Enhancement of Carrimycin Production Via Traditional Mutagenesis with Metabolic Engineering in *Streptomyces Spiramyceticus* 54IA

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Research Article

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Abstract

Background: Carrimycin is a new approved class I antibiotic in China. The novel carrimycin producing strain, *Streptomyces spiramyceticus* 54IA, was constructed by CRISPR-Cas9 editing system without insertion of antibiotics resistant gene. The problem of low yield limits this strain in large scale fermentation. In this study, the carrimycin production was significantly improved by strain mutagenesis coupled metabolic engineering.

Results: The *sspD* gene is responsible for degradation of triacylglycerol to provide precursors of the polyketide biosynthesis. The extra *sspD* gene controlled by the promoters of *pks* and *bsm42* genes could moderately enhance carrimycin production. The Bsm42 was identified to play a pathway-specific positive regulator for carrimycin biosynthesis. Due to production of carrimycin significantly enhanced by *bsm42* overexpression, the two different length promoters of *bsm42* individually ligated with two reporter genes were used to monitor *bsm42* expression for screening the higher carrimycin production mutants treated by plasma and ultraviolet. 47% of the 608 selected mutants had higher fermentation titer than the starting strain. The shorter promoter of *bsm42* displayed more appropriate for selection of the carrimycin production improved mutants. The F2R-15 mutant had highest titer (1010±30 μg/mL), which was about 9 times higher than that of 54IA strain. Comparative analysis of transcriptome profiles of F2R-15 mutant and 54IA strains found 158 differential expression genes with more than 2 fold-changes. The up-regulated genes were associated with macrolide precursor biosynthesis, macrolide-inactivation, antibiotics transporter, oxidative phosphorylation; while the most down-regulated genes were referring to the primary metabolites synthetic genes and biosynthetic genes of other secondary metabolites.

Conclusion: These results suggested that manipulation of the positive regulatory gene *bsm42* and traditional mutagenesis coupled with reporter-guided mutant selection method facilitated selection of carrimycin high-yielding mutants.

Background

Carrimycin, also known as bitespiramycin and shengjimycin, is a new, hybrid macrolide antibiotic approved by the China National Medical Products Administration in 2019. It is produced by recombinant *Streptomyces spiramyceticus*, which harbors a 4"-*O*-isovaleryltransferase gene (*ist*) from *Streptomyces thermotolerans* [1, 2]. Carrimycin mainly consisted of isovalerylspiramycins I, II, III and contained trace amount of other 4"-*O*-acylspiramycin components [3]. Compared to spiramycin, carrimycin has a longer half-life, higher potency, and greater tissue penetration [4, 5]; it shows potent inhibition to the G^+ pathogens, and recently was identified as an antiviral agent against a broad-spectrum of human coronaviruses, including the SARS-Cov-2 virus [6].

The carrimycin engineering strain, *Streptomyces spiramyceticus* WSJ-195 [7], was resistant to apramycin, thiostreptomycin and kanamycin, and it is difficult to perform directed genetic manipulation for this strain using the existing Streptomyces plasmids. So, a novel carrimycin-producing strain (*Streptomyces*

spiramyceticus 54IA) without resistant genes insertion into the genome was constructed by using CRISPR-Cas9 editing system [8]. The titer of carrimycin was improved through the ribosome engineering, but it was far from the requirement in the scale-up fermentation. Many efforts have been made to improve the production of carrimycin in *Streptomyces spiramyceticus* WSJ−195 in recent years, including traditional random mutagenesis [9, 10], medium optimization [11], exogenous feeding strategies [12, 13], fermentation process control [14], and genetic engineering [15]. Among these approaches, traditional mutagenesis is a powerful and easily operated method for strain improvement in *Streptomyces*, especially for microbes with less understanding of genomic information and metabolic mechanisms. Although the isovalerylspiramycins I (one component of carrimycin) high-producing strains have been developed with the composite mutagenesis of Plasma and UV [16], it is definitely laborious and time-consuming to improve carrimycin production by just relying on random mutagenesis and ordinary screen methods. Thus, traditional mutagenesis should combine with rational metabolic engineering strategies to shorten the process of strain breeding.

Metabolic engineering strategies have been widely used in the improvement of secondary metabolites in microbes, especially for the production of antibiotics. Currently, the improved production of antibiotics through metabolic engineering mainly focuses on investigation of regulatory genes [17], improvement of precursor supply [18, 19], and comparative metabolomics profiling analysis [20, 21]. Among those strategies, enhanced precursor supply has been indicated to be a direct way to increase the accumulation of desired natural products. For example, the increased supply of propionyl-CoA carboxylase along with the addition of propionate was found to be an effective way to increase the concentration of intracellular methylmalonyl-CoA for rapamycin biosynthesis in *S. hygroscopicus* [22]. Spiramycin's structural backbone is a polyketide, putative platenolide I including ethylmalonyl-CoA, methylmalonyl-CoA and methoxymalonyl-CoA, which depends on polyketides synthase (PKS) and malonyl-CoA for its biochemical composition [23]. Wang et al [24] applied multi-omics to reveal that intracellular triacylglycerols (TAGs) pool as an intracellular carbon source for polyketide biosynthesis during stationary phase, and the mobilization of cellular TAGs enables carbon flux to be redirected to polyketide biosynthesis. They devised a new dynamic degradation of TAG (ddTAG) strategy that increases polyketide titers and applied ddTAG in four Streptomyces species to increase yields of actinorhodin (Act), jadomycin B, oxytetracycline and avermectin B1a. The *sco6196* is the key gene involved in degradation of TAG in *S. coelicolor*. Given that accumulation of a TAG pool is widespread in actinomycetes [25]. The strategy of TAG appropriately degradation can be also widely used for improvement of polyketide antibiotics biosynthesis.

The positive regulatory genes of spiramycin biosynthesis, *srm22* and *srm40*, also were identified in this gene cluster. Srm40 is a pathway-specific activator in spiramycin biosynthesis, and Srm22 is required for *srm40* expression [26]. The *bsm23* and *bsm42* genes in *Streptomyces spiramyceticus*, homologous with *srm22* and *srm40*, also play the positive regulatory genes involved in spiramycin biosynthesis [27]. The Bsm42 plays the role in the activation of spiramycin biosynthesis, and its overexpression can improve the yield of spiramycin. However, we discovered that Bsm23 inhibited spiramycin production at high expression level although it was a necessary regulatory gene for spiramycin biosynthesis. The expression

level of the transcriptional activators can play as an indicator for high production of target metabolite. A double reporter-guided mutant selection method has been developed to facilitate selection of clavulanic acid high-yield mutants [28].

Carrimycin is a new potent drug for anti-infection and had antiviral activity. We constructed a new resistant marker-free strain *Streptomyces spiramyceticus* 54IA by CRISPR-Cas9 editing system. But the carrimycin production of 54IA is far lower than that of the WSJ-195 strain. In this study, the carrimycin production was improved by increasing expression of *sspD*, homologous to *sco6196*, through introducing extra *sspD* copy under the control of the promoter of carrimycin biosynthetic genes. The more concentration of SspD at appropriate time means the more the precursors degraded from of TAG for carrimycin biosynthesis. Furthermore, investigation and manipulation of the positive regulator is another effective way to enhance the yield of carrimycin.

Results

The analysis of SspD, homolog of SCO6196, in *streptomyces spiramyceticus* 54IA

SCO6196 is AMP-binding domain-containing protein to activate fatty acids by binding to coenzyme A in the *Streptomyces coelicolor* A3 (2). It can channel carbon flux from both intracellular TAGs and extracellular substrates into polyketide biosynthesis. SspD in *Streptomyces spiramyceticus* 54IA (533 amino acid residues) had high sequence similarity (80% identity and 88% similarity) to the SCO6196 from *Streptomyces coelicolor* A3 (2). However, its upstream and downstream genes display significantly difference with that of *sco6196* (Figure 1A).

In order to gain the improvement of expressing *sspD* to supply more precursors for carrimycin biosynthesis, the extra constructed *sspD* gene was driven by the P*pks* or P*bsm42* promoter in the carrimycin biosynthetic gene cluster, both of which were activated during the stationary phase of the host strain. Plasmids pSET-*Ppks-sspD* and pSET-*Pbsm42-sspD* were thus constructed (Figure 1B) and used to transform 54IA strain to obtain strain 54IA::*Ppks-sspD* and 54IA::*Pbsm42-sspD*.

Table 1 Fermentation titer of the wild-type strain and transformants

Strains	Fermentation titer（μg/mL）	Concentration of isovalerylspiramycins （μg/mL）
54IA	115±13.96	56.17±4.31
54IA::*Ppks-sspD*	253±12.75	93.63±6.63
54IA::*Pbsm42-sspD*	187±9.42	79.74±3.96
54IA::*kasOp*-bsm42*	964±34.87	325.04±19.45
54IA::*kasOp*-bsm23*	276±18.55	65.75±8.21

Date are means ± SE of three independent biological replicates

54IA::*Ppks-sspD* and 54IA::*Pbsm42-sspD* showed higher titer than that of the original strain 54IA. As shown in Table 1, the titer of 54IA::*Ppks-sspD* and 54IA::*Pbsm42-sspD* reached 253±12.75 μg/mL and 187±9.42 μg/mL, which are 2.2 and 1.6 times higher than that of 54IA, respectively. In order to calculate the yield of the isovalerylspiramycins, the three main active components of carrimycin, the fermentation broth extractions of the strains were analyzed by HPLC. Based on the standard curves of carrimycin, the isovalerylspiramycins yield of in strain 54IA*::Ppks-sspD* (93.63±6.63 μg/mL) and 54IA::*Pbsm42-sspD* (79.74 μg/mL) (Table 1) were significantly increased than that of 54IA (56.17±4.31 μg/mL). The results suggest that improved *sspD* expression at suitable time controlled by the appropriate promoter could effectively enhance the yield of carrimycin in 54IA strain.

Enhancement of carrimycin production by overexpression of Bsm23 and Bsm42 positive transcription regulators

It has been reported that Bsm23 and Bsm42 are two positive regulators of the spiramycin biosynthesis. To investigate the performance of Bsm23 and Bsm42 in the regulation of carrimycin biosynthesis, *bsm23* and *bsm42*were both overexpressed in 54IA strain. Plasmid pSET-*kasOp*-bsm23* containing *bsm23* assembled with *kasOp** promoter was constructed, and plasmid pSET-*kasOp*-bsm42* contained *bsm42* which was also driven by *kasOp** promoter (Figure 1B). The two recombinant plasmids were then transferred into 54IA to give strain 54IA:: *kasOp*-bsm23* and 54IA:: *kasOp*-bsm42*.

In Table 1, the results showed that the titer of strain 54IA::*kasOp*-bsm42* reached as high as 964±34.87 μg/mL, which was 7.4 times higher than that of the original strain 54IA. Meanwhile, its isovalerylspiramycins yield could improve to 325.04±19.45 μg/mL, about 5.8 fold higher than that of 54IA. In contrast, the fermentation titer of strain 54IA::*kasOp*-bsm23* was moderately improved 2.4 times than that of 54IA, but the isovalerylspiramycins production was similar with that of 54IA. Therefore, Bsm42 was the pathway-specific positive activator for carrimycin biosynthesis. So, the improved expression level of *bsm42* could play as an indicator for high production of carrimycin in 54IA strain.

Double reporter plasmids for efficient screening for carrimycin high-yield strain

Similar to other pathway-specific positive activator, the promoter region of *bsm42* could play a direct target for variety of regulatory proteins. We attempt to adjust the expression of *bsm42* through mutagenesis method combining with a reporter system to serve as a screening indicator. By protoplast transformation methods, the reporter plasmids pDR-42F1R and pDR-42F2R were transduced into 54IA to obtain the F1R and F2R transformants, respectively. It was found that the wild type strain 54IA could normally grow below 2 μg/mL of kanamycin (Km), however the F1R and F2R could maximally tolerate Km at 60 μg/mL. About 1×10^5 spores of F1R and F2R were separately treated with UV and plasma, followed by spreading spores on greater than or equal to 60 μg/mL of Km. The hundreds of mutants grew out for 7 days of incubation, and then the selection plates were sprayed with catechol. 608 yellow-colored mutants were randomly picked for carrimycin titer measurement. The 286 mutants (47%)

produced statistically higher carrimycin yield than the starting strain 54IA containing the pDR2 plasmid (Figure 2 and Table 2). The titers of positive F1R transformants (Figure 2A) were much lower than that of the selected F2R tranformants. Among F2R tranformants tested in Figure 2B, F2R-15 strain with the 200 μg/mL of Km resistance had the highest titer, reaching at a concentration of 1010±30 μg/mL.

Table 2 Positive rates of different F1R and F2R mutant pools

Mutant (Km μg/mL)	The number of positive strain/total	Positive rate
F1R (60)	71/147	48%
F2R (90)	99/111	89%
F2R (150)	87/174	50%
F2R (200)	18/89	20%
F2R (300)	11/87	13%
Total	286/608	47%

As shown in Figure 2C, only a few of F1R mutant colonies (Figure. 2C-a) was visually observable yellow color appeared on the lawns, but most of F2R mutant colonies (Figure 2C-b) displayed the significant yellow color. The XylE activities were tested in the highest yield mutants of F1R-4 and F2R-15 (Fig. 2C-c). When sprayed with substrate catechol, the colonies of F2R-15 displayed bright yellow color, at the same time the colonies of F1R-4 showed weak yellow color, while there was no visible yellow color appeared on the lawns of the starting strain 54I-A.

Differentially expressed genes between the high-yield strain F2R-15 and 54IA

Comparative analysis of transcriptome profiles from RNA-seq of the F2R-15 mutant and 54IA strains found 44 genes with obvious differences in expression, including 20 significantly up-regulated and 24 down-regulated genes (Table 3). The up-regulated genes are associated with carrimycin biosynthetic precursor, macrolide-inactivation, antibiotics transporter, oxidative phosphorylation, two sigma factors and three regulators. The improved genes of ID-6064 and ID-203 are related to the generation of acyl-CoA and Isovaleryl-CoA, which are the important building blocks of carrimycin biosynthesis. Gene of ID-803, macrolide-inactivating glycosyltransferase gene, could play an important role in self-protection to macrolide antibiotic inhibition. There are 7 enhanced expression genes involved in transporter system, especially the response to antibiotic. In addition, some increased genes referring to oxidative phosphorylation might provide more energy for the secondary metabolism. The improvement of sigma factors and three regulatory genes may be directly or indirectly involved in regulating the biosynthesis of carrimycin. The most of down-regulated genes were attributed to 10 secondary metabolites gene clusters referring to the biosynthesis of siderphore, terpene, lassopeptide, feglymycin, ectoine and some *t1pks*-

nrps compounds. Strangely, the deoxysugar biosynthetic genes of carrimycin in F2R-15 mutant also showed lower expression level than that in 54IA. As for genes in synthesis and metabolism of bio-macromolecules, some important genes involved in metabolism of amino acids and nucleotides were increased, however, the key biosynthetic genes of the two primary metabolites were decreased. These results are consistent with the general knowledge that the secondary metabolism initiation would inhibit the primary metabolism.

Table 3 Differentially expressed genes between the F2R-15 mutant and 54IA

ID	Gene description	Regulation	Fold change	Pathway description
Precursor biosynthesis				
6064	pyruvate dehydrogenase E1 component subunit alpha	UP	6.34	Glycolysis, acyl-CoA biosynthesis
203	DNA alkylation response protein,	UP	2.22	Isovaleryl-CoA dehydrogenase activity, the leucine degradation pathway
Macrolide-inactivating				
803	macrolide-inactivating glycosyltransferase	UP	3.68	Response to macrolide antibiotic
Transporter				
2176	ABC transporter permease	UP	5.17	Efflux transmembrane transporter activity
5715	Esterase	UP	4.49	ATPase activity, glycine betaine transport
711	ATP-binding protein DrrA	UP	4.43	Daunorubicin resistance ABC transporter
696	ABC-F family protein	UP	3.76	ATPase activity, response to antibiotic
518	MFS transporter	UP	2.91	Sporulation
1243	Efflux RND transporter permease subunit	UP	2.58	Transmembrane transporter activity, response to antibiotic
6822	ABC transporter, *srmB*(*bsm25*)	UP	2.00	ATPase activity, response to antibiotic
Oxidative phosphorylation				
5654	NADH-quinone oxidoreductase subunit L	UP	5.64	Oxidative phosphorylation
2106	NADH-quinone oxidoreductase subunit M	UP	5.50	
5817	NADH-quinone oxidoreductase subunit L	UP	4.38	
84	NADH-quinone oxidoreductase subunit G	UP	2.80	
Sigma factor				
1068	SigE family RNA polymerase sigma factor	UP	2.79	DNA-binding transcription factor activity

699	Sigma-70 family RNA polymerase sigma factor	UP	2.55	DNA-binding transcription factor activity
Regulator				
2796	VWA domain-containing protein	UP	6.11	ATPase activity, signal transduction
1928	AfsR family transcriptional regulator	UP	4.56	Regulation of transcription (Hyphal growth)
709	Helix-turn-helix transcriptional regulato	UP	2.05	Phosphorelay signal transduction system
Amino acid metabolism				
1738	4-hydroxyphenylpyruvate dioxygenase	UP	5.13	Tyrosine and phenylalanine metabolism
2137	glutamine synthetase	DOWN	5.81	Glutamine biosynthetic process
827	glutamate synthase large subunit	DOWN	3.48	Glutamate biosynthetic process
434	3-isopropylmalate dehydratase	DOWN	2.83	Valine, leucine and isoleucine biosynthesis
Nucleotide metabolism				
357	purine-nucleoside phosphorylase	DOWN	6.49	Purine metabolism, Pyrimidine metabolism
955	adenosine deaminase,	DOWN	5.00	Nucleotide metabolic process
Secondary metabolites gene clusters				
1832	IucA/IucC family siderophore biosynthesis protein	DOWN	8.33	Desferrioxamine B biosynthetic gene cluster (Siderophore)
1546	ABC transporter substrate-binding protein	DOWN	7.69	
2436	aspartate aminotransferase family protein	DOWN	5.92	
472	IucA/IucC family protein	DOWN	7.30	Kanamycin biosynthetic gene cluster
730	iron chelate uptake ABC transporter family permease subunit	DOWN	4.55	siderophore biosynthetic gene cluster
1398	serine hydroxymethyltransferase	DOWN	3.02	ectoine-butyrolactone biosynthetic gene cluster
503	ABC transporter permease subunit	DOWN	2.75	
422	purine permease	DOWN	3.83	t1pks-nrps metabolite biosynthetic gene cluster

1820	VWA domain-containing protein	DOWN	2.56	
16	NDP-aminohexose N-dimethyltransferase (*bsm22*)	DOWN	3.27	Deoxysugar biosynthesis of spiramycin
92	NDP-hexose dehydratase (*bsm26*)	DOWN	2.76	
74	GTPase (*bsm27*)	DOWN	2.39	
18	glycosyltransferase, *bsm28*-35	DOWN	2.26	
530	DUF350 domain-containing protein	DOWN	3.22	Terpene biosynthetic gene cluster
1046	iron-containing alcohol dehydrogenase family protein	DOWN	2.04	
873	diaminobutyrate–2-oxoglutarate aminotransferase	DOWN	2.98	Ectoine biosynthetic gene cluster
87	peptide-N4-asparagine amidase A	DOWN	2.83	Feglymycin biosynthetic gene cluster
857	AAA family ATPase	DOWN	2.24	ladderane-nrps biosynthetic gene cluster
1704	acetylornithine transaminase	DOWN	2.10	Lassopeptide biosynthetic gene cluster

The expressions of *bsm23* (ID-60), *bsm42* (ID-834) and the major up-regulated genes were further validated repeatedly by qPCR (Figure 3). The results showed that the expression level of these up-regulated genes were essentially in agreement with that of trancriptome profiles, only gene 6064, 2176 and 2796 showed much higher expression level. However, the *bsm25* (ID-6822), *bsm42* (ID-834) and *bsm23* (ID-60) located in carrimycin biosynthetic gene cluster had similar expression level between the F2R-15 mutant and 54IA starting strains.

Discussion

In this article, we explored the feasibility of increasing *sspD* expression and overexpression of positive transcriptional regulators, *bsm23* and *bsm42*, for improvement of the carrimycin production in the 54IA strain. The production of carrimycin was moderately increased in the plasmids inserted strain containing the *sspD* gene under control of the *pks* or *bsm42* promoters. The SspD, SCO6196 homolog, was considered to involve in degradation of TAG to produce the synthetic substrates for carrimycin biosynthesis in 54IA strain. The extra *sspD* gene copy was designed to expression at the starting period of carrimycin biosynthesis to increase substrate supply for PKS assemble line. This strategy just moderately improved carrimycin biosynthesis probably due to insufficient Ssp improved expression on the control of the *pks* or *bsm42* promoter in 54IA strain.

Generally, the antibiotic production is stringently and elaborately regulated by pyramidal transcriptional regulatory cascades, including signaling pathways, global regulators, pathway-specific regulator, and feedback regulation [29]. Combination of different strategies to manipulate regulatory genes can achieve higher antibiotic production in both the native and/or heterologous host. There are many reports proved that overexpression of pathway specific positive regulators can improve the production of antibiotics, such as TylS or (especially) TylR for Tylosin [30], ToyA for toyocamycin biosynthesis [31], SlnR modulated salinomycin biosynthesis [32], and so on. In this study, the Bsm42 plays the similar role in carrimycin biosynthesis, and its overexpression can significantly enhance the yield of carrimycin. Bsm23, however, is also a necessary regulator for carrimycin biosynthesis, but its higher expression cannot significantly enhance the antibiotics yield. The expression level of positive regulator is not always related to the production of antibiotic. These positive regulators also emerged in other antibiotics producing strain. For example, overexpression of *milR* with a strong constitutive promoter led to decreasing of milbemycin production in *S. bingchenggensis* [33]. In conclusion, the threshold of the over-expressed regulator was a key point to determine the production of antibiotic.

In view of the production of carrimycin correlated with the level of the *bsm42* expression, the two different length of promoter of *bsm42* was ligated to two reporter genes acting as indicator for screening carrimycin enhanced mutants. In our previous work, a stable isovalerylspiramycin I high-producing strain yielding 2000 μg/mL was obtained though atmospheric and room temperature plasma mutagenesis combined ultraviolet radiation [34]. In this study, this mutagenesis method was used to treat the 54IA containing the reporter plasmids. The *bsm42* promoter combined with two reporter genes is efficient selective marker for screening high production of carrimycin through Km resistant and color changes in mutants. The results demonstrated that the shorter promoter of the *bsm42* was more suitable for selection of the carrimycin high-yield strains. The efficiently selected strain F2R-15 produced carrimycin at a concentration of 1010±30 μg/mL, which was about 9 times higher than that of the original strain 54IA. The F2R-15 was selected from the plate of Km at 200 μg/mL, but not from the higher resistant concentration of Km. These results suggested the continuous high expression of *bsm42* was not always benefit for carrimycin biosynthesis.

The up-regulated genes in transciptome profiles of F2R-15 strain and 54IA were related to the macrolide resistant, transport, biosynthetic precursors from TCA or amino acid catabolism. The feedback regulation is often brought by antibiotic to coordinate antibiotic production and transport. Evidences have shown that antibiotic, as ligand for proper regulator, affects the final production in *Streptomyces*. The expression of antibiotic biosynthetic genes was modulated by the RedZ and undecylprodigiosin complex [35]. The activity of AtrA, which regulates primary and secondary metabolism, is reduced by lidamycin of *Streptomyces globisporus* and actinorhodin (ACT) of *S. coelicolor* [36]. Export of antibiotic is important for the producer to reduce the intracellular antibiotic concentration, which can relieve self-toxicity. In *Amycolatopsis mediterranei*, *ΔrifQ* mutant brought overexpression of RifP. The accelerated export of rifamycin may reduce the intracellular rifamycin concentration, relieve other possible feedback inhibition of rifamycin biosynthesis and finally lead to more than two-fold improvement of rifamycin B production [37]. Overexpression of DrrC, which provide self-resistance to DNR and DXR, achieved 5.1-fold increase in

DXR production in *S. peucetius* ATCC 27952 [38]. Transporters enhance the efflux of the self-produced antibiotics, which can be an important strategy for self-protection from self-toxicity.

The down-regulated genes were mainly located in the other secondary metabolites biosynthetic gene clusters, which might reduce competition for biosynthetic substrates and energy of carrimycin. The decreased genes involved in 10 secondary metabolites gene clusters for the biosynthesis of siderphore, terpene, lassopeptide, feglymycin, ectoine and some t1pks-nrps compounds. However, the most of carrimycin biosynthetic genes in F2R-15 were a little higher expression level than 54IA starting strain, and even the deoxysugar biosynthetic genes were decreased expression. The possible reason is the inappropriate harvesting time of the strains in which production of the carrimycin starts to decline.

The above data showed that manipulation of regulatory cascades can be an efficient way to enhance the production of antibiotic. It is obvious that the balance and synergy between primary and secondary metabolism is very important for the overproduction of antibiotic. Rewiring regulatory network combined with metabolic engineering will be a more powerful way to enhance the production of antibiotic in *Streptomyces* [39]. On the basis of understanding the regulation of antibiotic biosynthesis, rewiring the regulatory network is much more efficient to optimize antibiotic producers than the classical random mutagenesis methods.

Material And Methods

Strains, plasmids, culture conditions and primers

All strains and plasmids used in this study are listed in Table S1. The original strain *Streptomyces spiramyceticus* 54IA was generated from *S. spiramyceticus* 1941 by inserting *ist* and *acyB2* genes into the downstream of the spiramycin gene cluster [8]. The slant/plate medium, seed medium, and culture conditions for *S. spiramyceticus* were prepared according to previously described methods [39]. *Streptomyces* strains were cultivated at 28°C for 120 h in soluble fermentation medium for isolation of total RNA. Soluble fermentation medium (per 100 mL) contained: dextrin, 5.0 g; NaCl, 1g; $MgSO_4$, 0.55 g; $CaCO_3$, 0.5g; NH_4NO_3, 0.7g, KH_2PO_4, 0.065 g; $ZnSO_4$•$7H_2O$ 0.01g; $CoCl_2$, $5´10^{-5}$ g; with pH adjusted to 7.2 before autoclaving. The primers used are listed in Table S2.

DNA isolation, manipulation, sequencing, and bioinformatics analysis

Routine DNA manipulation with *E. coli* and recombinant DNA techniques in *Streptomyces* species were performed as described previously [40]. Routine DNA sequencing was carried out by The Beijing Ruibiotech (Beijing, China). Primers were synthesized by Sangong (Shanghai, China). Protein secondary structure predictions were performed using the NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequence alignments and homology comparisons were performed using Clustal W and BLAST software.

Genetic manipulation

All plasmids constructed in this study were introduced into *S. spiramyceticus* 54IA according to the PEG-assisted protoplast transformation method reported previously [41]. To investigate the availability of *sco6196* for improving carrimycin biosynthesis in *S. spiramyceticus* 54IA, the plasmids, pSET-*Ppks-sspD* and pSET-*Pbsm42-sspD*, were constructed to increase the expression of *sspD* under the control of *Ppks* and *Pbsm42* promoter, respectively. The *sspD* gene was amplified using primers *sspD*-F/*sspD*-R from the genomic DNA of *Streptomyces spiramyceticus.* The *Ppks* and *Pbsm42* promoter were amplified using primers *Ppks*-F/*Ppks*-R and *Pbsm42*-F/*Pbsm42*-R from the genomic DNA of *S. spiramyceticus* 54IA. The fragments containing promoter *Ppks* or *Pbsm42* with *sspD gene* were inserted into the *Bam*HI/*Xba*I sites of pSET152 to obtain the resultant plasmids *p*SET-*Ppks-sspD* and pSET-*Pbsm42-sspD*.

To confirm that the positive regulatory gene *bsm23* and *bsm42* can improve the carrimycin production *bsm23* and *bsm42* were amplified by the primers from the genomic DNA of *S. spiramyceticus* 54IA, and then inserted into *Nde*I/*Xba*I sites of pSET152, under the control of the constitutive promoter *kasOp** to get the plasmids pSET-*kasOp**-bsm23 and pSET-*kasOp**-bsm42 .

The construction of *xylE* and *neo* double-reporter genes controlled by promoters of *bsm42*

The promoter of *bsm42* could play the indicator for enhanced carrimycin biosynthesis. The 300 bp promoter of *bsm42* was amplified by the primer *Pbsm42*-F1 and *Pbsm42*-R, and the primer *Pbsm42*-F2 and *Pbsm42*-R for the 200 bp length promoter of *bsm42*. The two promoter fragments were digested by *Not* I, then ligated into the upstream of the two reporter genes *neo* (kanamycin resistance gene) and *xylE* gene at the pDR2 vector to obtain pDR-42F1R and pDR-42F2R plasmids. The *neo* was used to ensure the basic selection efficiency while *xylE* was used to detect target over-expression and visually display the over-expression differences among mutants. The activity of XylE could be detected by spraying catechol on colonies in a selection plate or quantitatively measured in cell-free extract [28]. pDR-42F1R and pDR-42F2R plasmids were individually transduced into the 54IA strain to obtain the resultant strains *S. spiramyceticus* F1R and F2R. The 54IA strain containing pDR2 was as the control strain.

Streptomyces spiramyceticus F1R and F2R mutated by UV and plasma

The lethality rates of *S. spiramyceticus* F1R and F2R in five different UV treating times were investigated. After treating for 60, 75 and 90 seconds in the UV, the lethality rates of the F2R spores increase to 74.43%, 79.15%, 95.00% respectively, and the lethality rates of the F1R spores could reach 55.48%, 67.77%, 91.54% respectively. As a result, to obtain the desirable lethality rates the exposure time employed in this study were 75-90 s for F1R and 60-90 s for F2R.

Quantitative analysis of carrrimycin bioproduction using *Streptomyces spiramyceticus* 54IA and mutants

S. spiramyceticus 54IA and mutants were grown on agar plates for 7 d at 30 °C. The spores were inoculated into 50 mL fermentation medium in an Erlenmeyer flask (250 mL) and incubated at 28°C and 200 rpm for 7 days. To obtain statistically significant results, three independent strains were selected and fermentations were repeated at least three times independently. The resulting fermentation broth was

extracted with ethyl acetate (100 mL), and the solvent was removed in vacuum. The extracts were dissolved in 1 mL CH_3OH and centrifuged at 13,000g for 10 min, and 10 μL of supernatant was subjected to HPLC analysis following the previously described system [15]. In order to calculate the yields of carrimycin in the mutants and the starting strain 54IA, a quantitative curve was established based on the relationship of integral area and the weight of carrimycin. To quantitatively analyze carrimycin titers in the plasmids transduced strains and that of the original strain, extracts of the mutant strains were diluted 10 times and then subjected to HPLC alongside analogously prepared WT-derived extract. The titers of carrimycin in different strains were calculated based on the established standard carrimycin curve. The t-test was used in the quantitative calculation of carrimycin titers in different strains.

Transcriptome analysis and quantitative real-time PCR of *streptomyces spiramyceticus* 54IA-2 and mutant strains

S. spiramyceticus 54IA and F2R-15 incubated for 120h in soluble fermentation medium were harvested and flash-frozen in liquid N_2. Frozen mycelia pellets were ground into a fine powder by using a pestle and a mortar, and total RNA was extracted using an RNAprep pure Micro Kit (TIANGEN) according to the manufacturer's instructions. Contaminating chromosomal DNA in the RNA samples was eliminated by treating with DNase I (Promega). The quantity and quality of RNA samples were assessed by measuring the A_{260} and A_{280} of the samples using Nanodrop (DeNovix), and the integrity of the purified RNA samples was determined by denaturing agarose gel electrophoresis. The transcriptome analysis was performed by OE Biotech (Shanghai, People's Republic China). *P* value <0.05 and foldChange >2 set as the threshold for significantly differential expression. Genes with more than 2-fold change and *P* value <0.05 were defined as significantly regulated genes.

Quantitative real-time PCR was performed on a Light Cycler 96 (Roche) with FastStart Essential DNA Green Master (Roche). All qPCR gene-specific primers were designed to produce ~150 bp long amplicons and all reactions were performed in triplicate for three different samples using gene specific primers (Table S3). The 16S RNA gene from *S. spiramyceticus* 54IA was used as the internal control to normalize samples. PCR program: 96 °C 1 min (96 °C 30 s, 61 °C 30 s, 72°C 1 min) 40 cycles, 72 °C 10 min. Melting-curve analysis was performed to check the specificity of PCR amplification. Melting-curve analysis was performed to check the specificity of PCR amplification. Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification and genes expression was normalized against the genes expression of 16S RNA to generate a ΔCt value (Ct of target gene–Ct of endogenous control). The change in the genes' expression was calculated using $2^{-\Delta\Delta Ct}$ method.

Statistics

Each experiment was replicated three times, with the error bars showing the standard deviations (SDs). To compare the difference between the test and control data, *P* values were calculated by Student's t test ($P < 0.001$).

The fragment of *sco6196* cloned from *streptomyces spiramyceticus* 54IA has been deposited in GenBank with the Accession OL616099.

Declarations

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Availability of data and materials

Not applicable.

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Authors' Contributions

WQH and TYH conceived the work and drafted the manuscript. KML and JLD, equally to this study, performed experiments and analyzed data. JJL participated in the experiment and collected the data. TYH and WQH wrote and revised the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interest.

References

1. Shang G, Dai J, Wang Y. Construction and physiological studies on a stable bioengineered strain of shengjimycin. J Antibiot (Tokyo). 2001;54:66–73.

2. Epp JK, Huber ML, Turner JR, Goodson T, Schoner BE. Production of a hybrid macrolide antibiotic in *Streptomyces ambofaciens* and *Streptomyces lividans* by introduction of a cloned carbomycin biosynthetic gene from *Streptomyces thermotolerans*. Gene. 1989;85(2):293–301.
3. Sun CH, Jiang W, Huang J, Jin WZ, Wang YG. Shengjimycins: a group of hybrid antibiotics, 4”-acylspiramycins. Actinomycetologica. 1999;13(2):120–125.
4. Shi XG, Sun YM, Zhang YF, Zhong DF. Tissue distribution of bitespiramycin and spiramycin in rats. Acta Pharmacol Sin. 2004;25(11):1396–401.
5. Shi XG, Fawcett JP, Chen XY, Zhong DF. Structural identification of bitespiramycin metabolites in rat: A single oral dose study. Xenobiotica. 2005;35 (4): 343–358.
6. Yan H, Sun J, Wang K, Wang H, Wu S, Bao L, He W, Wang D, Zhu A, Zhang T, Gao R, Dong B, Li J, Yang L, Zhong M, Lv Q, Qin F, Zhuang Z, Huang X, Yang X, Li Y, Che Y, Jiang J.Repurposing CFDA-approved drug carrimycin as an antiviral agent against human coronaviruses, including the currently pandemic SARS-CoV-2. Acta Pharm Sin B. 2021; 11(9):2850–2858.
7. Wang YG, Dai JL, Shang GD. Genetically engineered bacterial strain spiramycin *streptomycetespiramyceticus* WSJ-195 of bitespiramycin: CN, 02148771.5. 2004-10-06 (in Chinese).
8. Liu J, Zhang Y, He W. Construction of a novel carrimycin-producing strain by using CRISPR-Cas9 and ribosome engineering techniques. Chin J Biotechnol. 2021; 37(6):2116–2126.
9. Wang HY, Zhou HX, Dai JL, et al. Studies on space-flight breeding of genetically engineered strain of biotechmycin. Chin J Pharm Biotechnol. 2007;14(1):10–13 (in Chinese).
10. Dai JL, Li RF, Wu LZ, et al. Improvement of new generation of bitespiramycin producing strain by microwave radiation. Chin J Antibiot. 2009;34(7): 406–410, 428 (in Chinese).
11. Dai JL, Li RF, WANG YG, WU LZ, He WQ. Optimization of fermentation medium for bitespiramycin production by response surface methodology. Journal of Shenyang Pharmaceutical University. 2010;27(6):482–488 (in Chinese).
12. Li Z, Wang Y, Chu J, Zhuang Y, Zhang S. Leucine improves the component of isovalerylspiramycins for the production of bitespiramycin. Bioprocess Biosyst Eng. 2009;32:641-647.
13. Gao X, Wang Y, Chu J. A preliminary study on the impact of exogenous A-Factor analogue 1,4-butyrolactone on stimulating bitespiramycin biosynthesis. Bioprocess Biosyst Eng. 2019, 42(12):1903–1913.
14. Dai JL, Wang YH, Liu YW, Xiao CY, Tong ZM, Chu J, Zhang SL, Lv GY, Wang YG. Study on the fermentation of genetically engineered strain of biotechmycin in fermentor. Chin J Antibiot. 2005;30(6):324–327, 428 (in Chinese).
15. Lu Z, Zhang X, Dai J, Wang Y, He W. Engineering of leucine-responsive regulatory protein improves spiramycin and bitespiramycin biosynthesis. Microb Cell Fact. 2019;18(1):38.
16. Dai JL, Zhang XT, Lu ZL, et al. Breeding of high isomycin-I-producing strain by MPMS composite mutagenesis with plasma and UV. Chin J Antibiot. 2018;43(2):182–188 (in Chinese).

17. Xia H, Zhan X, Mao XM, Li YQ. The regulatory cascades of antibiotic production in Streptomyces. World J Microbiol Biotechnol. 2020;36(1):13.
18. Li L, Wei K, Liu X, Wu Y, Zheng G, Chen S, Jiang W, Lu Y. aMSGE: advanced multiplex site-specific genome engineering withorthogonal modular recombinases in Actinomycetes. Metab Eng. 2019;52:153–167.
19. Yu Z, Lv H, Wu Y, Wei T, Yang S, Ju D, Chen S. Enhancement of FK520 production in *Streptomyces hygroscopicus* by combining traditional mutagenesis with metabolic engineering. Appl Microbiol Biotechnol. 2019;103 (23–24):9593–9606.
20. Xia M, Huang D, Li S, Wen J, Jia X, Chen Y. Enhanced FK506 production in Streptomyces tsukubaensis by rational feeding strategies based on comparative metabolic profiling analysis. Biotechnol Bioeng. 2013;110(10):2717–2730.
21. Wang J, Liu H, Huang D, Jin L, Wang C, Wen J. Comparative proteomic and metabolomic analysis of Streptomyces tsukubaensis reveals the metabolic mechanism of FK506 overproduction by feeding soybean oil. Appl Microbiol Biotechnol. 2017;101(6):2447–2465.
22. JungWS, Yoo YJ, Park JW, Park SR, Han AR, Ban YH, Kim EJ, Kim E, Yoon YJ. A combined approach of classical mutagenesis and rational metabolic engineering improves rapamycin biosynthesis and provides insights into methylmalonyl-CoA precursor supply pathway in *Streptomyces hygroscopicus* ATCC 29253. Appl Microbiol Biotechnol. 2011;91(5):1389–1397.
23. Karray F, Darbon E, Oestreicher N, Dominguez H, Tuphile K, Gagnat J, Blondelet-Rouault MH, Gerbaud C, Pernodet JL, Organization of the biosynthetic gene cluster for the macrolide antibiotic spiramycin in *Streptomyces ambofaciens*. Microbiology. 2007;153:4111–4122.
24. Wang W, Li S, Li Z, Zhang J, Fan K, Tan G, Ai G, Lam SM, Shui G, Yang Z, Lu H, Jin P, Li Y, Chen X, Xia X, Liu X, Dannelly HK, Yang C, Yang Y, Zhang S, Alterovitz G, Xiang W, Zhang L. Harnessing the intracellular triacylglycerols for titer improvement of polyketides in Streptomyces. Nat Biotechnol. 2020;38(1):76–83.
25. Alvarez HM, Steinbuchel A. Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol. 2002;60:367–376.
26. Karray F, Darbon E, Nguyen HC, Gagnat J, Pernodet JL. Regulation of the biosynthesis of the macrolide antibiotic spiramycin in *Streptomyces ambofaciens*. J Bacteriol. 2010;192:5813–5821.
27. Dai J, Wang Y, Liu J, He W. The regulatory genes involved in spiramycin and bitespiramycin biosynthesis. Microbiol Res. 2020;240:126532.
28. Xiang SH, Li J, Hua Yin, Zheng JT, Yang X, Wang HB, Luo JL, Bai H, Yang KQ. Application of a double-reporter-guided mutant selection method to improve clavulanic acid production in *Streptomyces clavuligerus*. Metab Eng. 2009;11(4-5):310–318.
29. Xia H, Li X, Li Z, Zhan X, Mao X, Li Y. The Application of Regulatory Cascades in Streptomyces: Yield Enhancement and Metabolite Mining. Front Microbiol. 2020;11:406.
30. Cundliffe E. Control of tylosin biosynthesis in *Streptomyces fradiae*. J Microbiol Biotechnol. 2008;18(9):1485–1491.

31. Xu J, Song Z, Xu X, Ma Z, Bechthold A, Yu X. ToyA, a positive pathway-specific regulator for toyocamycin biosynthesis in *Streptomyces diastatochromogenes* Appl Microbiol Biotechnol. 2019;103(17):7071–7084.
32. Zhu Z, Li H, Yu P, Guo Y, Luo S, Chen Z, Mao X, Guan W, Li Y. SlnR is a positive pathway-specific regulator for salinomycin biosynthesis in *Streptomyces albus*. Appl Microbiol Biotechnol. 2017;101(4):1547–1557.
33. Zhang Y, He H, Liu H, Wang H, Wang X, Xiang W. Characterization of a pathway-specific activator of milbemycin biosynthesis and improved milbemycin production by its overexpression in *Streptomyces bingchenggensis*. Microb Cell Fact. 2016;15(1):152.
34. Dai JL, Zhang XT, Lu ZL, et al. Breeding of high isomycin-I-producing strain by MPMS composite mutagenesis with plasma and UV. Chin J Antibiot. 2018;43 (2):182–188 (in Chinese)
35. Wang L, Tian X, Wang J, Yang H, Fan K, Xu G, Yang K, Tan H. Autoregulation of antibiotic biosynthesis by binding of the end product to an atypical response regulator. Proc Natl Acad Sci U S A. 2009;106(21):8617–8622.
36. Li X, Yu T, He Q, McDowall KJ, Jiang B, Jiang Z, Wu L, Li G, Li Q, Wang S, Shi Y, Wang L, Hong B. Binding of a biosynthetic intermediate to AtrA modulates the production of lidamycin by *Streptomyces globisporus*. Mol Microbiol. 2015;96(6):1257–1271.
37. Lei C, Wang J, Liu Y, Liu X, Zhao G, Wang J. A feedback regulatory model for RifQ-mediated repression of rifamycin export in *Amycolatopsis mediterranei*. Microb Cell Fact. 2018;17(1):14.
38. Malla S, Niraula NP, Liou K, Sohng JK. Self-resistance mechanism in Streptomyces peucetius: overexpression of *drrA*, *drrB* and *drrC* for doxorubicin enhancement. Microbiol Res. 2010;165(4):259–267.
39. Xia H, Zhan X, Mao XM, Li YQ. The regulatory cascades of antibiotic production in Streptomyces. World J Microbiol Biotechnol. 2020;36(1):13.
40. Kieser T, Bibb MJ, Butter MJ, Chater KF, Hopwood DA (2000) Practical Streptomyces genetics: a laboratory manual. The John Innes Foundation, Norwich.
41. Wang YG, Jin LF, Jin WZ, Zhang XH, Zeng Y, Xu XM, Jun Y. Cloning and expression of midecamycin 4”-acylase gene in spiramycin producing strain. Chin J Biotechnol. 1992;8:1–14.

Figures

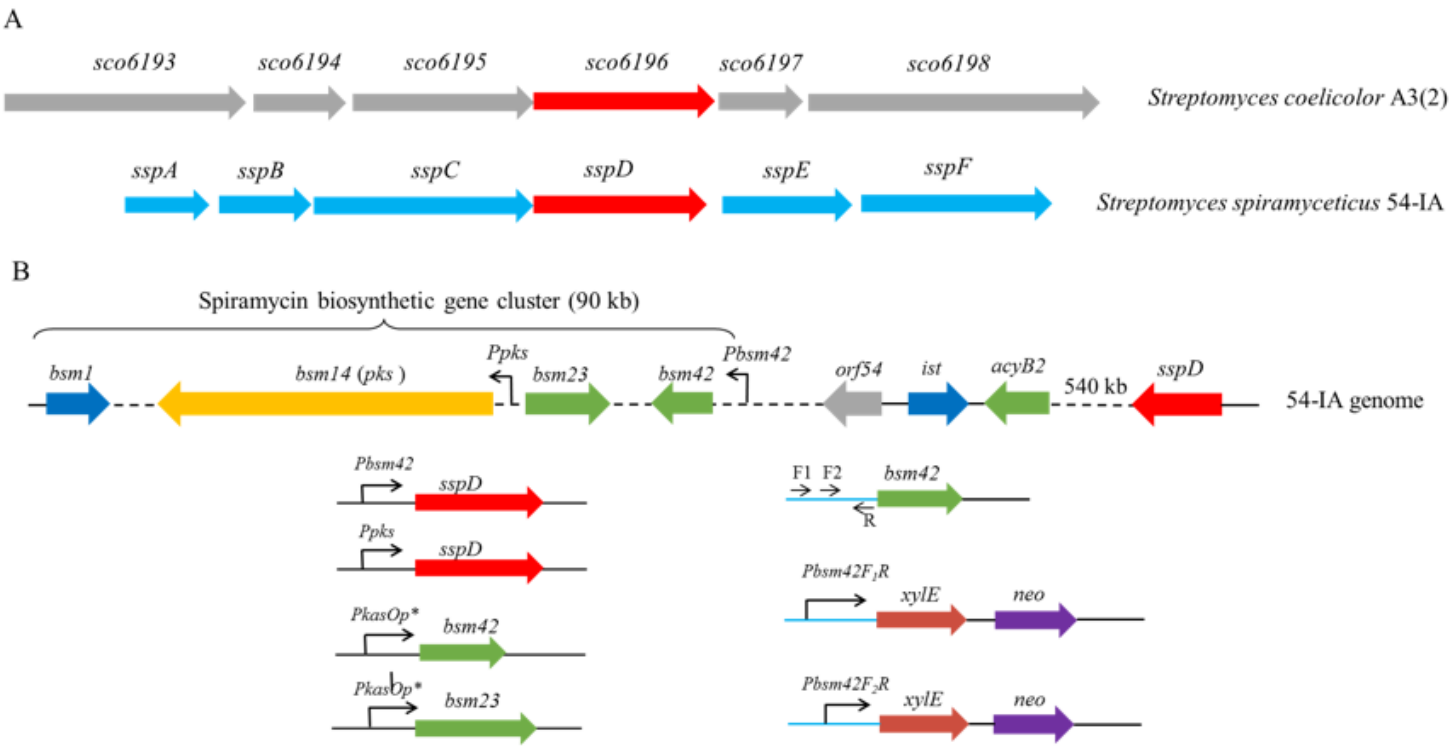

Figure 1

Bioinformatics analysis of *sspD* gene and construction of target genes under control different promoters. A: Alignment of the *sco6196* and *sspD* gene clusters in *Streptomyces coelicolor* A3(2) and *Streptomyces spiramyceticus* 54IA, respectively. B: Construction of target genes under control different promoters in *Streptomyces spiramyceticus* 54IA. The genes are indicated by various colors. The *sco6196* and *sspD* genes are in red color and the regulatory genes in green color. Promoters of interest (Ptarget) are indicated by black arrows.

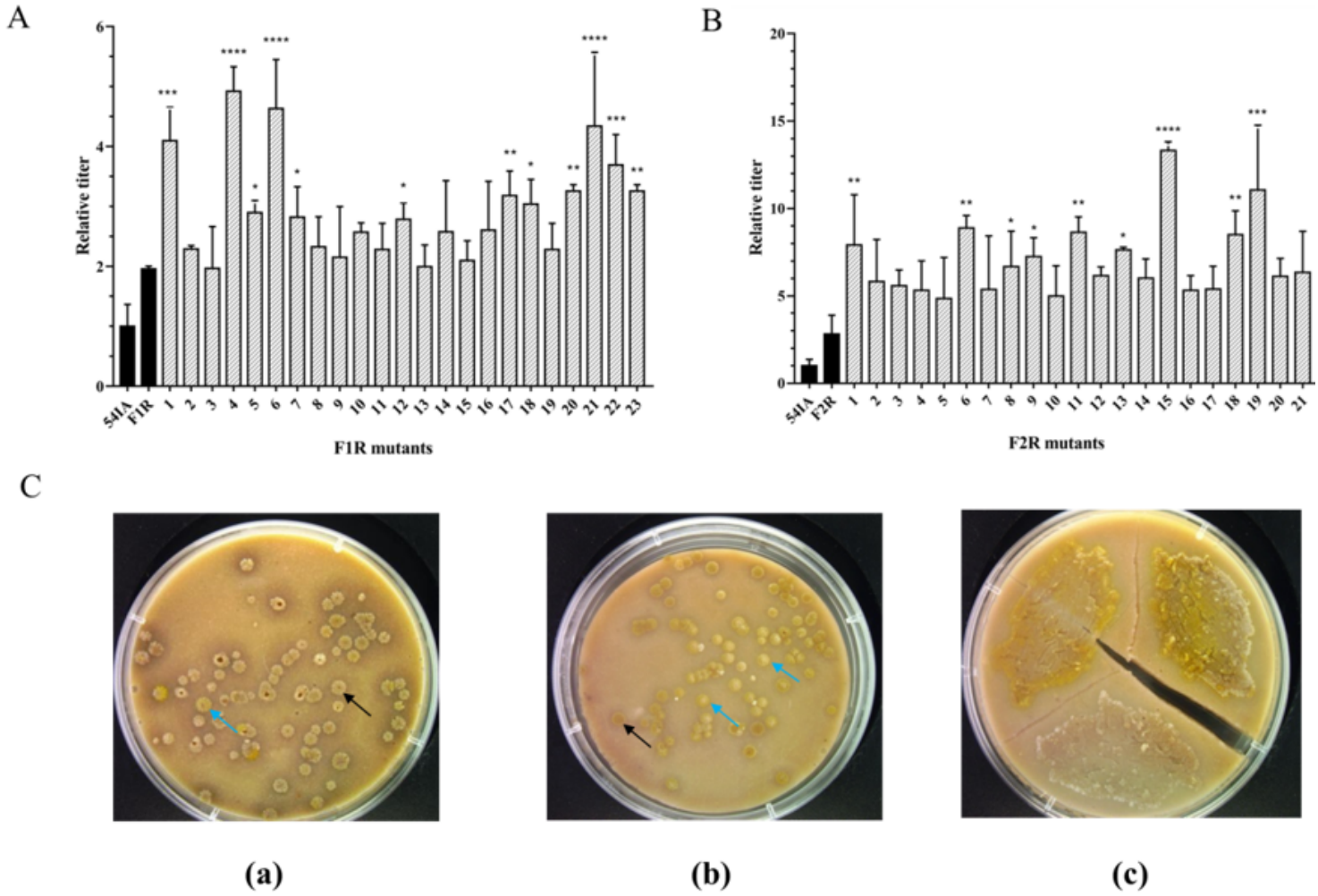

Figure 2

The characterization of F1R and F2R mutants. A: The titer of F1R mutants from pDR-42 F1R double-reporter system screening. 54IA: original strain *S. spiramyceticus* 54IA containing the pDR2 plasmid, F1R: the original pDR-42 F1R plasmid transformant, 1-23: the positive F1R mutants. B: The titer of F2R mutants from pDR-42F1R double-reporter system screening. 54IA: original strain 54IA, F1R: the original pDR-42F2R plasmid transformant, 1-21: the positive F2R mutants. The carrimycin titer is indicated as fold changes obtained by dividing mean value of each strain by the value of *S. spiramyceticus* 54IA. Error bars indicate the average standard deviations from the mean. C: F1R (a) and F2R (b) mutant colonies on selection plate sprayed with catechol. Black arrow: the typical false positive mutant, showing elevated kanamycin resistance but no observable XylE activity; blue arrow: the typical *xylE* over-expressing mutant (yellow). (c) Comparison of F1R-4 (left, No. 4 mutant), F2R-15 (right, No.15 mutant) and 54IA strain containing the pDR2 plasmid (under).

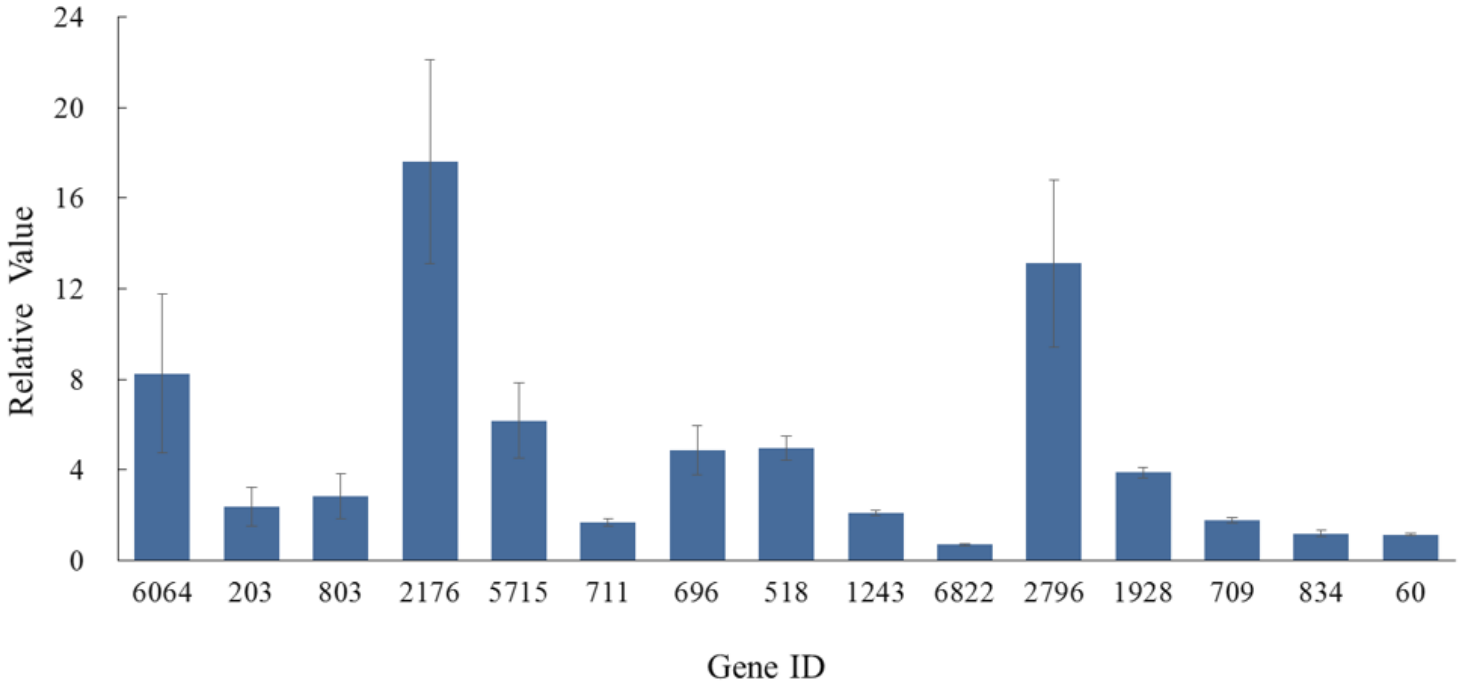

Figure 3

qPCR of some up-regulated genes in the F2R-15 mutant and 54IA starting strains (n=3).

Supplementary Files

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