However, the method using RGMS to engineer the M527 strain to enhance rimocidin production has not been performed.

Therefore, in this study, the single-reporter RGMS method was employed to improve the rimocidin production of *S. rimosus* M527 (Fig. 2). First, the gene *rimA* was chosen as target, and the kanamycin resistance gene (*neo*) was used as a reporter gene. A promote-reporter fusion plasmid harboring the *rimA* promoter (P_{rimA}) and the *neo* gene was constructed and introduced into *S. rimosus* M527 to monitor *rimA* expression through the kanamycin-resistance (Kan^r) level. The initial conjugants were treated and mutagenized by using atmospheric and room temperature plasma (ARTP). Kan^r mutants were selected and fermented to determine rimocidin production, antifungal activity, cell growth, and the transcriptional level of *rimA* gene.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

The plasmids and strains used in this study are listed in Table 1. *Fusarium oxysporum* f. sp. *cucumerinum*, a plant-pathogenic fungus, was employed as an indicator in the antifungal activity assay.

Streptomyces rimosus M527 and its derived strains were cultured on a mannitol soya flour (MS) solid medium at 28°C for sporulation and conjugation as described by Zhao et al. (2019). Samples for analysis were taken every 24 hr. *Escherichia coli* was incubated in an LB medium at 37°C. The antibiotics apramycin, kanamycin, and chloramphenicol were added, if required.

DNA Manipulations

The procedures of DNA manipulation were carried out based on Sambrook and Russel's description (Sambrook & Russel, 2001). The genetic manipulations of *Streptomyces* were performed according to the operation manual by Kieser et al. (2000).

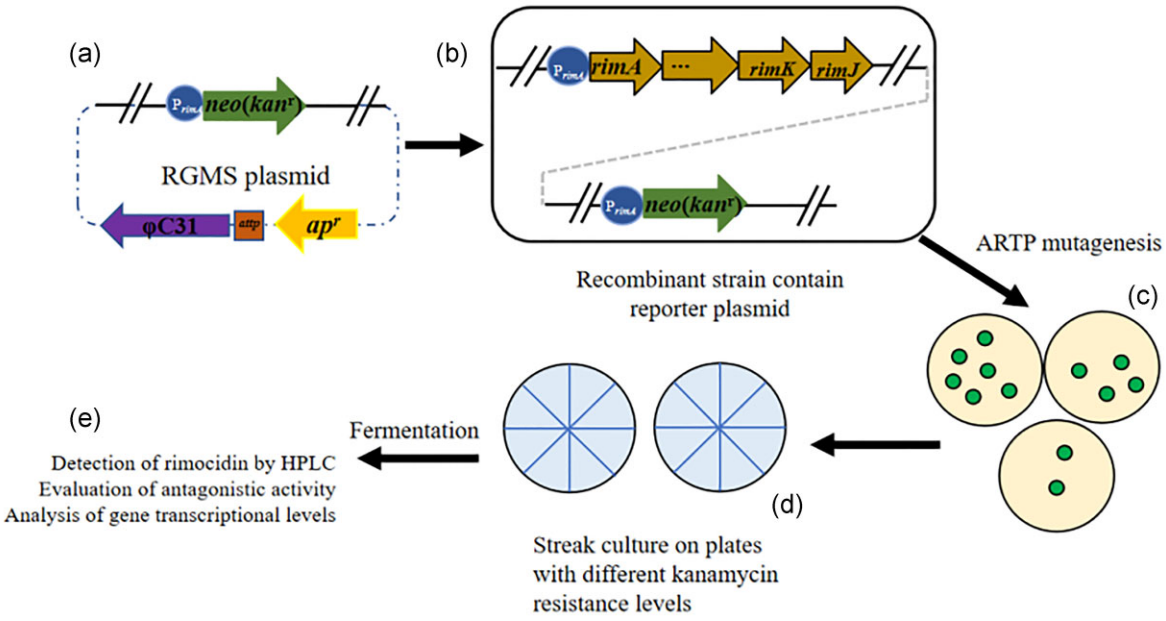

Fig. 2. Workflow of the reporter-guided mutant selection method (RGMS) used for detection of *rimA* gene expression and further improvement of rimocidin production. Key steps include: (A) Gene *rimA* was chosen as the target, kanamycin-resistance (Kan^r) gene (*neo*) as reporter gene was placed under the control of targeted promoter P_{rimA} to generate a single-reporter plasmid; (B) The constructed single-reporter plasmid was integrated into *S. rimosus* M527 by conjugation to yield initial strain M527-pAN; (C) Spore suspensions of M527-pAN were treated and mutagenized by using atmospheric and room temperature plasma (ARTP), then were diluted to a suitable concentration and plated on GYM solid medium for 7 days at 28°C; (D) Mutants of M527-pAN were streaked on plates containing different concentrations of kanamycin; (E) Kan^r mutants were selected to investigate rimocidin production, antifungal activity, cell growth and transcriptional level of *rimA* gene.

Table 1. Strains, plasmids and primers used in this study

Strains or plasmids or primers	Description	Source or reference
Strains		
Escherichia coli JM109	A general cloning host	TaKaRa
Escherichia coli ET12567 (pUZ8002)	*Cm*^{r}, *Km*^{r}, donor strain for conjugation	Our lab
Fusarium oxysporum f. sp. *cucumerinum*	A plant-pathogenic fungus, used as indicator in antifungal activity assay	Our lab
Streptomyces rimosus M527	Wild-type strain, rimocidin producer, CCTCC2013270	Our lab
M527-pAN	Wild-type strain with integrative single-reporter plasmid pAN	This work
M527-pAN-S34	Kan^r mutant of M527-pAN by ARTP mutagenesis	This work
Plasmids		
pSET152	Integrative plasmid, harboring *Apr*^{r}, $oriT_{RK2}$, ΦC31 *int/attP*	Our lab
pSET152-P_{rimA}-*neo* (pAN)	The reporter gene *neo* under the control of promoter P_{rimA}	This work
Primers		
P_{rimA}-F	5′-gggctgcaggtcgac*tctaga*CTCCGACCGCTGCGT-3′(*Xba* I)	This work
P_{rimA}-R	5′-tcccgttgaatatggctcatCACTCCCGGATCGCTCTGA-3′	This work
P_{neo}-F	5′-gcgatccgggagtgATGAGCCATATTCAACGGGA-3′	This work
P_{neo}-R	5′-tgattacgaattc*gatatc*TTAGAAAAACTCATCGAGCATCAAA-3′(*Eco*R V)	This work
P_{AP}-F	5′-CTCAACCTTGGGGTTACCC-3′	Our lab
P_{AP}-R	5′-ACGTCAGCCAATCGACTGG-3′	Our lab

Primers P_{rimA}-F/R were used for amplification of 255-bp upstream sequence of *rimA* gene (P_{rimA}). Primers P_{neo}-F/R were used for amplification of 816-bp kanamycin resistance gene (*neo*). Primers P-AP-F/R were used for amplification of 750-bp apramycin resistance gene (*aac(3)IV*). In primers P_{rimA}-F/R and P_{neo}-F/R, lowercase letters are reserved for introducing homologous sequences of linearized vector ends or upstream/downstream genes, and uppercase letters homologous sequences of P_{rimA} and *neo*.

Construction of Single-Reporter Plasmid

The primers used for PCR amplification in this study are listed in Table 1. The 255-bp upstream sequence of *rimA* gene (P_{rimA}) and kanamycin-resistance gene (*neo*) were amplified by using the genomic DNA of *S. rimosus* M527 and plasmid pET28a as the template, respectively. High-fidelity PCR was performed to obtain DNA fragments that were used for plasmid construction.

The reporter plasmid was assembled based on the shuttle vector pSET152. Two amplified fragments, a 255-bp P_{rimA} and a 816-bp *neo* gene fragment, were inserted into the *Xba* I and *Eco*R V sites of plasmid pSET152 based on DNA seamless cloning technology by the ClonExpress® Ultra One Step Cloning Kit C115. This yielded the single-reporter plasmid pSET152-P_{rimA}-*neo* (pAN), in which *neo* is transcribed and translated under the control of P_{rimA}. The identity of the two fragments P_{rimA} and *neo* in plasmid pAN was verified by sequencing at Tsingke Biotechnology Co., Ltd. Subsequently, plasmid pAN was introduced into the WT strain *S. rimosus* M527 by intergeneric conjugation from donor

Table 2. Screening of kanamycin resistance (Kan^r) levels of M527-pAN mutants yielded by ARTP

Total number of kanamycin-resistance mutants	Concentration of Kan^r (μg/ml)	Name of strain	Maximum production of rimocidin (mg/l)	Inhibitory zone size (mm)
12[a]	0–100		100–150	
19[a]	100–200		190–200	
45[a]	300–400		220–230	
79				
		S34	256.2	23.5 ± 0.44
3	500–700	S38	284.6	25.5 ± 0.29
		S52	273.1	24.8 ± 0.36

[a]These mutants were not named.
Spore suspensions of initial strain *S. rimosus* M527-pAN were treated and mutagenized by using ARTP. After ARTP mutagenesis, the spore solution was mixed and diluted to a proper concentration, and then cultured on GYM solid medium for 7 days at 28°C. The initial strain *S. rimosus* M527-pAN has a minimum inhibitory concentration of 100 μg/ml Kan^r level, which was determined as an indicator of the transcriptional level of the promoter P_{rimA} before ARTP mutagenesis. The kanamycin concentration used for the selection of Kan^r mutants should be elevated properly. Mutants of M527-pAN were randomly picked and inoculated on MS solid media containing 100–700 μg/ml concentration of kanamycin for 4–5 days at 28°C.

E. coli ET12567/pUZ8002, and then exconjugants were selected by the phenotype of apramycin-resistance and confirmed by the PCR analysis of apramycin-resistance gene (*ap^r*).

ARTP Mutagenesis of *S. rimosus* M527-pAN

Spore suspensions of the initial strain *S. rimosus* M527-pAN were treated and mutagenized by using ARTP on a system (College of Life Science, Huzhou University, China), which was utilized to generate radio frequency. The power input of the radio frequency was set at 100 W. The flow rate of high-purity helium (He) as the working gas was set at 10 l/min. The spore suspension (10^6–10^8 cells/ml) was evenly spread on a sterilized metal slide and exposed to the ARTP jet for 20, 40, and 60 s, respectively. After ARTP mutagenesis, the spore solution was mixed and diluted to the appropriate concentration, and then cultured on GYM solid medium (Kieser et al., 2000) for 7 days at 28°C.

Determination of the Kanamycin-Resistance (Kan^r) Levels of Mutants of M527-pAN

The WT strain M527, the initial strain M527-pAN, and mutants of M527-pAN were randomly picked and inoculated on MS solid media containing 100–700 μg/ml kanamycin for 4–5 days at 28°C. The concentration of kanamycin that yielded no observable growth of colonies was considered as an indicator of the transcriptional level of promoter P_{rimA} before ARTP mutagenesis. The concentration of kanamycin would be increased appropriately for the selection of Kan^r mutants.

Analysis of Gene Transcriptional Levels by qRT-PCR

The extraction of RNA and analysis of transcriptional levels of *rimA* and *neo* genes in the WT strain M527, the initial strain M527-pAN and three mutants M527-pAN-S34, S38, and S52 were performed as described by Liao et al. (2020).

Production of Rimocidin and Subsequent High-Performance Liquid Chromatography Analysis

The fermentation of rimocidin and related HPLC analysis was performed as described by Zhao et al. (2019). All experiments were performed in two independent triplicates.

Evaluation of Antifungal Activity

The determination of antifungal activity was carried out according to methods described by Ma et al. (2016). *Fusarium oxysporum* f. sp. *cucumerinum* was used as an indicator, and its inhibition zone was measured as antifungal activity. All strains (WT strain M527, initial strain M527-pAN and mutants pAN-S34, S38, and S52) were tested in two independent triplicate experiments.

Statistical Analysis

All assays were performed in two independent triplicates, and the results were expressed as mean ± standard deviations (SD). Statistical analysis was performed using Student's *t*-test.

Results

Construction of Single-Reporter Plasmid and Recombinant Strain

The gene *rimA* as loading module in the rimocidin biosynthesis was chosen as target; P_{rimA}, a 255-bp upstream sequence of the *rimA* gene, was predicted by PromPredict Software (a web-based tool to identify promoter regions in the genomic DNA sequence). P_{rimA} was chosen as target for the elevated expression level of gene *rimA* and increase in rimocidin production. The single-reporter plasmid pAN was constructed, in which the expression of reporter gene *neo* was driven by P_{rimA} (Fig. S1). The single-reporter plasmid pAN was confirmed by using *Xba* I and *Eco*R V digestion (Fig. S2). Plasmid pAN was transferred into *S. rimosus* M527 by intergeneric conjugation to yield *S. rimosus* M527-pAN, in which the integration of pAN was verified by PCR amplification of the *ap^r* gene (Fig. S3).

Determination of Kanamycin Resistance Levels of Mutants Isolated by ARTP

Seventy-nine mutants obtained by ARTP mutagenesis were streaked on plates with different concentrations of kanamycin by Kan^r screening, mutants derived from Kan^r screening are shown in Table 2. Among 79 mutants of *S. rimosus* M527-pAN, a total of 67 mutants showed Kan^r, and could be grown on plates containing different kanamycin concentrations between 100–700 μg/ml, whereas 12 mutants exhibited no/low Kan^r same as WT strain *S. rimosus* M527 that could grow on plates with maximum kanamycin concentration of 100 μg/ml (Table 2). The Kan^r concentrations of 64 mutants mainly fell in the range of 100–400 μg/ml. Notably, three Kan^r mutants (M527-pAN-S34, S38,

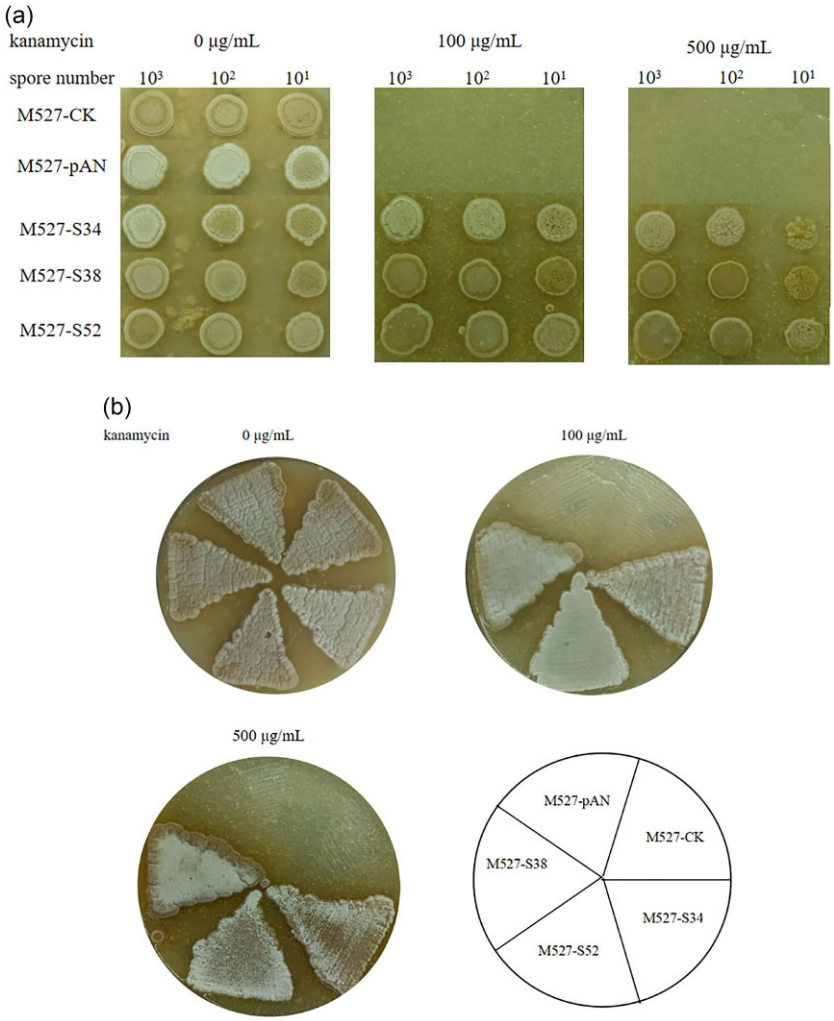

Fig. 3. Screening of high Kan^r mutants. (a) Verification of Kan^r levels of the WT strain M527, initial strain M527-pAN, and mutants M527-pAN-S34, S38, and S52. About 1×10^8 spores of the tested strain were plated on the selection medium containing 0, 100, 500 μg/ml kanamycin. (b) Mutants S34, S38, and S52 showed visible resistance to 500 μg/ml kanamycin. All strains were grown on the solid MS medium and cultured for 5 days at 28°C.

S52) exhibited high Kan^r level, and their Kan^r concentrations reached 500–700 μg/ml (Fig. 3).

Subsequently, the antifungal activity of these Kan^r mutants was determined by agar block screening. By using *F. oxysporum* f. sp. *cucumerinum* as indicators, there was no obvious difference between most Kan^r mutants and the WT strain M527 with respect to antifungal activity, whereas three mutants, M527-pAN-S34, S38 and S52, produced larger diameters of inhibition zone compared with the WT strain M527 (Fig. 4). Therefore, these three Kan^r mutants were chosen for further analyses.

Characterizations of Kan^r Mutants M527-pAN-S34, S38 and S52

Rimocidin production

To confirm whether the high antifungal activity of mutants was the result of increased rimocidin production, the rimocidin production profiles of mutants M527-pAN-S34, S38, and S52 (Fig. S4) were examined. As shown in Fig. 5a, the highest level (284.6 mg/l) determined in M527-pAN-S38 exhibited a 44.1% increase in the amount of rimocidin produced by WT strain M527 (197.5 mg/l). The concentration of rimocidin produced by mutants S52 (273.1 mg/l) and S34 (256.2 mg/l) exhibited 38.3% and 29.7% increase in the rimocidin yield compared with *S. rimosus* M527, respectively. These results suggested that the increase in the expression level of P_{rimA} led not only to the enhancement of Kan^r but also to the improvement of rimocidin production in these three Kan^r mutants, and that the increasing extent of rimocidin production was positively correlated with their Kan^r levels. In addition, there was no difference in rimocidin production between the initial strain M527-pAN and WT strain M527, suggesting that the integration and expression of plasmid pAN in the M527 strain had no obvious effect on rimocidin production.

Cell growth

Mutants M527-pAN-S34, S38, S52, and the initial strain M527-pAN as well as WT strain M527, were grown for shake-flask fermentation. There was no obvious distinction in cell dry weight (DCW) (Fig. 5b) and morphological differentiation among M527, M527-pAN and M527-pAN-S34, S38, S52 (Fig. 2). This result demonstrated that ARTP mutagenesis did not have an adverse effect on the DCW of three mutants M527-pAN-S34, S38, and S52, and that the integration and expression of plasmid pAN had no effect on the growth behavior of M527.

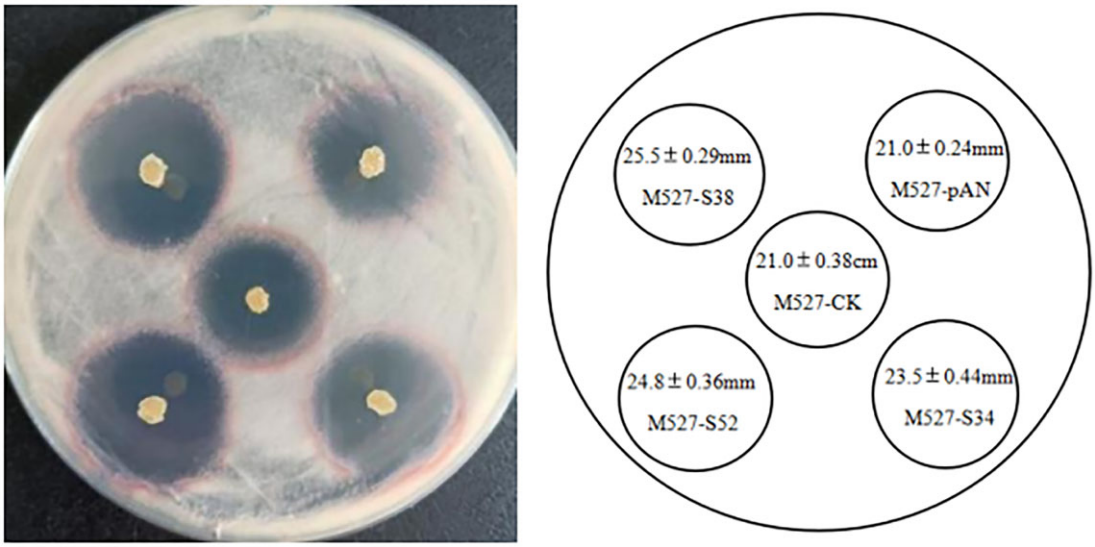

Fig. 4. Detection and comparison of antifungal activities of WT strain M527, initial strain M527-pAN, and mutants M527-pAN-S34, S38, and S52 against *F. oxysporum* f. sp. *cucumerinum*. Spore suspension (500 μl) of *F. oxysporum* f. sp. *cucumerinum* (1×10^6 cfu ml^{-1}) was spread and inoculated on PDA medium at 28°C for 1 day. A agar block (4 mm in diameter) containing actively growing WT strain M527, initial strain M527-pAN, and mutants M527-pAN-S34, S38, and S52 was aseptically placed on aforementioned PDA medium containing pathogenic fungus at 28°C for 3–4 days. The diameter of inhibition zone was measured as antagonistic activity. Plant-pathogenic fungus *F. oxysporum* f. sp. *cucumerinum* was used as indicator strain in antifungal activities assay.

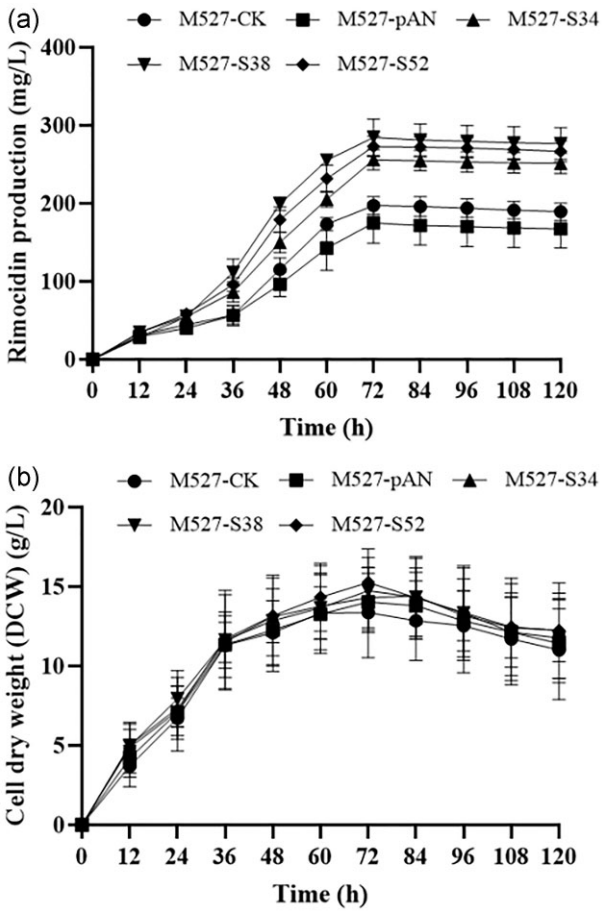

Fig. 5. Detection and comparison of rimocidin concentration (a) and cell growth (b) of WT strain M527(●), initial strain M527-pAN(▲), mutant strains M527-S34(▲), M527-S38(▼), and M527-S52(◆) in shake-flask culture experiment. All shake-flask fermentations were carried out in 250 ml flasks with a working volume of 40 ml at 200 rpm and 28°C. The medium was inoculated at 5% (v/v). The error bars were calculated from three different batches of fermentation.

Expression analysis of neo and rim genes

To test whether the expression level of P_{rimA} was increased in mutants M527-pAN-S34, S38, and S52, quantitative real-time RT-PCR analysis was performed to evaluate the transcriptional level of *rimA* gene in mutants M527-pAN-S34, S38, S52, and control strain M527-pAN, as well as WT strain *S. rimosus* M527, after 36 and 72 hr of fermentation. The transcriptional level of *rimA* gene had no significant difference between the M527-pAN as well as the WT strain *S. rimosus* M527 (Fig. 6), suggesting that the integration and expression of plasmid pAN did not perturb the transcription of *rimA* gene in strain M527. In terms of mutants, increase in the transcription levels of *rimA* and *neo* genes corresponded to elevated rimocidin production, suggesting that the enhancement of P_{rimA} expression led to the improvement of both *neo* gene expression and rimocidin production in mutants caused by ARTP mutagenesis. In addition, the transcriptional levels of other *rim* genes of mutants M527-pAN-S34, S38, and S52 were also higher than those of strain M527 (Fig. 6).

Discussion

In this work, RimA, a loading module of rimocidin biosynthesis, was chosen as a target gene. To enhance the expression level of RimA, the RGMS strategy was employed to increase the rimocidin production of *S. rimosus* M527. Our RGMS technique was based on ARTP mutagenesis and reporter-guided selection. ARTP mutagenesis has been proven to be a novel and efficient approach to generate high-yield mutants for breeding microorganisms. Although ARTP has been successful in increasing the productivity of desired target secondary metabolites in *Streptomyces* (Jiang et al., 2017; Liu et al., 2021), it is a laborious and time-consuming method as it requires the isolation of many mutants from an enormous mutagenized population. To facilitate the process of mutant selection, a targeted selection method based on the reporter gene *neo* was employed, in which it was placed under the control of P_{rimA} and used for evaluating the expression strength of *rimA* and screening the high rimocidin-producing mutant strains. A total of 79 mutants

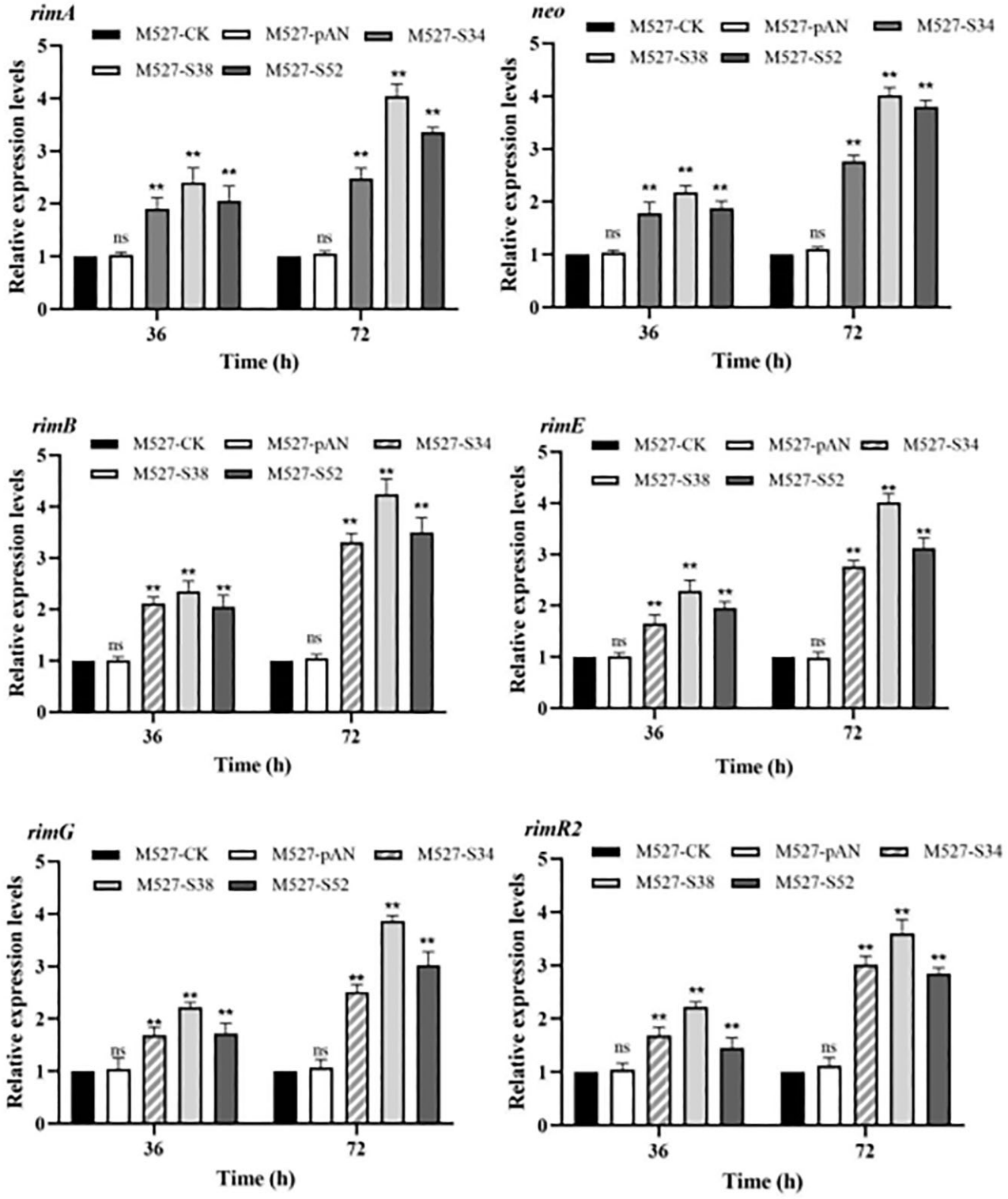

Fig. 6. Comparison of the transcriptional levels of *neo* and *rim* genes by using quantitative reverse transcription-PCR in WT strain *S. rimosus* M527, initial strain M527-pAN and mutants M527-pAN-S34, S38, and S52. The error bars were produced by calculating the standard deviation of the data from three replicates of each sample. 'ns' indicates no statistically significant results (p value >.05). *Indicates statistically significant results (.01 < p value <.05). **Indicates highly statistically significant results (p value <.01).

were isolated by ARTP mutagenesis. Among them, 12 mutants showed no Kan^r, of which 9 mutants lost the ability of sporulation. Sixty-seven mutant strains could grow on the MS media containing different concentrations of Kan. Among them, 45 mutant strains, which showed Kan^r in a range of 300–400 μg/ml, displayed an 10–12% increase in rimocidin production compared with the initial strain M527-pAN. Meanwhile, 19 mutant strains showed Kan^r in the range of 100–200 μg/ml but had no difference in rimocidin production compared with the initial strain M527-pAN. These 19 mutants may produce spontaneous Kan^r mutagenesis and their high Kan^r was not due to the enhancement of *neo* gene expression. Notably, another three Kan^r mutants (M527-pAN-S34, S38, S52) exhibited the highest Kan^r level (500–700 μg/ml) and rimocidin production, and these showed 30%–40% higher rimocidin production compared with the initial strain M527-pAN. The experimental data of qRT-PCR also confirmed that the transcriptional levels of *rimA* and *neo* genes were significantly improved in the three mutants M527-pAN-S34, S38, and S52 compared with the initial strain M527-pAN, indicating that the expression level of P_{rimA} was strongly enhanced. It is worth mentioning that rimocidin production in mutants M527-pAN-S34, S38, and S52 was increased along with elevated transcription levels of the *rimA* gene. The result confirmed that the increased rimocidin production was due to the enhanced expression of P_{rimA}. Furthermore, the expression and integration of single-reporter plasmid pAN into the chromosome of M527 had no effects on cell growth, rimocidin production, expression of *rimA*, and level of kanamycin resistance.

In this study, by using a single-reporter plasmid combined with ARTP mutagenesis, the rate of false positive mutants that showed elevated kanamycin resistance (19/79) was not due to the high expression of the reporter gene *neo*, and was clearly numerous. On the one hand, the optimal parameters of ARTP including treatment time (seconds), energy input (Watt), and helium gas flow rate were beneficial to obtaining positive mutants (Ottenheim et al., 2018; Zhang et al., 2014; Zhu et al., 2022). In fact, the experimental parameter of ARTP was optimized to ensure a suitable lethal rate for the efficient mutation and selection of mutants. The lethal rate was increased to 41.5, 92.3, and 99.9%, after a treatment time of 20, 40, and 60 s, respectively (Fig. S5). When the samples were treated for 60 s or even longer, no spores were able to survive. According to previous researches (Cai et al., 2021; Jiang et al., 2017; Liu et al., 2021), the lethal rate of 90% is suitable and applicable for ARTP mutation. Therefore, in this study, the treatment time of ARTP mutation was selected as 40 s. In addition, a single ARTP treatment was unusually performed, and this was routinely combined with the optimization of fermentation (Yu et al., 2022a; Yu et al., 2022b; Yu et al., 2019b) or ribosome engineering (Zhang et al., 2019) to improve efficiency. Nonetheless, a double-reporter RGMS strategy was recently developed to decrease the frequency of false positives caused by the single-reporter method (Xiang et al., 2009). Therefore, future research will concentrate on the further optimization of ARTP and the development of a double-reporter system for the further enhancement of rimocidin production based on the RGMS strategy.

The rimocidin production enhancement in mutant M527-38 was limited and thus, cannot meet the requirement of industrial production. Therefore, we will focus on the isolation of strains that produce abundant quantities of rimocidin by fine-tuning gene regulation or traditional mutation breeding from mutant S38. Although the transcriptional level of *rim* gene was promoted in mutants, the rimocidin biosynthesis is not only regulated by the rim gene biosynthetic cluster, but is also affected by other internal characteristics of the host strain. Comparing the genome sequences of hyper-strains with that of the wild-type strain will contribute to further analysis in this field. Moreover, the regulatory mechanisms at the molecular level and its key nodes involved in the rimocidin biosynthesis pathway can be elucidated with the obtained hyper-strains and the WT strain by using RNA-seq or omics technologies. Our prospective follow-up studies are in progress and the results will be reported in a separate paper.

Moreover, another tetraene that was also identified in the fermentation broth of *S. rimosus* M527 is a structural analog of rimocidin and is called CE-108. Rimocidin and CE-108 differ in the aglycone moiety, with a methyl side chain in CE-108 instead of the propyl group in rimocidin (Pérez-Zúñiga et al., 2004). Therefore, because of same elongation module during formation in CE-108 and rimocidin biosynthesis, the effects of RimA on their biosynthesis are nearly identical. However, rimocidin exhibits much better antifungal activity compared with CE-108 (Escudero et al., 2015). Therefore, the individual targeted increase of rimocidin and decrease of CE-108 is worth studying. Further studies are needed to improve the specificity of RimA by modifying the acyle-CoA transferase domain of RimA, in order to increase the concentration of rimocidin and decrease the amount of CE-108.

In summary, our findings confirm that the RGMS strategy based on single-reporter plasmid pAN and ARTP is a feasible and effective method for the targeted improvement of rimocidin yield in *S. rimosus* M527.

Supplementary Material

Supplementary material is available online at JIMB (*www.academic.oup.com/jimb*).

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Conflict of Interest

The authors declare no conflict of interest.

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