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Neomycin biosynthesis is regulated positively by AfsA-g and NeoR in *Streptomyces fradiae* CGMCC 4.7387

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Neomycins are a group of aminoglycoside antibiotics with both clinical and agricultural applications. To elucidate the regulatory mechanism of neomycin biosynthesis, we completed draft genome sequencing of a neomycin producer *Streptomyces fradiae* CGMCC 4.7387 from marine sediments, and the neomycin biosynthesis gene cluster was identified. Inactivation of the *afsA-g* gene encoding a γ-butyrolactone (GBL) synthase in *S. fradiae* CGMCC 4.7387 resulted in a significant decrease of neomycin production. Quantitative RT-PCR analysis revealed that the transcriptional level of *neoR* and the *aphA-neoGH* operon were reduced in the *afsA-g::aac(3)IV* mutant. Interestingly, a conserved binding site of AdpA, a key activator in the GBL regulatory cascade, was discovered upstream of *neoR*, a putative regulatory gene encoding a protein with an ATPase domain and a tetratricopeptide repeat domain. When *neoR* was inactivated, the neomycin production was reduced about 40% in comparison with the WT strain. Quantitative RT-PCR analysis revealed that the transcriptional levels of genes in the *aphA-neoGH* operon were reduced clearly in the *neoR::aac(3)IV* mutant. Finally, the titers of neomycin were improved considerably by overexpression of *afsA-g* and *neoR* in *S. fradiae* CGMCC 4.7387.

neomycin, regulation, γ-butyrolactone, *Streptomyces*

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INTRODUCTION

Gram-positive filamentous bacteria *Streptomyces* are producers of numerous secondary metabolites with fascinating bioactivities, including many important drugs and agricultural antibiotics (Bérdy, 2005). Biosynthesis of these compounds is typically under stringent regulation at different levels and usually activated in a growth-phase-dependent manner (Chater, 2006; Liu et al., 2013). The signals from the environment or elaborated by *Streptomyces* are usually transferred from the global regulators to the pathway-specific regulators, which control the transcription of genes involved in secondary metabolite biosynthesis and self-protection (Liu et al., 2013; Bibb, 2005; Niu et al., 2016). A fundamental comprehension of the regulatory systems will undoubtedly deepen our understanding of the biosynthesis of secondary metabolites and facilitate their titer improvements by rational metabolic engineering (Chen et al., 2008; Chao et al., 2015).

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The aminoglycoside family is a group of very important antibiotics with both clinical and agricultural applications. Streptomycin, isolated by Waksman and colleagues in 1943 (Schatz et al., 1944) was the first aminoglycoside to be identified and the first drug for tuberculosis treatment. The regulation of streptomycin biosynthesis in *Streptomyces griseus* IFO 13350 has been well studied and is the prototype of how γ-butyrolactone (GBL) signals control both secondary metabolism and morphological differentiation (Niu et al., 2016; Takano, 2006). The GBL signal involved in streptomycin regulation is named A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), which is biosynthesized by AfsA, a key enzyme catalyzing the condensation of a fatty acyl-acyl carrier protein with dihydroxylactone phosphate to build the core structure of A-factor (Ando et al., 1997; Kato et al., 2007), and some tailoring enzymes. The signal transduction cascade from A-factor to streptomycin biosynthesis is through three intermediate regulators ArpA, AdpA and StrR. In *S. griseus* IFO 13350, A-factor reaches a threshold near the middle of exponential growth phase and binds to its receptor ArpA to de-repress the transcription of a key regulatory gene *adpA* (Ohnishi et al., 2005); AdpA then activates a number of genes modulating morphological differentiation and secondary metabolite biosynthesis, including *strR*, a streptomycin pathway activator encoding gene, which starts the biosynthesis of streptomycin (Retzlaff and Distler, 1995; Vujaklija et al., 1993). Besides the A-factor system, several other GBL regulatory systems have been previously identified in diverse *Streptomyces* species. In those cases, the GBL signals are mostly transferred through orthologs of ArpA and AdpA in a similar manner as the A-factor system, revealing the widespread conservation of the GBL regulatory cascade.

The second aminoglycoside antibiotic, neomycin, was also discovered by the Waksman laboratory (Waksman et al., 1949). Neomycin is mainly used as a topical antibiotic to treat bacterial infections in humans, but has also been used as a veterinary antibiotic in some countries. As an important and early discovered antibiotic, neomycin has been intensively studied and its biosynthetic pathway has already been delineated (Kudo et al, 2009). However, little is known about its regulation mechanism.

In this study, we sequenced and analyzed the genome of a neomycin producer (*Streptomyces fradiae* CGMCC 4.7387) and confirmed the neomycin gene cluster by inactivation of the *neoE* gene encoding a 2-deoxyinosamine dehydrogenase. Based on additional gene inactivation and RT-PCR analysis, it was proposed that neomycin biosynthesis is modulated positively by the GBL synthase gene *afsA-g*, and a putative regulatory protein NeoR is involved in the GBL cascade from AfsA-g to the neomycin cluster. Subsequently, genes *afsA-g* and *neoR* were overexpressed in *S. fradiae* CGMCC 4.7387 respectively, resulting in two recombinant strains with improved neomycin titers.

RESULTS

Identification of neomycin and its biosynthetic gene cluster in *S. fradiae* CGMCC 4.7387

When *S. fradiae* CGMCC 4.7387 was fermented in soybean meal medium, its supernatant displayed strong inhibitory activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The main bioactive component was determined to be neomycin B by the fact that it had the same HPLC retention time and the same molecular formula $C_{23}H_{46}N_6O_{13}$ (HR-ESI-MS, $[M+H]^+$ ion at *m/z* 615.3213; calculated 615.3196) as the neomycin B standard (Figure 1A and B).

After draft genome sequencing of *S. fradiae* CGMCC 4.7387, a 2-deoxyinosose synthase encoding gene was used as a probe to identify a 31-kb gene cluster, which has the same gene organization as the two known neomycin gene clusters from *S. fradiae* DSM 40063 (GenBank accession No. AJ629247) and *S. fradiae* NBRC 12773 (GenBank accession No. AB211959) (Figure 1C) (Kudo et al., 2009). The proteins encoded by genes in the three *neo* clusters displayed very high homologies (>86% identities) (Table 1). To confirm the identity of the neomycin gene cluster in *S. fradiae* CGMCC 4.7387, the *neoE* gene encoding a 2-deoxyinosamine dehydrogenase was inactivated to generate the *neoE::aac(3)IV* mutant CIM4001 (Figure 2A). As expected, CIM4001 lost the ability to produce neomycin and it could be successfully complemented by constitutive expression of *neoE* in *trans* (Figure 1A).

Neomycin production is modulated positively by *afsA-g*

Production of many antibiotics is modulated by GBLs in *Streptomyces*, including the aminoglycoside antibiotic streptomycin. Careful analysis of the *S. fradiae* CGMCC 4.7387 genome revealed one putative GBL synthase gene *afsA-g*, encoding a protein with 28.9% identity to AfsA from *S. griseus* IFO 13350 (Ohnishi et al., 2008). Two putative GBL receptor encoding genes *arpA-g1* and *arpA-g2* were linked to *afsA-g*. ArpA-g1 and ArpA-g2 display 27% and 23% identities to the characterized A-factor receptor ArpA, respectively (Onaka and Horinouchi, 1997). To study the influence of the GBL regulatory cascade on neomycin production, the *afsA-g* gene was inactivated by gene replacement to generate the *afsA-g::aac(3)IV* mutant CIM4002 (Figure 2C). The titer of neomycin B in CIM4002 was decreased to 179.7±7.7 mg L^{-1}, about 40% of the titer of the wild-type strain (420.6±16.7 mg L^{-1}) (Figure 3A), indicating that the GBLs modulate neomycin production positively. Complementation of the *afsA-g* mutation was achieved by introducing a plasmid, pCIM4005 carrying *afsA-g* under the

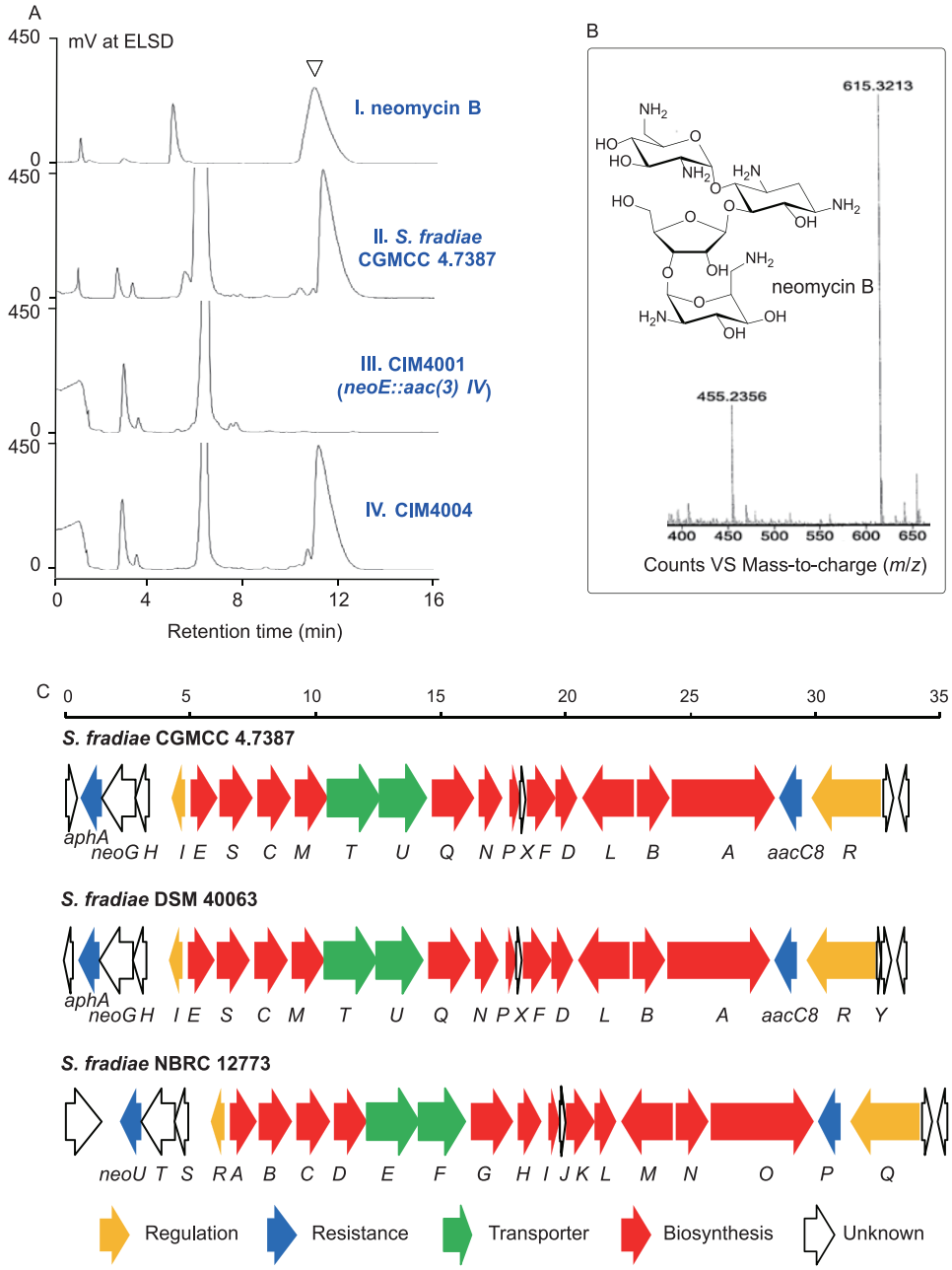

Figure 1 Identification of the neomycin biosynthetic gene cluster in *S. fradiae* CGMCC 4.7387. A, HPLC-ELSD analysis of neomycin production. I, neomycin B standard; II, the wild-type strain *S. fradiae* CGMCC 4.7387; III, the *neoE* inactivated mutant CIM4001; IV, the CIM4001 complemented strain CIM4004. B, High resolution ESI-MS of the neomycin component isolated from *S. fradiae* CGMCC 4.7387. C, Organization of the neomycin biosynthetic gene cluster from *S. fradiae* CGMCC 4.7387.

control of the *ermE** promoter into CIM4002, to generate CIM4005, in which the production of neomycin B was partially restored to 334.7±19.4 mg L^{-1} (Figure 3A).

The effect of *afsA-g* inactivation on *S. fradiae* CGMCC 4.7387 morphological differentiation was examined on five different media (Mannitol soya flour medium (MS), Minimal medium (MM), R2YE medium, Gause's synthetic agar and Nutrient agar) and no distinct change was observed between *S. fradiae* CGMCC 4.7387 wild-type and the *afsA-g::aac(3)IV* mutant CIM4002 (data not shown).

Transcriptional analysis of *neoR* and the *aphA-neoGH* operon in the *afsA-g::aac(3)IV* mutant CIM4002

We suppose that the GBL signaling pathway modulates the production of neomycin possibly through transcriptional regulation of target genes in the *neo* cluster. In order to test this, total RNAs from CIM4002 and the wild-type strain were extracted, and quantitative RT-PCR analysis was carried out to examine the transcriptional levels of 14 genes (*aphA*, *neoG*, *neoH*, *neoI*, *neoE*, *neoC*, *neoT*, *neoQ*, *neoP*, *neoL*, *neoB*, *neoA*, *aacC8* and *neoR*), which covered all the possible

Table 1 Homologous proteins of ORFs in the neomycin biosynthetic gene clusters[a)]

Genes	Sizes[a]	Proposed functions	Protein homologs in *S. fradiae* DSM 40063[b]	Protein homologs in *S. fradiae* NBRC 12773[b]
aphA	272	Aminoglycoside 3′-Phosphotransferase	AphA (98.5/98.5)	NeoU (100/100)
neoG	431	Regulator	NeoG (99.5/99.8)	NeoT (99.5/99.8))
neoH	173	Regulator	NeoH (100/100)	NeoS (100/100)
neoI	175	Regulator	NeoI (100/100)	NeoR (100/100)
neoE	340	2DOIA dehydrogenase	NeoE (100/100)	NeoA (100/100)
neoS	424	L-Gln:2DOI aminotransferase	NeoS (100/100)	NeoB (100/100)
neoC	430	2DOI synthase	NeoC (99.8/99.8)	NeoC (99.8/99.8)
neoM	421	UDP-GlcNAc:2DOS GlcNAc transferaseNeoM (419) CDS16 (421,99.5%) NeoD(421,99.5%)	NeoM (99.5/99.5)	NeoD (100/100)
neoT	666	ABC transporter	NeoT (99.5/99.5)	NeoE (96.5/96.5)
neoU	594	ABC transporter	NeoU (99.8/99.8)	NeoF (99.8/99.8)
neoQ	541	Paromamine 6′-dehydrogenase	NeoQ (100/100)	NeoG (100/100)
neoN	299	Neomycin 5‴-epimerase	NeoN (100/100)	NeoH (100/100)
neoP	233	5″-phosphoribostamycin phosphataseNeoP (233) CDS21(141,59.7%)[1] NeoI(233,100%)	NeoP (98.7/99.1)	NeoI (98.7/99.1)
neoX	83	Hypothetical protein	NeoX (98.8/98.8)	NeoJ (98.8/98.8)
neoF	366	UDP-GlcNAc:ribostamycin GlcNAc transferase	NeoF (99.5/99.5)	NeoK (99.5/99.5)
neoD	279	2′-N-Acetylparomamine deacetylase	NeoD (98.9/99.3)	NeoL (98.9/99.3)
neoL	660	PRPP:neamine 5-phosphori-bosyltransferase	NeoL (98.5/98.9)	NeoM (98.8/99.2)
neoB	358	L-Gln:6′-Oxoparomamine aminotransferase	NeoB (86.1/86.1)	NeoN (86.1/86.1)
neoA	1,253	Unknown	NeoA (95.4/95.5)	NeoO (93.3/93.4)
aacC8	287	Aminoglycoside N-acetyltransferase	AacC8 (100/100)	NeoP (100/100)
neoR	886	Regulator	NeoR (99.5/99.5)	NeoQ (99.5/99.5)

a) a, numbers are in amino acids; b, given in brackets are percentage identity/ percentage positive.

operons in the *neo* cluster. Total RNAs from *S. fradiae* CGMCC 4.7387 wild-type and CIM4002 were isolated from mycelia grown for 60 h in soybean meal medium, at the point where neomycin production was beginning to increase exponentially. As shown in Figure 3B, the transcriptional levels of *neoR* and the three genes in the *aphA-neoGH* operonwere clearly reduced in CIM4002; while the transcriptional levels of the rest 10 genes were quite similar between wild type and CIM4002 (data not shown).

NeoR influences neomycin production positively

Among the four genes regulated by AfsA-g, *neoR* encodes a putative regulatory protein showing moderate similarity with some regulators from the LAL family (large ATP-binding regulators of the LuxR family), such as NysR1 (25.3% identity), an activator from the nystatin gene cluster of *Streptomyces noursei* ATCC 11455 (Sekurova et al., 2004). Notably, a distinct difference between NeoR and the LAL family regulators is that the former one consists of only an N-terminal ATPase-domain and a C-terminal tetratricopeptide repeat (TPR) domain, while the latter ones have an extra DNA binding domain in their C terminal regions. Interestingly, a DNA sequence (5'-GGGCCGCTTC-3'), which is very similar to the consensus binding sequence of AdpA-like proteins (Yamazaki et al., 2004), was found 139-bp upstream of the translation start codon of *neoR* (Figure 4A), and this finding suggests that *neoR* is regulated by GBL signals, which is consistent with the RT-PCR results of the *afsA-g::aac(3)IV* mutant CIM4002.

To determine the role of *neoR* in the production of neomycin, a *neoR* inactivated mutant CIM4003 was constructed by replacing it with the apramycin resistance cassette

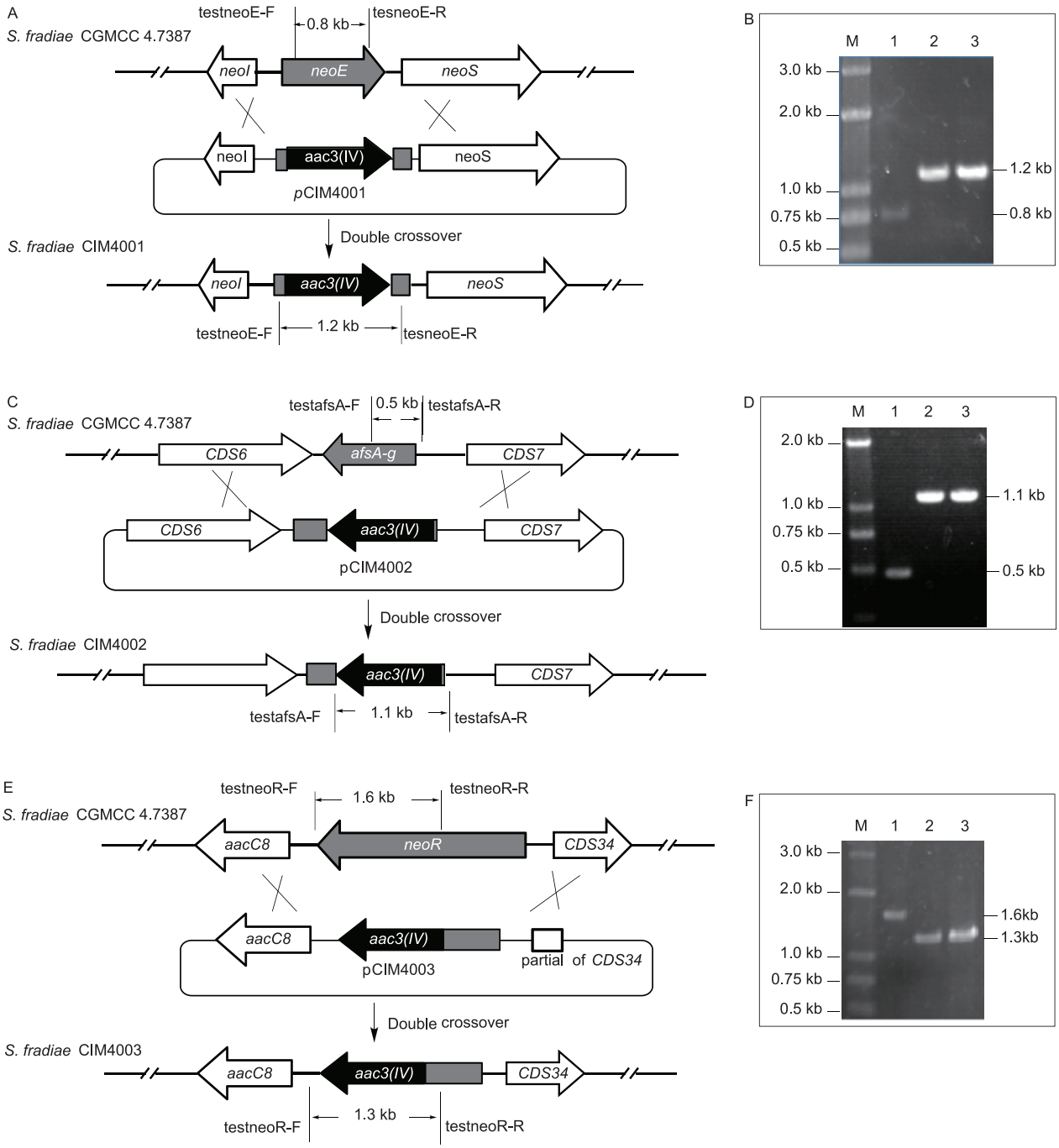

Figure 2 Construction of the *neoE::aac3(IV)* mutant CIM4001, the *afsA-g::aac3(IV)* mutant CIM4002 and the *neoR::aac3(IV)* mutant CIM4003. A, Diagram illustrating the construction of CIM4001 by replacing *neoE* with the apramycin-resistance gene cassette (*aac(3)IV*). B, PCR confirmation of the *neoE* inactivation. Lane M, DNA ladder; Lane 1, fragment obtained by PCR using *S. fradiae* CGMCC 4.7387 as a template; Lane 2, fragment obtained by PCR using pCIM4001 as a template was used as a positive control; Lane 3, fragment obtained by PCR using *S. fradiae* CIM4001 as a template. C, Diagram illustrating the construction of CIM4002 by replacing *afsA-g* with *aac(3)IV*. D, PCR confirmation of the *afsA-g* inactivation. Lane M, DNA ladder; Lane 1, fragment obtained by PCR using *S. fradiae* CGMCC 4.7387 as a template; Lane 2, fragment obtained by PCR using pCIM4002 as a template was used as a positive control; Lane 3, fragment obtained by PCR using *S. fradiae* CIM4002 as a template. E, Diagram illustrating the construction of CIM4003 by replacing *neoR* with *aac(3)IV*. F, PCR confirmation of the *neoR* inactivation. Lane M, DNA ladder; Lane 1, fragment obtained by PCR using *S. fradiae* CGMCC 4.7387 as a template; Lane 2, fragment obtained by PCR using pCIM4003 as a template was used as a positive control; Lane 3, fragment obtained by PCR using *S. fradiae* CIM4003 as a template.

aac(3)IV (Figure 2E). HPLC analysis showed that the production of neomycin B in CIM4003 was reduced to about 60% (277.4±16.4 mg L^{-1}) relative to the wild-type strain. Complementation of CIM4003 was carried out by putting *neoR* under the control of a constitutive promoter *ermE** on plasmid pUWL201-oriT to generate pCIM4006 and then it was

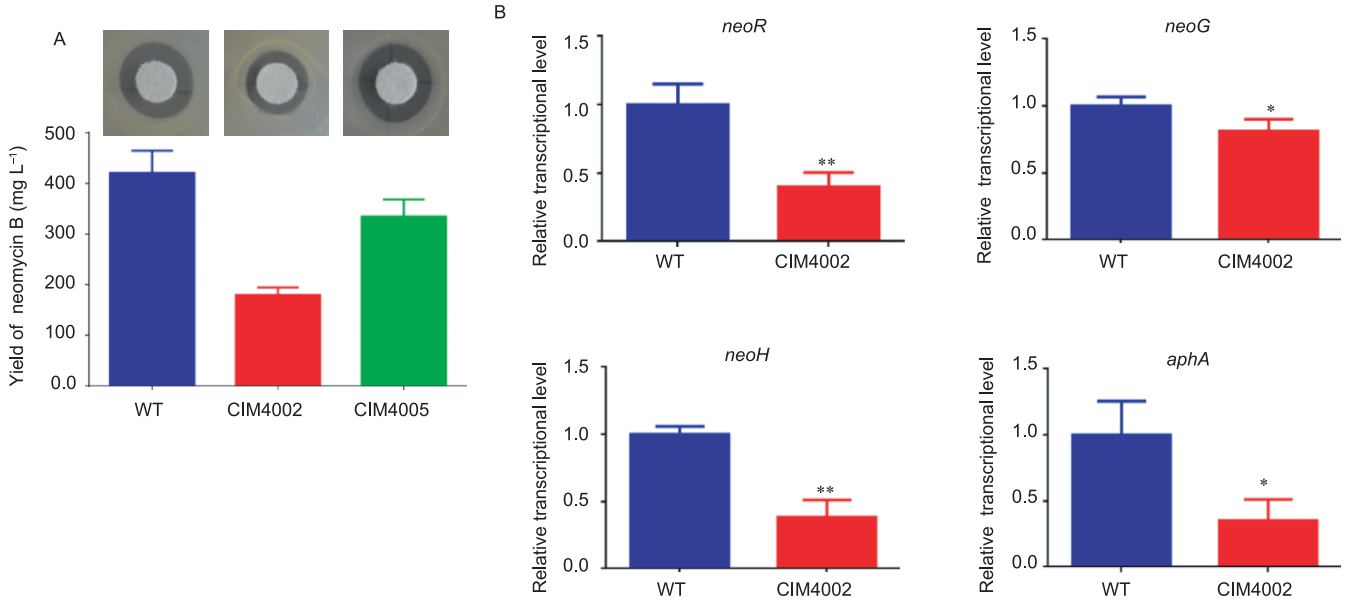

Figure 3 Phenotype and qRT-PCR analysis of the *afsA-g* inactivated mutant CIM4002. A, Production of neomycin in *S. fradiae* CGMCC 4.7387 (WT), the *afsA-g* inactivated mutant CIM4002 and its complemented strain CIM4005. B, Quantitative RT-PCR analysis of the transcriptional levels of *neoR*, *neoG*, *neoH* and *aphA* in the *afsA-g* inactivated mutant CIM4002. The results are presented relative to the transcript values of the wild-type *S. fradiae* CGMCC 4.7387. Mean values of at least three independent experiments with SD indicated by error bars. *, $P<0.05$; **, $P<0.01$.

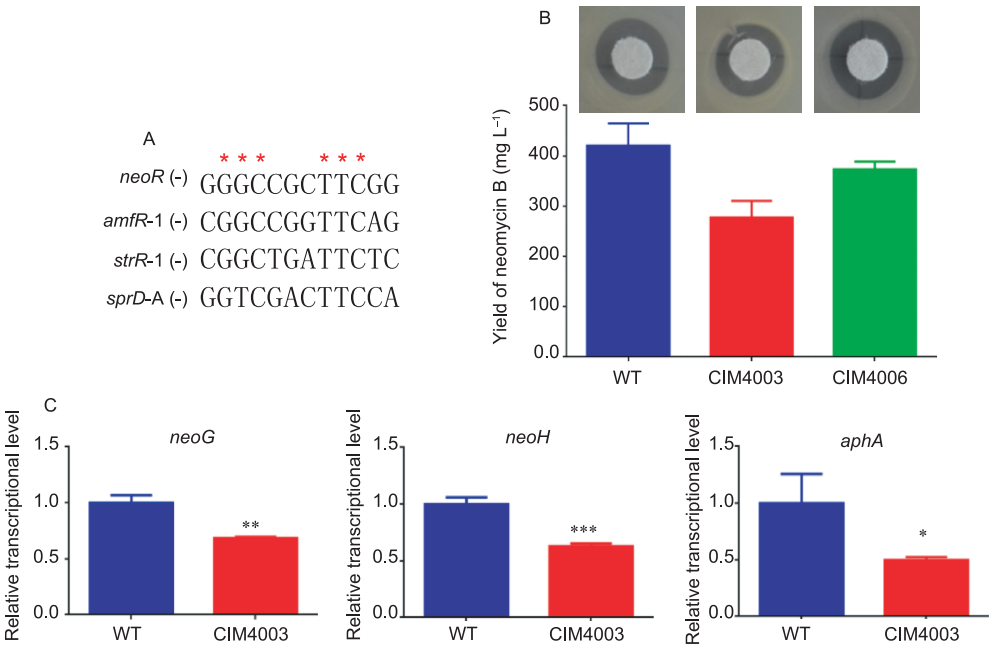

Figure 4 Phenotype and qRT-PCR analysis of the *neoR* inactivated mutant CIM4003. A, Alignment of the characterized AdpA binding sequences from *S. griseus* IFO 13350 and the putative AdpA-like protein binding sequence upstream of the *neoR* gene. The conserved nucleotides are marked with asterisks. The consensus AdpA binding sequence is TGGCSNGWWY (S: G or C; W: A or T; Y: T or C; N: any nucleotide) (Yamazaki et al., 2004). B, Production of neomycin in *S. fradiae* CGMCC 4.7387 (WT), the *neoR* inactivated mutant CIM4003 and its complemented strain CIM4006. The antibacterial assay results are showed at the top of each strain. C, Quantitative RT-PCR analysis of the transcriptional levels of *neoG*, *neoH* and *aphA* in the *neoR* inactivated mutant CIM4003. The results are presented relative to the transcript values of the wild-type *S. fradiae* CGMCC 4.7387. Mean values of at least three independent experiments with SD are indicated by error bars. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

introduced into CIM4003 to generate CIM4006. The production of neomycin B in CIM4006 was partially restored to 373±7.8 mg L^{-1}, indicating that NeoR has a positive influence on neomycin production (Figure 4B).

Transcription of the *aphA-neoGH* operon is reduced in the *neoR::aac(3)IV* mutant CIM4003

Quantitative RT-PCR analysis was carried out to examine the 14 aforementioned genes in the *neoR::aac(3)IV* mutant

CIM4003. The result of RT-PCR showed that the transcriptional levels of three adjacent genes (*aphA*, *neoG* and *neoH*) were significantly reduced in CIM4002 when compared with those in the wild-type (Figure 4C), while no clear difference was observed for the transcription of the other 10 genes tested (data not shown).

Improvement of neomycin production in *S. fradiae* CGMCC 4.7387

Both *afsA-g* and *neoR* influence the production of neomycin positively, which encouraged us to overexpress the two genes individually in *S. fradiae* CGMCC 4.7387 to increase the neomycin titers. The aforementioned plasmid pCIM4005, in which *afsA-g* is under the control of the constitutive promoter *ermE**, was introduced into the wild-type strain to generate an *afsA-g* overexpression strain CIM4007. The titer of neomycin B in CIM4007 was increased to 722.9±20.1 mg L^{-1}, which is about 1.7 times of that of wild-type strain (Figure 5). The *neoR* gene was overexpressed in *S. fradiae* CGMCC 4.7387 in a similar way as that of *afsA-g* to generate *S. fradiae* CIM4008, in which the neomycin B titer was increased to 564.7±32.5 mg L^{-1}. The control strain CIM4009 with the empty plasmid pUWL201-oriT had a similar neomycin B titer (466.8±21.7 mg L^{-1}) as the wild-type strain, excluding the possible influence on neomycin production from the plasmid.

DISCUSSION

Although the regulation of streptomycin by A-factor is a prototype of GBL regulatory cascades, the regulatory mechanisms of many other aminoglycoside antibiotics are still mysterious, including neomycin, one of the earliest discovered and utilized antibiotics. On the basis of results shown herein, we propose the biosynthesis of neomycin in *S. fradiae* CGMCC 4.7387 is modulated by the GBL signals and NeoR through the transcriptional regulation of the three genes *aphA*, *neoG* and *neoH* in the *neo* cluster. An obvious question arising from this study is that how neomycin production is affected by *aphA*, *neoG* and *neoH*. Among the three genes, the proteins encoded by *neoG* and *neoH* are both hypothetical proteins with unknown functions, whose influence on neomycin production is unclear at this stage, whereas, the protein encoded by *aphA* displays 82.4% identity to Rph from the ribostamycin cluster, an aminoglycoside phosphotransferase conferring resistance to its producer *Streptomyces ribosidificus* ATCC 21294. In many cases, the titers of antibiotics are related to the resistance tolerance of their producers, e.g., the titers of neomycin in *S. fradiae* ATCC 10745 and kanamycin in *Streptomyces kanamyceticus* ATCC 1285 were substantially improved by amplification of a resistance gene encoding 6'-N-acetyltransferase from *S. kanamyceticus* M1164 (Crameri and Davies, 1986). One plausible explanation of neomycin titer changes is that the resistance of *S. fradiae* CGMCC 4.7387 toward neomycin is related to the expression level of *aphA*, which influences neomycin production.

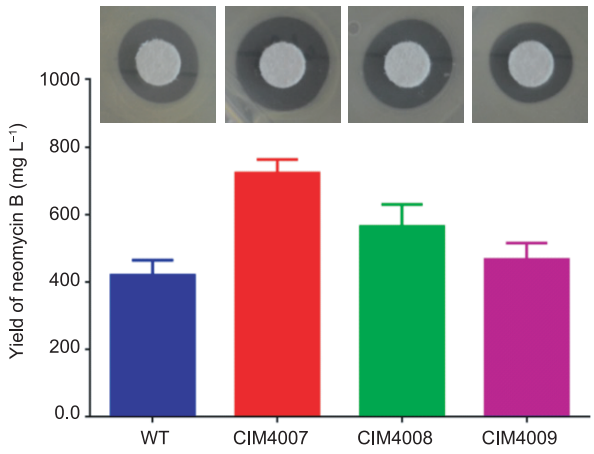

Figure 5 Improvement of neomycin production by overexpression of *neoR* and *afsA-g*. Neomycin B yields of the wild-type strain *S. fradiae* CGMCC 4.7387 (WT), the *afsA-g* overexpressed strain CIM4007, the *neoR* overexpressed strain CIM4008 and the control strain CIM4009 harboring the empty plasmid pUWL201-oriT are presented. The antibacterial assay results are showed at the top of each strain.

NeoR was proposed to be a positive regulatory protein based on the facts that (i) inactivation of *neoR* reduced neomycin titer; (ii) overexpression of *neoR* increased neomycin titer; (iii) the transcriptional levels of *aphA*, *neoG* and *neoH* were reduced in the *neoR::aac(3)IV* mutant CIM4002. However, the lack of a DNA binding domain in NeoR implies that it may not work as a typical activator, which binds to the operator region of promoter and increase the binding efficiency of RNA polymerase. We overexpressed His_6-tagged NeoR in *E. coli*, purified the protein and checked its binding affinity to the upstream region of the *aphA-neoGH* operon by electrophoretic mobility shift assays, which showed that NeoR cannot bind to the promoter region of the *aphA-neoGH* operon (data not shown). NeoR has an N-terminal ATPase-domain and a C-terminal TPR domain, which are present in many *Streptomyces* regulators, e.g., the aforementioned LAL family regulators (De Schrijver and De Mot, 1999) and some SARP regulators like AfsR (Tanaka et al., 2007) and PolY (Li et al., 2010). The ATP hydrolysis function of the ATPase domain was proved in PolY *in vitro*, and it was proposed that the ATPase-domain of PolY might be a sensor to detect the endogenous ADP/ATP pool of *Streptomyces cacaoi* (Li et al., 2010). The TPR domain is known to exist in a wide variety of proteins from both prokaryotic and eukaryotic organisms, which acts as a structural motif to mediate protein-protein interactions in multiprotein complexes (Sekurova et al., 2004). The domain

structure of NeoR implies that NeoR might be able to sense the cellular ADP/ATP pool and exert its regulatory function by interacting with other regulators.

Overexpression of defined positive regulatory genes was used successfully to enhance secondary metabolite production in many cases (Chen et al., 2008; Lombó et al., 1999). After demonstration of *afsA-g* and *neoR* regulating neomycin production in positive ways, the two genes were overexpressed under the control of the constitutive *ermE** promoter in a high-copy-number plasmid in *S. fradiae* CGMCC 4.7387, respectively. The titers of neomycin were improved to 722.9±20.1 mg L^{-1} in CIM4007 (*afsA-g* overexpressed) and 564.7±32.5 mg L^{-1} in CIM4008 (*neoR* overexpressed), consistent with the activator characters of AfsA-g and NeoR.

Conclusively, it was proposed that the production of neomycin is controlled positively by the GBL regulatory cascade and NeoR in *S. fradiae* CGMCC 4.7387. The titer of neomycin B was increased to about 1.7 times relative to the wild-type strain by overexpression of the *afsA-g* gene. Meanwhile, there are still many questions about the regulation of neomycin biosynthesis, such as the signal transduction pathway from AfsA-g to NeoR, the regulatory strategy of NeoR on the *aphA-neoGH* operon and the mechanism whereby the *aphA-neoGH* operon influences neomycin production, which need to be addressed in further investigation.

MATERIALS AND METHODS

Strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* JM109 (Sambrook et al., 1989) and *E. coli* ET12567/pUZ8002 (Paget et al., 1999) were cultured in Luria-Bertani (LB) broth. The neomycin producer *S. fradiae* CGMCC 4.7387 (originally named *S. fradiae* GB-2) was grown on solid MS agar for sporulation (Lu et al., 2009). For neomycin production, *S. fradiae* CGMCC 4.7387 was cultured in soybean meal medium containing soluble starch (20 g L^{-1}), soybean powder (15 g L^{-1}), glucose (5 g L^{-1}), yeast extract (2.5 g L^{-1}), $CaCO_3$ (1 g L^{-1}) and sea salt (35 g L^{-1}). When needed, antibiotics were added at a final concentration of 40 μg mL^{-1} for apramycin, 50 μg mL^{-1} for kanamycin, 4 μg mL^{-1} for thiostrepton and 25 μg mL^{-1} for nalidixic acid.

Table 2 Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference and source
Escherichia coli		
JM109	General cloning host	(Sambrook et al., 1989)
BL21(DE3)	Host strain for protein expression	Novagen
ET12567/pUZ8002	Strains for intergeneric conjugation	(Paget et al., 1999)
Streptomyces		
S. fradiae CGMCC 4.7387	Wild-type neomycin producer	(Lu et al., 2009)
S. fradiae CIM4001	*S. fradiae* CGMCC 4.7387 *neoE*:: *aac3(IV)*	This work
S. fradiae CIM4002	*S. fradiae* CGMCC 4.7387 *afsA-g*:: *aac3(IV)*	This work
S. fradiae CIM4003	*S. fradiae* CGMCC 4.7387 *neoR*:: *aac3(IV)*	This work
S. fradiae CIM4004	*S. fradiae* CIM4001 completed with cloned *neoE*	This work
S. fradiae CIM4005	*S. fradiae* CIM4002 completed with cloned *afsA-g*	This work
S. fradiae CIM4006	*S. fradiae* CIM4003 completed with cloned *neoR*	This work
S. fradiae CIM4007	Overexpression of *afsA-g* in S. *fradiae* CGMCC 4.7387	This work
S. fradiae CIM4008	Overexpression of *neoR* in S. *fradiae* CGMCC 4.7387	This work
S. fradiae CIM4009	*S. fradiae* CGMCC 4.7387 harboring pUWL201PW-oriT	This work
Plasmids		
pUWL201PW-oriT	Tsr^r/Amp^r, replicating vector in *Streptomyces*	(Li et al., 2013b)
pCIMt002	Apr^r/Amp^r, *oriT*, *ermE**, *sfp*, *idgS*, non-replicative in *Streptomyces*	(Li et al., 2015)
pCIM4001	pCIMt002 with *neoE*:: *aac3(IV)*	This work
pCIM4002	pCIMt002 with *afsA-g*:: *aac3(IV)*	This work
pCIM4003	pCIMt002 with *neoR*:: *aac3(IV)*	This work
pCIM4004	pUWL201PW-oriT with *neoE* under control of *ermE**	This work
pCIM4005	pUWL201PW-oriT with *afsA-g* under control of *ermE**	This work
pCIM4006	pUWL201PW-oriT with *neoR* under control of *ermE**	This work

DNA manipulation

General DNA manipulations were performed with standard procedures (Kudo et al., 2009). The ligation-independent cloning strategy was recruited in plasmid construction (Li, 1997). PCRs were performed with PrimeSTAR HS DNA polymerase (Takara, Shiga, Japan) according to the manufacturer's instructions. All primers used in this study are listed in Table 3. Intergeneric conjugation between *E. coli* ET12567/pUZ8002 and *S. fradiae* CGMCC 4.7387 was performed as standard protocol (Kieser et al., 2000).

Sequencing and bioinformatics analysis

The draft genome sequencing of *S. fradiae* CGMCC 4.7387 was carried out with Illumina in Majorbio Corporation (Shanghai, China). Sequencing of PCR products was performed by Invitrogen Co. Ltd. (Shanghai, China). The open reading frame prediction was performed with Prodigal (http://compbio.ornl.gov/prodigal/). The gene functions were annotated based on the search results of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the comparison with published neomycin biosynthetic gene clusters was carried out (Table 1).

Construction of the gene inactivation mutant strains of *neoE*, *afsA-g* and *neoR*

An allelic replacement strategy was used to inactivate *neoE* in *S. fradiae* CGMCC 4.7387 (Figure 2A). Briefly, the two 1.4-kb fragments flanking *neoE* were amplified using primers neoEL-F/neoEL-R and neoER-F/neoER-R and cloned into the *Bln* I and *Mun* I sites of pCIMt002 by ligation independent cloning strategy to generate pCIM4001, which was then introduced into *S. fradiae* CGMCC 4.7387 by *E. coli*-*Streptomyces* conjugation. The white exconjugant clones with apramycin resistance were selected and verified by PCR with primer pair testneoE-F/testneoE-R (Figure 2B). The double-crossover *neoE::aac(3)IV* mutant strain was designated as CIM4001.

Gene *afsA-g* and *neoR* were inactivated individually by a similar allelic replacement strategy. The 2.0-kb upstream fragment and the 2.1-kb downstream fragment of *afsA-g* and the two 1.7-kb fragments flanking *neoR* were PCR cloned with primers afsAL-F/afsAL-R and afsAR-F/afsAR-R, neoRL-F/neoRL-R, neoRR-F/neoRR-R respectively. The fragments were then cloned into the *Bln* I and *Mun* I sites of pCIMt002 to generate pCIM4002 and pCIM4003. After introducing pCIM4002 and pCIM4003 into *S. fradiae* CGMCC 4.7387 via *E. coli*-*Streptomyces* conjugation, exconjugants that conferred white and apramycin resistance were selected as the desired *afsA-g::aac(3)IV* mutant strain CIM4002 and the *neoR::aac(3)IV* mutant strain CIM4003 (Figure 2C and E). The genotypes of CIM4002 and CIM4003 were confirmed by PCR with primers testafsA-F/testafsA-R and testneoR-F/testneoR-R (Figure 2D and F).

Complementation of CIM4001, CIM4002 and CIM4003

To complement the *neoE::aac(3)IV* mutant strain CIM4001, a 1.0-kb fragment harboring the whole *neoE* was amplified with primer pair cneoEF/cneoER and cloned into pMD19T (Takara). The *neoE* was verified by sequencing, confirmed with *Nde* I/*Hin*d III digestion and inserted into the same sites of pUWL201pw-oriT to generate plasmid pCIM4004, which was then introduced into CIM4001 by *E. coli*-*Streptomyces* conjugation to obtain the *neoE* complemented strain CIM4004.

For complementation of the *afsA-g::aac(3)IV* mutant CIM4002 and the *neoR::aac(3)IV* mutant CIM4003, a 0.9-kb fragment for *afsA-g* and the 2.7-kb fragment for *neoR* were PCR-amplified from *S. fradiae* CGMCC 4.7387 with primers afsA-F/afsA-R and cneoEF/cneoER, verified by sequencing and inserted into the *Nde* I/*Hin*d III sites of pUWL201pw-oriT to generate the complementation plasmids pCIM4005 and pCIM4006, respectively. Plasmid pCIM4005 was then introduced into the *afsA-g::aac(3)IV* mutant CIM4002 to give CIM4005. The *neoR::aac(3)IV* mutant complemented strain CIM4006 was obtained by introduction of pCIM4006 into CIM4003.

Overexpression of AfsA-g and neoR in *S. fradiae* CGMCC 4.7387

Plasmid pCIM4005 was introduced into *S. fradiae* CGMCC 4.7387 to generate the *afsA-g* overexpressed strain CIM4007. The *neoR* overexpressed strain CIM4008 was generated by introduction of pCIM4006 into the wild-type strain *S. fradiae* CGMCC 4.7387. A control strain CIM4009 was obtained by introduction of the empty plasmid pUWL201-oriT into *S. fradiae* CGMCC 4.7387.

Production and detection of neomycin

For neomycin production, 20 μL of *S. fradiae* CGMCC 4.7387 spores (about 10^8 spores per mL) were inoculated into 50 mL soybean meal medium and incubated at 28°C for 3 days as seed culture, and 4 mL seed culture was then inoculated into 50 mL soybean meal medium in 250 mL flask and cultivated at 28°C for 7 days. The fermentation broth was adjusted to pH 3.0 and the supernatant was collected by filtration. After adjusting the pH value to 7.0, the supernatant was applied to 6 mL cation exchange resin Dowex 50WX2 in hydrogen form (Sigma-Aldrich, St. Louis, MO, USA) and washed with 80 mL water. Neomycin was then eluted with 60 mL 0.5 mol L^{-1} ammonia water, concentrated in vacuum, diluted to 1 mL by water and subjected to HPLC analysis.

HPLC analysis of neomycin was carried out with a reverse-phase C18 column (5 μm, 4.6×250 mm, Thermo, Waltham, MA, USA) on a HPLC system with ELSD detector (Scientific Systems Inc. State College, PA, USA). The column was developed using 7% methanol in water with 0.1% trifluoroacetic acid as the mobile phase at a flow rate of 0.6 mL min^{-1} for

Table 3 Primers used in this study

Primers	Sequences (5'→3')
neoEL-F	CTTCCATGGGCACGCCCTAGGCCTCGGTCAGAACCCGAGA
neoEL-R	ATCCCTTAACGTGAGCCTAGGAGGTGAAGCCGTGCCAGAC
neoER-F	CAGTCGATTGGCTGACAATTGCTGGTCGGCTACCGCATCG
neoER-R	CTTGCTAGCAGATGTCAATTGTGTAGCGCCTGCGGGTCTGC
testneoE-F	TCCGCGTCGCCTACAACTCC
testneoE-R	CGATGCGGTAGCCGACCAG
cneoEF	CATATGATGAAGGCTCTGGTGTTCGAG
cneoER	GGATCCTCAGGCCCGGAGGTTGA
afsAL-F	AAAAGCTTCCATGGGCACGCCCTAGGGCGGGGCAGCGAGGTGAAGAGTAT
afsAL-R	CCAAAATCCCTTAACGTGAGCCTAGGGCGGGTCGAGCGTGGTGTCGT
afsAR-F	CTCGCCAGTCGATTGGCTGACAATTGCTCCGCATGACCTTCCACGTCCAG
afsAR-R	CCAAGCTTGCTAGCAGATGTCAATTGGCATCCACTCGTACCCGAACCCTC
testafsA-F	CGCTCGTACACCCGCTCG
testafsA-R	CGTTGCCGTGCCCTTCAG
cafsAF	CATATGATGTTGTACGACACCACGCTCGACC
cafsAR	AAGCTTTCAGGGACGGAAGTCGACCCGGAAC
neoRL-F	CTTCCATGGGCACGCCCTAGGCCGTCCAGGGCCGAACAC
neoRL-R	ATCCCTTAACGTGAGCCTAGGCCCAGTTCGTACAGGACCTCGG
neoRR-F	CAGTCGATTGGCTGACAATTGGCGCTTTCGGAGCTGCCC
neoRR-R	CTTGCTAGCAGATGTCAATTGCTGACCACAGCGGGTGCG
testneoR-F	TCCACCCCGACGACGACC
testneoR-R	CCCAGTCAAGTGCGCTACCAA
cneoRF	CATATGATGCTGCCGTCCAGGGCCGAACACC
cneoRR	AAGCTTTCACCGGGCGGGCAGCTCCGAAAG
201F	CGTGCCGGTTGGTAGGAT
201R	GAGCGAGGAAGCGGAAGA
hrdBqF	TTGATGACCTCGACCATGTG
hrdBqR	GACTACACCAAGGGCTACAAG
neoRqF	GGACGGTCGCCAGGAGTTCG
neoRqR	GCAGGCCAGGCTCACCAGCA
neoGqF	ACGGCCATCAGGTAGTAGT
neoGqR	CGGGTCTGCTGGAGGTA
neoHqF	TCAGGTCGCCGAAGAGA
neoHqR	ACCGACGACCACATCTTCTA
aphAqs	GTCGAAGGCGGAGTTCTC
aphAqa	GCAGTGAACGAAGGAGACTC
neoIqF	CGTCTCCTCGTTGAGGAAGTA
neoIqR	CATCTCCGGCACCTATCTCT
neoEqF	ATGAAGGCTCTGGTGTTCG
neoEqR	CCGCAGACGGAGTTGTAG
neoCqF	TCGGTCCTCTCGCTCAAG
neoCqR	ACGGGCTGGTAGAAGGTC
neoTqs	CCACCGTCTTCGAGCAG
neoTqa	AACACCTTCAGCACCCG
neoQqF	TGCAACGCGATCCAGAG
neoQqR	CGTACTCGCTCAGCTTCATC
neoPqF	GTGCTCGTGTCGCACTC
neoPqR	CACCGGTGTTGCTGATCTT
neoLqF	GGAGTGCTACGGCTACGA
neoLqR	TATGCCCACGACGAGCA
neoBqF	GGGTGGTGGACTTCTTCTTC
neoBqR	CGACAGGTGCATGTGGT
neoAqF	TCGTTCCTCACCTCCGT
neoAqR	CTCCTGGCGGAACATCAC
aacC8qF	TCAGCAGCGGGTCGTAG
aacC8qR	TACTGCGGCTGGAACGA

40 min (Li et al., 2013a). Liquid chromatography-mass spectroscopy analysis was performed using Aligent 1200 HPLC system and 6520 QTF-MS system (Agilent, Santa Clara, CA, USA) with the electrospray ionization source.

Antibacterial assay

The antibacterial assay was carried out against *Staphylococcus aureus* ATCC 6538 on LB agar plate. Three microliters of the concentrated samples for HPLC detection were used for each assay. After incubation at 37°C for 12 h, each inhibiting zone was measured with a vernier caliper.

RNA isolation and real-time PCR

For *S. fradiae* CGMCC 4.7387 and the mutant strains, culture samples were collected after incubation for 60 h at 28°C in soybean meal medium. Total RNAs were isolated using the Total RNA Isolation Kit (CWBIO, Beijing, China) following the manufacturer's instructions and were treated subsequently with RNase-free DNase I (Thermo) and examined by PCR to exclude possible DNA contamination. First strand cDNA synthesis was carried out using 1 μg of each RNA sample with Maxima H Minus Reverse Transcriptase (Thermo) according to the manufacturer's procedure. Real-time PCR of selected genes was performed in LightCycle 480 System (Roche Diagnostics, Basel, Switzerland) using SYBR® Premix Ex TaqTM (Takara) with primers listed in Table 3. The primers were named as gene name with qF or qR for forward or reverse directions, such as neoGqF/neoGqR for detection of *neoG* and neoHqF/neoHqR for detection of *neoH*. *hrdB* was used as an internal control. The real-time PCR was set as follows: 5 min pre-denaturation at 95°C, followed by 45 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C and 30 s extension at 72°C. The results were analyzed by the LightCycle 480 software and the relative transcriptional levels of tested genes were quantified using the $2^{-\Delta\Delta C_T}$ method and normalized to the hrdB level (Livak and Schmittgen, 2001). Data shown here are the averages of three independent experiments analyzed by the software GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Nucleotide sequence accession numbers

Gene cluster containing afsA-g and its flanking genes and the neo gene cluster from *S. fradiae* CGMCC 4.7387 were deposited in GenBank under accession numbers KX871905 and KX871906, respectively.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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