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## Article

# Multicopy Chromosome Integration and Deletion of Negative Global Regulators Significantly Increased the Heterologous Production of Aborycin in *Streptomyces coelicolor*

Jia-Yi Li <sup>1</sup>, Jun-Yu Liang <sup>1</sup>, Zhao-Yuan Liu <sup>1</sup>, Yue-Zhao Yi <sup>1</sup>, Jing Zhao <sup>1,2,3</sup>, Zhi-Yong Huang <sup>4,5,\*</sup> and Jun Chen <sup>1,2,3,\*</sup>

<sup>1</sup> Department of Marine Biological Science & Technology, College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, China; 694889973@qq.com (J-Y. L.); 359284320@qq.com (J-Y. L.); 925653989@qq.com (Z-Y. L.); 913076462@qq.com (Y-Z. Y.); sunnyzhaoj@xmu.edu.cn (Z. J.)

<sup>2</sup> State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen University, Xiamen 361102, China

<sup>3</sup> Xiamen City Key Laboratory of Urban Sea Ecological Conservation and Restoration, Xiamen University, Xiamen 361102, China

<sup>4</sup> Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China;

<sup>5</sup> National Technology Innovation Center of Synthetic Biology, Tianjin 300308, China

\* Correspondence: chenjun@xmu.edu.cn (J. C.); huang\_zy@tib.cas.cn (Z-Y. H.)

**Abstract:** Aborycin is a type I lasso peptide with a stable interlocked structure, offering a favorable framework for drug development. The aborycin biosynthetic gene cluster *gul* from marine sponge-associated *Streptomyces* sp. HNS054 was cloned and integrated into the chromosome of *S. coelicolor* hosts with different copies. The 3-copy *gul*-integration strains *S. coelicolor* M1346::3gul showed better production than one-copy or 2-copy *gul*-integration strains, and the total titer reached approximately 10.4 mg/L, i.e., 2.1 times that of the native strain. Then, five regulatory genes, *phoU* (SCO4228), *wblA* (SCO3579), *SCO1712*, *orrA* (SCO3008) and *gntR* (SCO1678), which were reported to have negative effects on secondary metabolism, were further knocked out from the M1346::3gul genome by CRISPR/Cas9 technology. While the  $\Delta$ SCO1712 mutant showed a significant decrease (4.6 mg/L) and the  $\Delta$ phoU mutant showed no significant improvement (12.1 mg/L) in aborycin production, the  $\Delta$ wblA,  $\Delta$ orrA and  $\Delta$ gntR mutations significantly improved the aborycin titers to approximately 23.6 mg/L, 56.3 mg/L and 48.2 mg/L, respectively, which were among the highest heterologous yields for lasso peptides in both *Escherichia coli* systems and *Streptomyces* systems. Thus, this study provided important clues for future studies on enhancing antibiotic production in *Streptomyces* systems.

**Keywords:** RiPP; marine streptomyces; phoU (SCO4228); wblA (SCO3579); SCO1712; orrA (SCO3008); gntR (SCO1678)

## 1. Introduction

Aborycin is a class I lasso peptide and was first isolated from *Streptomyces* sp. SP9440 as a novel anti-HIV metabolite with the origin name RP 71955 [1,2]. It was independently rediscovered from soil *S. griseoflavus* Tü 4072 as an antibiotic [3], then from deep-sea *Streptomyces* sp. SCSIO ZS0098 as an anti-infective natural product [4] and from marine sponge-associated *Streptomyces* sp. MG010 as an antibacterial marker for screening gain-of-function mutants [5]. Aborycin has a typical lasso topology in which the N-terminal 9 amino acids form a macrocyclic ring, and the C-terminal 12 amino acids form a tail that folds back and threads through the ring [2]. Two disulfide bonds between the ring and the tail further increase the structural stability and distinguish the class I lasso peptides from others [6,7].

Lasso peptides are a growing class of bioactive bacterial peptides with unique lasso topology, which differentiates them from other members within the much larger ribosomally synthesized and



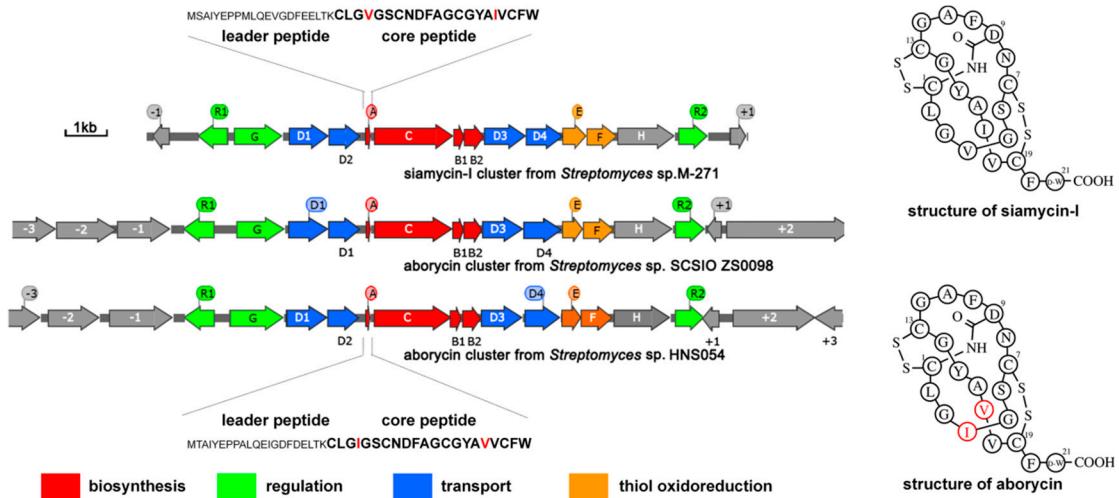
posttranslationally modified peptide (RiPP) superfamily [8]. The compact and constrained topology endows most lasso peptides with remarkable thermal and proteolytic stability and favors peptide-protein interactions, accounting for the diverse biological activities of lasso peptides, mainly as enzyme inhibitors and receptor antagonists [6,9]. The robust scaffolds of lasso peptides have attracted attention for drug development, such as epitope grafting [10], the incorporation of noncanonical amino acids [11] and protein fusion [12]. While these modifications provide the opportunity to develop novel biological activities with therapeutic potential, they always lead to lower production levels [7]. Moreover, genome mining approaches have greatly accelerated lasso peptide discovery in recent years. Since the first lasso peptide was isolated by genome mining in 2008 [13], the number of lasso peptides discovered by such approaches has steadily increased [9]. By applying the RODEO algorithm, > 1,400 lasso peptide biosynthetic gene clusters (BGCs) were identified from DNA sequence databases, a great increase over the previously known numbers [14]. Although lasso peptide BGCs are widely distributed among bacteria, only approximately 80 lasso peptides were previously characterized [15]. Therefore, both drug development and functional characterization demand effective production systems to explore this rich source of lasso peptides and their modifications.

The heterologous production of lasso peptides in *Escherichia coli* often provides higher yields than the use of native producers. However, this production advantage in *E. coli* seems to be confined to lasso peptides from proteobacteria and is less viable for clusters from other phyla [7,9]. Recently, *Streptomyces* hosts, especially *S. coelicolor*, *S. lividans*, and *S. albus*, have shown significant potential for producing lasso peptides from actinobacteria [14,16,17]. Several technological advances were also achieved for *Streptomyces* systems, for example, CRISPR/Cas9 genome editing technology [18,19] and the multiplexed site-specific genome engineering (MSGE) method [20]. Genome editing offers a rapid way to modify regulatory elements involving secondary metabolism and therefore impact heterologous production. The MSGE method led to the successful development of a panel of *S. coelicolor* heterologous hosts, in which up to five copies of BGCs could be integrated into the specific sites of the host chromosome in a single step, leading to significant yield improvements [20]. In our previous study, an aborycin BGC was identified from a marine sponge-associated *Streptomyces* sp. HNS054 [5]. This provided an opportunity to produce this lasso peptide heterologously in *Streptomyces* systems. Thus, in this study, a *Streptomyces* system for aborycin production that is compatible with up-to-date technologies was established. By increasing the integrated copy numbers of the aborycin BGC in the host chromosome and by deleting the negative global regulatory genes involved in secondary metabolism by genome editing, the production of aborycin was significantly improved. This study provides a useful reference to improve *Streptomyces* systems for the production of lasso peptides and their modifications and thus benefits drug development and functional characterization.

## 2. Results

### 2.1. General information on the aborycin gene cluster from *Streptomyces* sp. HNS054

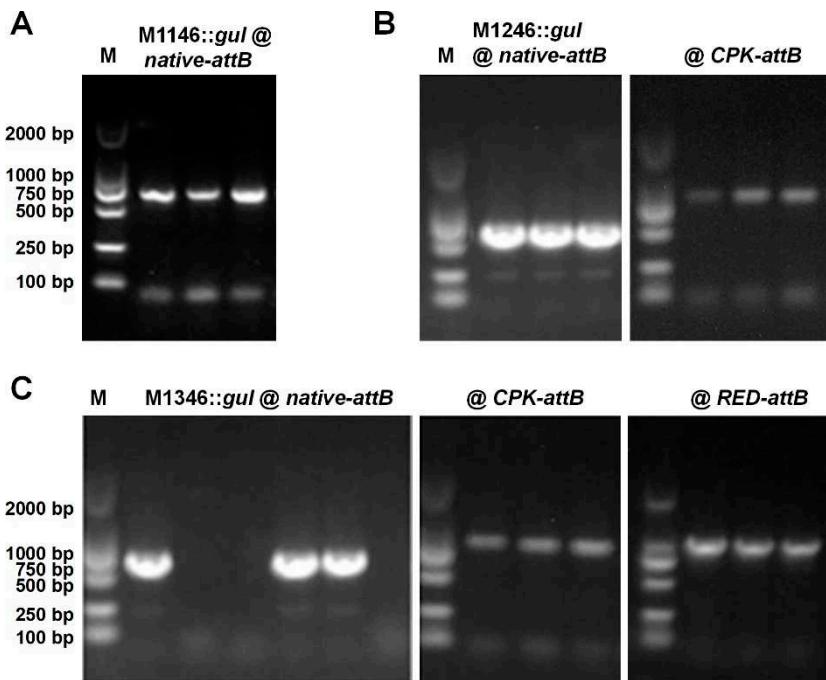
As previously reported [5], the aborycin gene cluster *gul* (named after Gulei Town, where the host marine sponge was collected from its coastal sea) from marine sponge-associated *Streptomyces* sp. HNS054 consists of 14 ORFs spanning an approximately 12 kb region from *gulR1* to *gulR2* (Figure 1). Sequence alignment showed that the DNA sequence of this region shares 98% identity to the aborycin gene cluster *abo* from *Streptomyces* sp. SCSIO ZS0098 [4]. The *gul* cluster also shows high identity to the siamycin-I gene cluster *msl* from *Streptomyces* sp. M-271 [21] in gene sequence and gene organization (Figure 1). *GulA* encodes a 42-residue peptide with a leader peptide at its N-terminus and a 21-residue core peptide (CLGIGSCNDFAGCGYAVVCFW) at its C-terminus. The primary and secondary structures of aborycin and siamycin-I are almost the same, except that the residues at the 4th and 17th positions are switched (Figure 1). Due to the high similarity between aborycin and siamycin-I, commercial reagents of siamycin-I could be used as a reference to locate the high performance liquid chromatography (HPLC) signal of aborycin.



**Figure 1.** General information on aborycin and siamycin-I, and their BGCs, sequences and secondary structures. Sequence differences between aborycin and siamycin-I are marked in red.

## 2.2. Cloning of the *gul* BGC and construction of strains for heterologous expression of aborycin

The cloning procedure of the *gul* BGC is shown in Figure S1. The 14 kb fragment containing the *gul* BGC was amplified from *Streptomyces* sp. HNS054 by high-fidelity PCR and subsequently cloned and inserted into the integrative vector *pSAT209*, yielding *pSAT-GUL* (Figure S2). The plasmid was fully sequenced, and the results confirmed that no mutation occurred within the plasmid frame and the *gul* ORFs. Then, the plasmid *pSAT-GUL* was transferred to *S. coelicolor* strains M1146, M1246, M1346, M1446 and M1546 by conjugation, respectively. As a blank control, the empty plasmid *pSAT209* was also transferred to *S. coelicolor* M1146. The numbers of successful *attP*-*attB* recombinations in exconjugants were determined by PCR. The results showed that strains M1146::*gul* (Figure 2A), M1246::2*gul* (Figure 2B) and M1346::3*gul* (Figure 2C) were successfully constructed. However, four-copy and five-copy integrations were unsuccessful after four rounds of conjugation and screening.

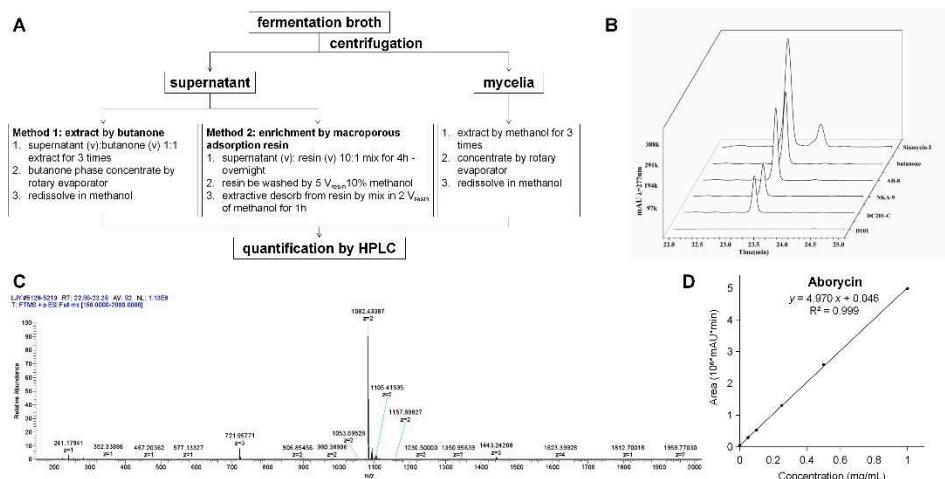


**Figure 2.** PCR validation of *gul* cluster integration in the *attB* loci. A. Exconjugants were checked for M1146::*gul* integration at the native *attB* locus. Positive integration resulted in a 557 bp band. B.

Exconjugants were checked for M1246::2gul integration at the native and *CPK attB* loci. Positive integration resulted in a 557 bp and a 773 bp band, respectively. C. Exconjugants were checked for M1346::3gul integration at the native, *CPK* and *RED attB* loci. Positive integration resulted in a 557 bp, a 773 bp and an 887 bp band, respectively.

### 2.3. Extraction and detection of aborycin

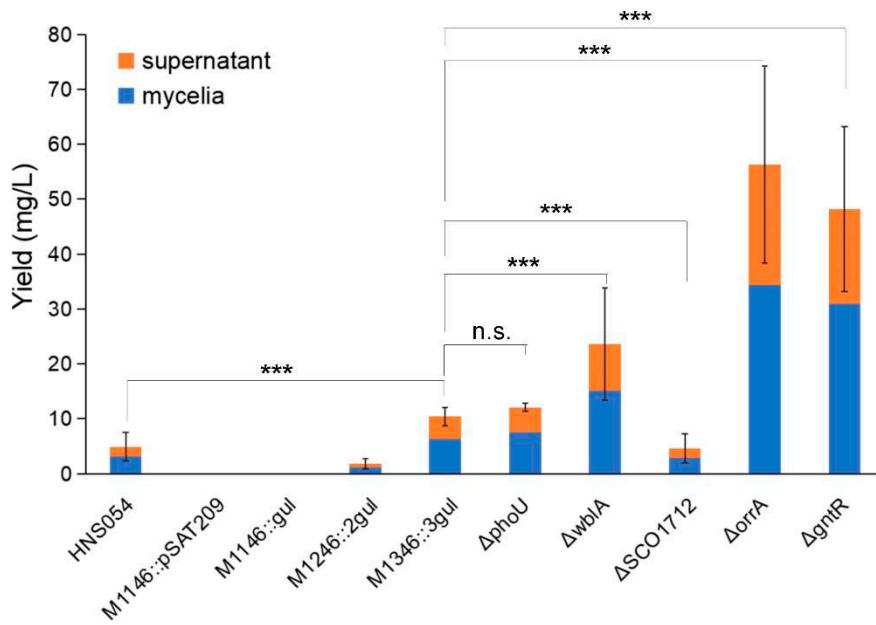
With siamycin-I as the control, the M1346::3gul strain was used to test whether aborycin was successfully produced. Aborycin was extracted as shown in Figure 3A. The butanone extract had an HPLC signal at 23.2 min, the same as the signal of siamycin-I (Figure 3B). In the following test of the macroporous adsorption resin method, the AB-8 resin showed high enrichment efficiency for aborycin, equivalent to that of the butanone method. Mass spectrum analysis confirmed that a substance with a relative molecular mass of  $m/z = 1082.43$  ( $z = 2$ ) accounted for the majority of the composition at the 23.2 min peak of the AB-8-enriched samples (Figure 3C). The MS data of this substance were perfectly matched to the aborycin data from a previous report [5]. Thus, two facts were confirmed by these tests. First, the M1346::3gul strain can express aborycin. Second, compared with the butanone method, the AB-8 resin method offered a safer, easier and cheaper method to enrich aborycin from the fermentation supernatant. This method was thus applied in the following quantification studies. After pure aborycin was isolated from 5.6 L culture broth of the M1346::3gul strain, a precise standard curve of concentrations to the HPLC peak areas of aborycin was obtained (Figure 3D). By applying this curve, aborycin production from different strains or conditions could be quickly quantitated by HPLC.



**Figure 3.** Extraction of aborycin. A. The extraction procedure. B. The 23.2 min HPLC peaks of extractives obtained from the supernatant of M1346::3gul by butanone or by macroporous adsorption resins. Siamycin-I was used as a control. The loading quantities of each test were adjusted to represent the same amount of the supernatant. C. Mass spectrum analysis of the matter abundance in the  $m/z$  range [150-2000] of the 23.2 min collection from the sample enriched by the AB-8 resin. A peak at  $m/z = 1082.43$  ( $z = 2$ ) accounted for approximately 90% of the total mass. D. The standard curve of concentrations to the HPLC peak areas of aborycin.

### 2.4. Yield comparison among different strains

Titers of aborycin from different strains are shown in Figure 4. Adding the supernatant and mycelial products, the native HNS054 strain average yielded  $4.9 \pm 2.6$  mg/L aborycin. No aborycin signal was detected from the blank control M1146::*pSAT209* and the one-copy strain M1146::gul. Signals became obvious for the M1246::2gul and M1346::3gul strains, which had average titers of  $1.8 \pm 1.0$  mg/L and  $10.4 \pm 1.6$  mg/L, i.e., 0.4 times and 2.1 times that of the native strain, respectively. Both supernatant and mycelia contributed to the production, accounting for approximately 38 percent and 62 percent, respectively.



**Figure 4.** Aborycin titers from different strains. HNS054, the wild-type strain *Streptomyces* sp. HNS054.  $\Delta phoU$  -  $\Delta gntR$ , gene knockout strains from *S. coelicolor* M1346::3gul. Orange block, average production from supernatant extracted by the AB-8 method. Blue block, average production from mycelia extracted by the methanol method. Error bar, standard deviation of total titers (supernatant + mycelia). Data were counted from triplicate experiments. \*\*\*,  $p < 0.001$ . n.s.,  $p > 0.05$ .

Then, the M1346::3gul strain was selected for genetic modification to verify whether aborycin production could be further improved by deleting negative regulatory genes (Table 1), which were reported to act at higher levels of the gene-regulatory networks to control secondary metabolism, whose knockout mutants always resulted in an increase in secondary metabolism. The  $\Delta phoU$ ,  $\Delta wblA$ ,  $\Delta SCO1712$ ,  $\Delta orrA$  and  $\Delta gntR$  mutants were successfully constructed (Figure S3, S4). A titer study showed that the aborycin production of the M1346::3gul  $\Delta phoU$  strain was  $12.1 \pm 0.7$  mg/L, which was slightly higher than that of the M1346::3gul strain but not significantly different ( $P > 0.05$ ). The  $\Delta wblA$  strain was significantly ( $P < 0.01$ ) higher than the M1346::3gul strain, with a titer of  $23.6 \pm 10.2$  mg/L, i.e., 4.8 times that of the native strain or 2.3 times that of the strain before *wblA* knockout. Greater improvements were displayed in the  $\Delta orrA$  and  $\Delta gntR$  mutants, which had titers of  $56.3 \pm 18.0$  and  $48.2 \pm 15.1$  mg/L, respectively (Figure 4).

**Table 1.** Information on the genes to be knocked out.

Trial	Gene Code	Symbol	Relevant Features	References
J01	SCO4228	<i>phoU</i>	$\Delta phoU$ mutant showed 6-fold increase in ACT production when phosphate starvation	[22]
J04	SCO3579	<i>wblA</i>	$\Delta wblA$ mutant showed 1.5-fold increase in doxorubicin production	[23]
J05	SCO1712	<i>SCO1712</i>	$\Delta SCO1712$ mutant showed 1.62-fold or 1.22-fold increase in ACT or RED production, respectively	[24]
J07	SCO3008	<i>orrA</i>	$\Delta orrA$ mutant showed great increase in ACT and RED production	[25]
J08	SCO1678	<i>gntR</i>	$\Delta gntR$ mutant altered the secondary metabolite profile of <i>S. coelicolor</i>	[26]

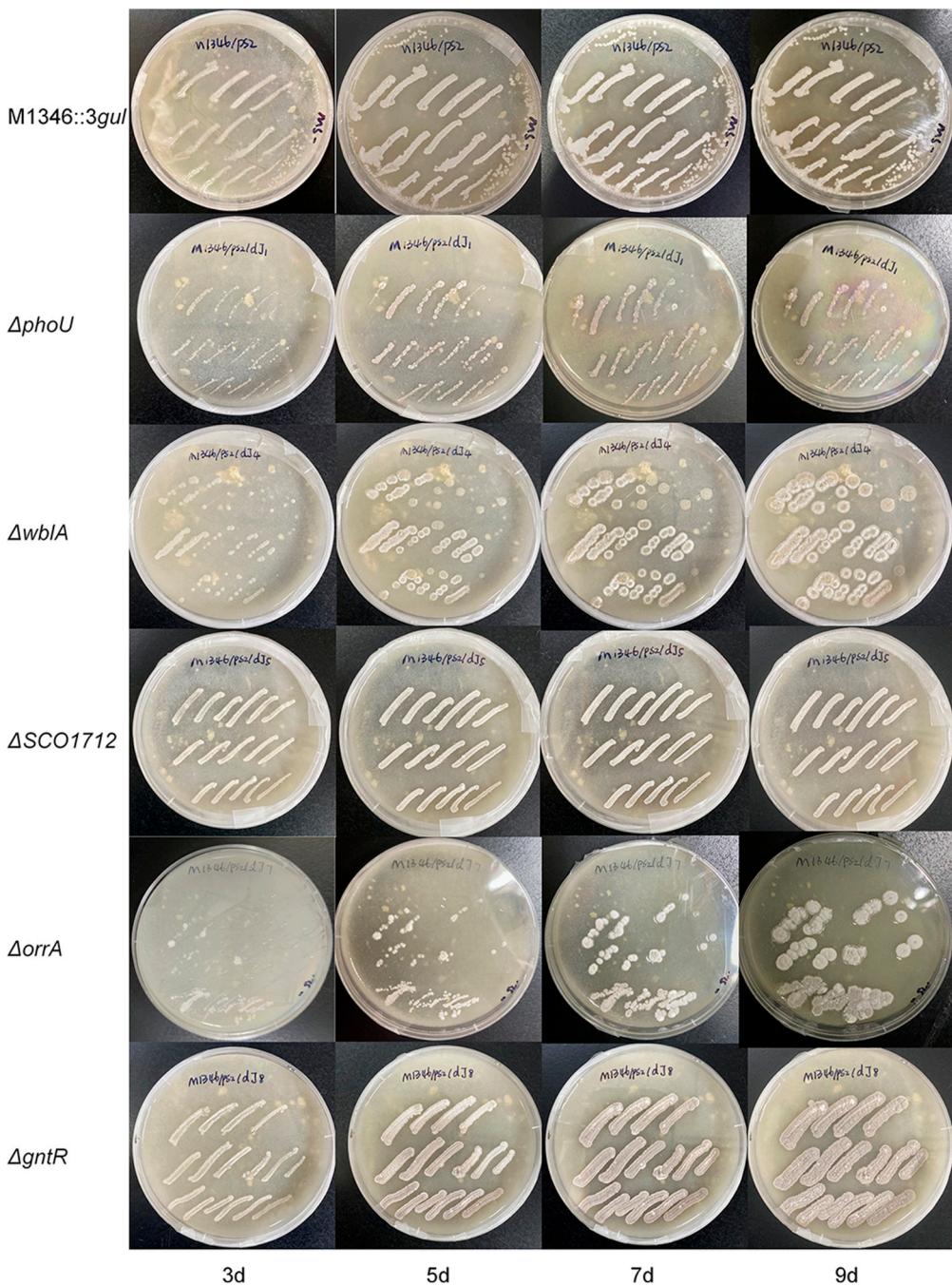
### 3. Discussion

In this study, efforts were made to use the *Streptomyces* system for the heterologous production of aborycin. First, the cloning procedure was simplified. The 14 kb genomic fragment containing the *gul* gene cluster was amplified by high-fidelity PCR, and sequencing confirmed its accuracy. High-fidelity PCR techniques have been developed to amplify long DNA fragments with lengths as long as 15-20 kb. The cloning of RiPP gene clusters could benefit from these developments because a large portion of RiPP gene clusters have lengths below 15 kb (Table S3). In a recent review on newly discovered RiPPs, out of 33 listed gene clusters, 25 (76%) had lengths below 15 kb ([27], Table S4). Second, to facilitate foreign DNA integration following genome editing, the integrative vector *pSET152* was modified to *pSAT209* to prevent antibiotic resistance conflict. Third, the extraction of aborycin was optimized. Aborycin is a peptide with amphiphilic characteristics and thus reversible affinity to certain macroporous adsorption resins. In this case, AB-8 resin was found to be the matched resin to adsorb aborycin in water solution and desorb it in methanol. By simply adsorbing and desorbing, approximately 40% of the total production was recovered from the culture supernatant. A similar situation occurred with the heterologous expression of svicucin, where approximately 1/3 of the product was released in the culture supernatant [16]. Large-scale production would discard the supernatant because of the high cost of solvent extraction. With this optimization, the product in the supernatant could be recovered at a low cost. We speculated that this theory and operation could be applied to other RiPPs due to their peptide nature. Finally, multiple chromosomal integration of foreign gene clusters following CRISPR/Cas9 genome editing was successfully achieved, and the best case resulted in a 25-fold increase in aborycin production compared to the native strain.

Multiple chromosomal integration of foreign BGC was proven to be a mature technique to increase heterologous expression. The approach was successfully demonstrated in *S. coelicolor* [20], *S. albus* J1074 [28] and *S. lividans* [29]. In this study, one-copy integration showed no signal, while three-copy integration showed a 2.1-fold increase compared with the native strain. However, more advanced techniques were needed to obtain 4- or 5-copy integration in one single step. In our attempts to transfer other BGCs into these M1146-M1546 hosts, the number of exconjugants decreased rapidly with increasing integrated copy number (data not shown). It was previously speculated that a high copy number of chromosomal integration caused the accumulation of target products, endangering bacterial growth [20]. However, the titer of aborycin was further improved 5.4-fold by *orrA* gene knockout, implying that the *Streptomyces* actually tolerate higher concentrations of aborycin.

CRISPR/Cas-based genome editing tools provide swift, accurate and traceless ways to modify the genomes of *Streptomyces* [30]. A straightforward way to exploit the CRISPR/Cas9 tools was to delete negative regulator genes. This study proposed to delete genes that were reported to have a negative impact on secondary metabolism at a high level of the regulation networks. Five mutants,  $\Delta phoU$ ,  $\Delta wblA$ ,  $\Delta SCO1712$ ,  $\Delta orrA$  and  $\Delta gntR$ , were successfully constructed from the M1346::3*gul* strain.

Although the detailed function of the *phoU* gene was unclear, it was speculated to be involved in the pho regulon, which responds to phosphate starvation. PhoR senses such conditions, and then PhoP is phosphorylated following PhoP-P binding to specific sequences named PHO boxes, thus activating or repressing a set of genes [31]. Under phosphate starvation conditions, the  $\Delta phoU$  mutants showed an approximately 6-fold increase in the production of actinorhodin [22]. Our study showed that aborycin production in the  $\Delta phoU$  mutants was not significantly improved from that in the strain before mutation when strains were cultured in the R5 medium. Moreover, the growth of the  $\Delta phoU$  mutants on MS-agar was retarded in a 9-day morphology observation (Figure 5). This type of the mutants requires more optimization before it can be applied in antibiotic production.



**Figure 5.** The growth of strains on MS-agar plates from 3d to 9d.  $\Delta phoU$  -  $\Delta gntR$ , gene knockout strains from *S. coelicolor* M1346::3gul.

The *wblA* gene was reported as a pleiotropic downregulator of antibiotic biosynthesis in *S. coelicolor*.  $\Delta wblA$  mutants exhibited a defect in sporulation, achieved higher biomass than the wild-type, and overproduced secondary metabolites [32]. Overproduction of antibiotics by disruption of the *wblA* orthologs was also observed in other *Streptomyces* bacteria [33]. This study confirmed that the production of aborycin in the  $\Delta wblA$  mutant was significantly improved by 2-fold. Higher growth and defects in sporulation were also observed (Figure 5).

SCO1712 is a member of the TetR family. TetR family transcriptional regulators are among the most common prokaryotic transcriptional regulators. When SCO1712 was overexpressed or disrupted, ACT production decreased or increased compared with that in *S. coelicolor* M145, respectively, suggesting that SCO1712 is a pleiotropic downregulator of antibiotic biosynthesis in *S. coelicolor* [24]. It was further speculated that there is a synergistic effect between SCO1712 and precursor flux pathways in antibiotic production [34]. Unfortunately, in this study, after SCO1712

knockout, the production of aborycin decreased significantly. This provides ideas for future optimizations on precursor flux pathways or mediums for this mutant.

OhkA (SCO1596) - OrrA (SCO3008) is a group of prokaryotic two-component regulatory systems with highly similar transcriptomic features.  $\Delta$ orrA mutants lead to significant overproduction of antibiotics and downregulation of *bld*, *chp*, *rdl*, and *wbl* genes associated with morphological development [25]. Correspondingly, we found that  $\Delta$ orrA mutants overproduced approximately 5.4-fold of aborycin than the strains before mutation (Figure 4). Their morphological development also was similar to  $\Delta$ wblA mutants (Figure 5). These results implied that the OrrA regulatory system likely covered the *wbl* regulatory system and controls a wider range of resources for antibiotic production.

The bacterial GntR family is one of the most abundant groups of helix-turn-helix transcription factors that respond appropriately to metabolite micro-environments [35]. It was reported that deletion of a GntR-like gene allowed platensimycin and platencin overproduction in *S. platensis* [36]. *GntR* (SCO1678) of *S. coelicolor* encodes a repressor protein to control the gluconate operon, which enable *Streptomyces* to utilize gluconate in the mediums [26]. No obvious evidences linked *GntR* (SCO1678) to antibiotic overproduction to date. Interestingly, both overproduction of aborycin (Figure 4) and overgrowth (Figure 5) were observed from the  $\Delta$ GntR mutants in this study. No additional gluconate were added in the R5 or MS-agar mediums to obtain these results. These observations provide a significant gene that is worthy of further studies to discover underlying metabolic regulation mechanisms.

Although great production was achieved by these genetic modifications, we believe the *Streptomyces* systems could be further improved. With more global regulators that govern secondary metabolism being characterized [37], it is worth manipulating them one by one to obtain further knowledge.

#### 4. Materials and Methods

##### 4.1. Strains, plasmids and primers

The strains and plasmids used in this study are listed in Table S1, and the primers used in this study are listed in Table S2. The construction of the related vectors is shown in Figure S1 and Figure S2.

##### 4.2. Construction of heterologous expression strains to produce aborycin

Foreign DNA integration could be fulfilled by the integrative plasmid pSET152 [38], and genome editing could be executed by the suicide plasmid pKCcas9 [19]. However, both vectors contain the apramycin resistance gene *aac(3)IV*. To achieve BGC integration following genome editing, the antibiotic-resistance markers between the two genetic manipulations should be different. Thus, the *aac(3)IV* gene in pSET152 was replaced by the ampicillin resistance gene *bla* from the pUC57 plasmid and the thiostrepton resistance gene *tsr* from the pGM1190 plasmid. The resulting plasmid was named pSAT209 (Figure S1). Genomic DNA of *Streptomyces* sp. HNS054 was extracted from the later logarithmic phase cells by a bacterial genomic DNA extraction kit (Cat # DP2001, Biotake, Beijing, China). Using primers 054LasF and 054LasR, a 14 kb DNA fragment containing the gul BGC (located at 15932-29864 nt of GenBank: AC003\_RS35325) was amplified from the *Streptomyces* sp. HNS054 genome (Assembly: GCF\_001044185.1). After gel purification, the gul fragment was assembled with *Eco*RV-linearized pSAT209 by a one-step cloning kit (Cat # C113, Vazyme, Nanjing, China). The resulting vector *pSAT-GUL* (Figure S1) was sequenced to confirm the accuracy. Then, it and the control plasmid *pSAT209* were transferred into *E. coli* ET12567/pUZ8002 competent cells and conjugated into *S. coelicolor* M1146-M1546 hosts by standard protocols (Kieser et al. 2000). Exconjugants were randomly picked to check *attP*-*attB* recombination by PCR with the following primers. The forward primer ID-oriT-fw targets a position approximately 400 bp upstream of the *attP* locus in the vector *pSAT-GUL*. The reverse primers, ID-native-*attB*-Rev, ID-CPK-*attB*-Rev, ID-RED-*attB*-Rev, ID-CDA-*attB*-Rev and ID-ACT-*attB*-Rev, each target a position downstream of an

individual *attB* locus in the *S. coelicolor* M1546 genome. The integrated copy number of the *gul* gene cluster in the host genome was determined by the number of positive *attP*-*attB* recombinations [20].

#### 4.3. Gene knockout in *S. coelicolor* M1346::3gul by the CRISPR/Cas9 method

The *phoU* (SCO4228), *wbla* (SCO3579), *SCO1712*, *orrA* (SCO3008) and *gntR* (SCO1678) genes (Table 1) in *S. coelicolor* M1346::3gul were independently knocked out by the CRISPR/Cas9 genome editing method [19]. To generate the  $\Delta$ *phoU* mutant, the sgRNA expression cassette (f1 fragment) was PCR amplified with the plasmid *pKCCas9dO* as the template and f1J01-fwd/f1gRNA-R as primers. The upstream (f2 fragment) and downstream (f3 fragment) regions of the *phoU* gene were PCR amplified with the primer pairs f2J01-fwd/rev and f3J01-fwd/rev, respectively. Then, three DNA fragments were assembled by overlapping PCR using primers f1J01-fwd and f3J01-rev. The resulting DNA fragment was cloned and inserted into *pKCCas9* using the one-step cloning kit (Cat # C113, Vazyme, Nanjing, China) to yield *pKCCas9-dJ01*. The obtained plasmid was introduced into the M1346::3gul strain by conjugal transfer. The correct knockouts were verified by PCR using primers ID-dJ01-fwd/rev. By using the J04, J05, J07 and J08 sets of primers and the same procedure, *pKCCas9-dJ04*, -dJ05, -dJ07 and -dJ08 were generated and yielded the  $\Delta$ *wbla*,  $\Delta$ *SCO1712*,  $\Delta$ *orrA* and  $\Delta$ *gntR* mutants after successful conjugation into the M1346::3gul strain, respectively. The positions of the spacers, primers and target genes are shown in Figure S3.

#### 4.4. Metabolite analysis

The wild-type *Streptomyces* sp. HNS054 and the *S. coelicolor* strains were grown on MS solid medium to achieve sporulation. Approximately  $10^8$  spores (or 40  $\mu$ L mycelia store) were transferred to 40 mL R5 medium with appropriate antibiotics and cultured at 200 rpm and 28 °C for 3 d as the seed solution. Ten milliliters of the seed was transferred to 500 mL of R5 medium and cultured at 200 rpm and 28 °C for 9 d to complete the fermentation. Aborycin was extracted as shown in Figure 3A. The fermented broth was centrifuged to separate the supernatant from the mycelia and then independently extracted. The supernatant was extracted by two methods. The first method was butanone extraction three times, and then the butanone phase was concentrated by rotary evaporation. Then, four macroporous adsorption resins, AB-8, DC201-C, NK-9 and D101 (Hecheng New Material, Zhengzhou, China), were tested for their aborycin recovery efficiencies. It was found that the AB-8 resin had a high enrichment ratio for aborycin (Figure 3B), and AB-8 was therefore used as the second method to extract aborycin. Because the AB-8 resin method was safer, easier and cheaper, all aborycin titers of the supernatants in this study were obtained by this method. The mycelium was extracted three times with 300 mL methanol and then concentrated by rotary evaporation. Extracts from both the supernatant and mycelium were adjusted to a final concentration of approximately 5 mg/mL in methanol and filtered with 0.22  $\mu$ m filter membranes before HPLC or LC-MS analysis. Analytical HPLC was performed with a Shimadzu Prominence LC-20A (Shimadzu, Tokyo, Japan) using a 2.6×250 mm Ultimate XB-C18 column (Welch, Shanghai, China). The elution conditions were 1% solvent B for 0-7 min and then a linear gradient to 95% solvent B (solvent B: 0.1% formic acid + 100% CH<sub>3</sub>CN; solvent A: 0.1% formic acid in H<sub>2</sub>O) for 7-30 min at a flow rate of 1 mL/min and monitored at 277 nm. To locate the aborycin signal, the commercial siamycin-I reagent (Adipogen Life Sciences, San Diego, USA) was used as the control. Because the amino acid sequences between aborycin and siamycin-I were the same except that the residues at the 4th and 17th positions were switched (Figure 1), it is reasonable to expect the HPLC signal position of aborycin to be slightly different from that of siamycin-I. LC-MS analysis was performed with a Q Exactive spectrometer (Thermo Scientific, USA) by the same elution program as HPLC. The software MZmine 2 [39] was used to assess the chromatograms.

#### Purification of aborycin and preparation of the calibration curve

A 5.6 L culture broth of the M1346::3gul strain was prepared as described in the previous section. The crude extract was enriched by the AB-8 resin method from the supernatant and then purified by

semipreparative HPLC with a YMC-PACK ODS-A column (10×250 mm,  $\varphi$  5  $\mu$ m, YMC, Kyoto, Japan). F1-F4 fractions near the target position were collected, and the F2 fraction was determined by analytical HPLC to be the major fraction containing aborycin. Then, approximately 22 mg of the F2 fraction was obtained after rotary evaporation. Ten milligrams of the F2 fraction was dissolved in 2 mL of methanol, and analytical HPLC was run as described in the previous section. The 23.2 min peaks were collected and concentrated to approximately 4 mg white powder of pure aborycin. A methanol solution of aborycin was prepared with a precise concentration of 1.00 mg/mL and then diluted to a series of concentrations. These dilutions were subjected to analytical HPLC, and the 23.2 min peak areas were recorded. A standard curve of concentrations to HPLC peak areas of aborycin was then constructed (Figure 3D), and the following formula was deduced.

$$y = 4.970x + 0.046 \quad (R^2 = 0.999) \quad (1)$$

where  $y$  ( $10^6 \cdot \text{mAU} \cdot \text{min}$ ) is the peak area of the HPLC signal of aborycin, and  $x$  (mg/mL) is the concentration.

## 5. Conclusions

This study demonstrated improvements in the *S. coelicolor* system for aborycin overproduction. The maximum shake flask titer was over 50 mg/L, comparable to the best examples of *E. coli* systems or *Streptomyces* systems for lasso peptide heterologous expression [7]. We confirmed that increasing the copy number of chromosome integration and disrupting the negative global regulators governing secondary metabolism were effective ways to improve antibiotic production in *S. coelicolor* systems. *GntR* (SCO1678) was proposed to be a significant target that is worthy of further studies on metabolic regulation mechanisms. The optimized extraction method, which could efficiently recover aborycin from the supernatant, also contributed to the total titer. Due to the similar chemical properties of RiPPs to aborycin, this tactic is also suitable for the development of other RiPP relatives. By applying these modifications to *Streptomyces* systems, the drug development and functional characterization of new lasso peptides from genome mining will be greatly improved.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Construction of the integrative vector for aborycin expression; Figure S2: Clone of the *gul* gene cluster; Figure S3: CRISPR/Cas9 constructions of the five gene-knockout mutants from M1346::3gul; Figure S4: PCR screening for positive mutants; Table S1: Information of plasmids and strains; Table S2: Primer sequence used in this study; Table S3: The length of RiPP biosynthetic gene clusters; Table S4: RiPP examples reported by Montalbán-López et al.

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