Improvement of Nemadectin Production by Overexpressing the Regulatory Gene *nem*R and Nemadectin Biosynthetic Gene Cluster in *Streptomyces Cyaneogriseus*

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Abstract

Nemadectin, a 16-member macrocyclic lactone antiparasitic antibiotic, is produced by *Streptomyces cyaneogriseus* subspecies *noncyanogenus*. Moxidectin, a C-23 oximate derivative of nemadectin, is widely used as a pesticide due to its broad-spectrum, highly efficient, and safe anthelmintic activity. NemR, a LAL family regulator, is encoded by *nem*R and is involved in nemadectin biosynthesis in *S. cyaneogriseus*. In this report, gene disruption and complementation experiments showed that *nem*R plays a positive role in the biosynthesis of nemadectin. The transcription level of nemadectin biosynthetic genes in the *nem*R knockout strain was significantly decreased compared with those in the wild-type strain MOX-101. However, overexpression of *nem*R under the control of native or strong constitutive promoters resulted in the opposite, increasing the production of nemadectin by 56.5 or 73.5%, respectively, when compared with MOX-101. In addition, the gene cluster of nemadectin biosynthesis was further cloned and overexpressed using a CRISPR method, which significantly increase nemadectin yield by 108.6% (509 mg/L) when compared with MOX-101.

Keywords

- nemadectin
- overexpression
- biosynthesis gene cluster
- *Streptomyces cyaneogriseus*

Introduction

Nemadectin produced by *Streptomyces cyaneogriseus* subspecies *noncyanogenus* is a 16-member macrocyclic lactone antiparasitic antibiotic with broad-spectrum endectocidal and nematocidal activity.[1,2] The structure of nemadectin is similar to those of milbemycin, avermectin, and meilingmycin.[3–5] Moxidectin, a C-23 oximate derivative of nemadectin (►**Fig. 1**), showed stronger insecticidal activity than nemadectin.[6] Moxidectin has many advantages over avermectin, ivermectin, and other insect-repellent antibiotics, such as low toxicity, high efficiency, and a broader spectrum.[7–10] In 2018, the U.S. Food and Drug Administration approved moxidectin for the treatment of onchocerciasis (river blindness) in patients aged 12 years and older.

The preliminary research on nemadectin was mainly focused on strain breeding and optimization of fermentation technology. In 2009, the yield of the strain was increased to 172 mg/L by ultraviolet (UV) mutagenesis combined with the screening of precursor resistance.[11] In 2014, the fermentation process of nemadectin was optimized by investigating various factors, such as time, temperature, and dissolved oxygen.[12] In 2015, Li et al reported a method for improving nemadectin production by screening a mutant strain DC18–01 and adding the precursor sodium acetate for one or more times in the middle and later stages of fermentation.[13] Although the

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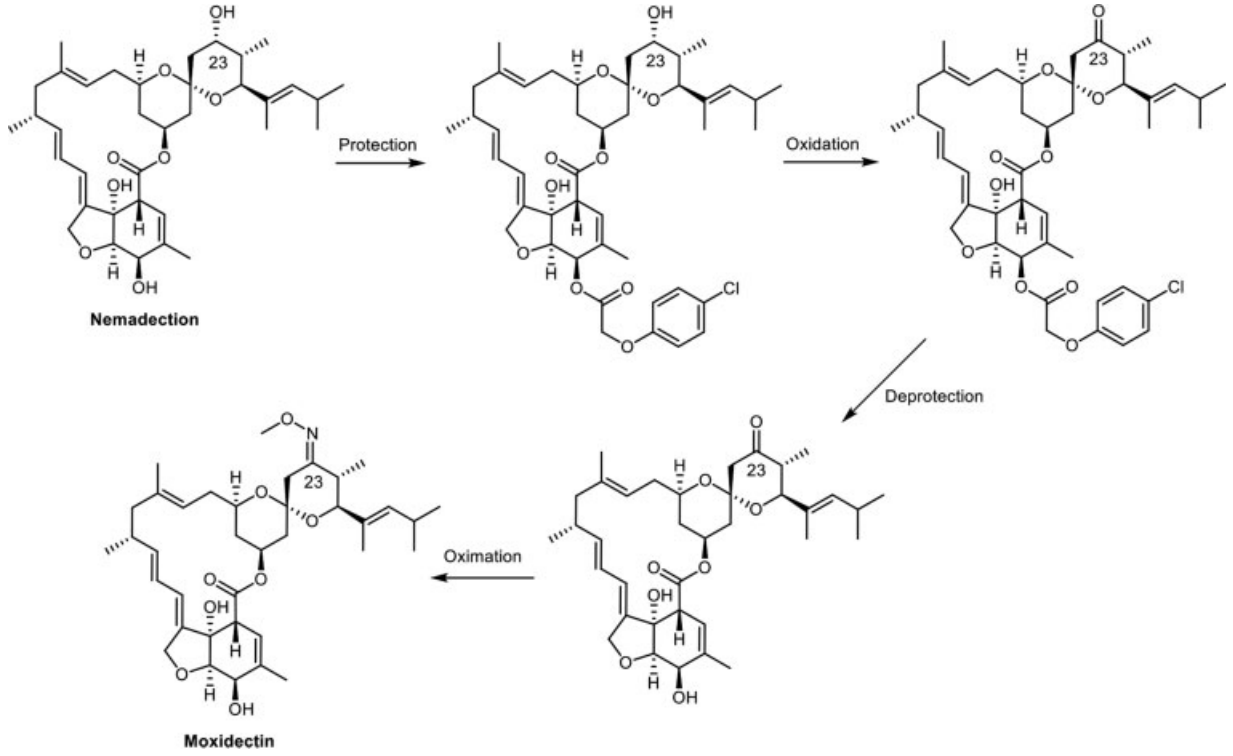

Fig. 1 The semi-chemical synthesis of moxidectin.

biosynthetic gene cluster of nemadectin has been identified, and its complete sequence is also available,[14] the study on genetic modification to nemadectin-producing strain remains largely unknown. In 2019, the function of the gene *nem*R was characterized, which is a positive regulatory gene encoding a LAL family transcriptional regulator within the nemadectin biosynthesis gene cluster of the strain NMWT1.[15] The low fermentation yield of nemadectin resulted in the high production cost of moxidectin. In this study, the LAL family regulatory gene *nem*R, which serves as an activator for nemadectin biosynthesis, was confirmed and was employed to improve nemadectin production by overexpression of *nem*R in the strain MOX-101. The nemadectin biosynthesis gene cluster in MOX-101 was successfully cloned and used to increase nemadectin production by duplicating of the biosynthesis gene cluster in *S. cyaneogriseus*.

Materials and Methods

Strains, Plasmids, and Primers

Strains and plasmids used in this study are listed in ►**Table 1**. Primers are listed in ►**Table 2**.

Construction of Gene Deletion, Complementation, and Overexpression Strains

The construction process for the *Δnem*R mutant with deletion of *nem*R is as follows. A 1.5-kb fragment upstream of the *nem*R start codon and a 1.5-kb fragment downstream of the *nem*R stop codon were amplified from MOX-101 genomic DNA (gDNA) using the primer pairs nemR-up-F/R and nemR-down-F/R, respectively. Two polymerase chain reaction (PCR) fragments were spliced together through overlapping PCR using the primers nemR-up-F and nemR-down-R. The resulting product was cloned into a 3.1-kb DNA fragment that was amplified from pSET152 using the primers pSET152-F/R to obtain the *nem*R-deleted plasmid p*Δnem*R using the seamless ligation method (ClonExpress II One Step Cloning kit) (►**Fig. 2A**). The plasmid p*Δnem*R was transferred from S17–1 into MOX-101 by intergeneric conjugation. After selecting single-crossover recombinant strains and double-crossover recombinant strains, the resulting mutant with deletion of *nem*R was named *Δnem*R.

The *nem*R complementation strain *Δnem*R/p*nem*R was constructed as follows. Using the gDNA of MOX-101 as the template, a 3.7-kb DNA fragment containing the gene *nem*R and its promoter region was amplified by PCR with the primers nemR-N-F/R. The PCR product was inserted into the *Xba*I/*Bam*HI site of pSET152 to generate the integrative plasmid p*nem*R (►**Fig. 2B**). The sequence of *nem*R in p*nem*R was confirmed by DNA sequencing. After conjugal transfer, p*nem*R was transferred into the *Δnem*R strain to obtain the *nem*R complementation strain *Δnem*R/p*nem*R. As a control, the control plasmid pSET152 was transferred into MOX-101 to generate the strain MOX-101/pSET152.

The gene *nem*R was amplified from MOX-101 gDNA using the primers nemR-Q-F/R and cloned into the *Nde*I/*Asc*I sites of pSET152 to generate the overexpression plasmid p*nem*R-ermEp*, in which *nem*R is driven by the strong constitutive promoter ermEp* (►**Fig. 2C**). Next, p*nem*R bearing the native promoter and p*nem*R-ermEp* were transferred into MOX-101 to obtain overexpression strains MOX-101/p*nem*R and MOX-101/p*nem*R-ermEp*.

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant characteristic	Reference or source
Strains		
E. coli		
DH5α	F$^-$, ϕ80*lacZ*Δ*M15*, Δ(*lacZYA-argF*) *U169, deoR, recA1, endA1, hsdR17 (rk$^-$, mk$^+$), phoA, supE44, λ$^-$, thi-1, gyrA96, relA1*	CWBIO
S17–1	*supE44,* Δ*lacU169 (FlacZ*Δ*M15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1*, par phage lysogenic	Gibco-BRL
EPI300	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*dlacZ*ΔM15Δ*lacX74 recA1 endA1 araD139* Δ (*ara, leu*)*7697 galU galK λ- rpsL* (*StrR*) *nupG trfA tonA*	Epicentre
S. cerevisiae		
VL6–48	*MATα his3-D200 trp1-D1 ura3–52 ys2ade2–101 met14 psi+ cir0*	ATCC MYA-366
S. cyaneogriseus		
MOX-101	Nemadectin producer	This laboratory
Δ*nem*R	MOX-101 with deleted *nemR*	This study
Δ*nem*R/p*nem*R	Δ*nem*R mutant with p*nem*R	This study
MOX-101/pSET152	MOX-101 strain with pSET152	This study
MOX-101/p*nem*R	MOX-101 strain with p*nem*R	This study
MOX-101/p*nem*R-ermEp*	MOX-101 strain with p*nem*R-ermEp*	This study
MOX-101/pCL01	MOX-101 strain with pCL01	This study
MOX-101/pCL-MOX	MOX-101 strain with pCL-MOX	This study
Plasmids		
pSET152	*E. coli* replicon, *Streptomyces* ØC31 attachment site, Apr^R	This laboratory
pKCCas9(*tipAp*)	pKC1139 with the scocas9 gene under the control of the inducible promoter *tipAp*	[16]
pCL01	Large gene cluster capture vector: derived from pCC1BAC containing a single copy F-factor replicon and a high-copy origin of replication, ΦC31 *int/attP*, *aac(3)IV*, *oriT* RK2, ARSH4/CEN6, and TRP1	[17]
pΔ*nem*R	pSET152 with the upstream and downstream DNA fragments of *nemR*	This study
p*nem*R	pSET152 with the expression of *nemR* under the control of native promoter	This study
p*nem*R-ermEp*	pSET152 with the expression of *nemR* under the control of strong promoter *ermEp**	This study
pCL-M1	pCL01 with the upstream and downstream DNA fragments of the gene cluster MOX1	This study
pCL-M2	pCL01 with the upstream and downstream DNA fragments of the gene cluster MOX2	This study
pCL-M	pCL01 with the upstream and downstream DNA fragments of the gene cluster MOX	This study
pCL-MOX1	pCL-M1 with the biosynthetic gene cluster MOX1	This study
pCL-MOX2	pCL-M2 with the biosynthetic gene cluster MOX2	This study
pCL-MOX	pCL-M with the biosynthetic gene cluster MOX	This study

Construction of Nemadectin Biosynthetic Gene Cluster Overexpression Strain

Cloning nemadectin biosynthetic gene cluster was conducted following the CRISPR-TAR method reported previously.[18] The nemadectin biosynthetic gene cluster MOX was divided into two parts, namely, MOX1 (50 kb) and MOX2 (41.7 kb). MOX1 and MOX2 were cloned respectively, and then two modules were spliced to generate the complete nemadectin biosynthetic gene cluster.

CRISPR-digested gDNA was prepared as follows. First, four gRNA minigenes encoding gRNA were amplified from the plasmid pKCCas9 (*tipAp*) by the primers gRNA-MOX1-up-F/gRNA-R, gRNA-MOX1-down-F/gRNA-R, gRNA-MOX2-up-F/gRNA-R, and gRNA-MOX2-down-F/gRNA-R designed at each end of MOX1 and MOX2 gene clusters, respectively. Next, the resulting products were transcribed *in vitro* by MEGAScriptTMT7 Kit (Thermo Fisher Scientific, China) to obtain gRNAs used to guide Cas9 nuclease to cleave the gDNA of MOX-101. These gRNAs were purified using MEGAClear Kit (Thermo Fisher Scientific, China). Then, the gDNA isolated by the phenol–chloroform method was digested *in vitro* overnight with the help of the Cas9 nuclease and gRNAs.

Plasmids pCL-M1, pCL-M2, and pCL-M were constructed to capture MOX1, MOX2, and MOX gene clusters, respectively.

Table 2 Primers used in this study

Primers	Sequences (5′→3′)[a]
nemR-up-F	AAAGATCCGTCGACCTGCAGAAGCTTGCCCTACGCCATGACCAAGG
nemR-up-R	GTTCAGACCGGGCTTTCGATAATCTCCCTCTGACTCCCTCCCC
nemR-down-F	ATCGAAAGCCCGGTCTGAACG
nemR-down-F	TCGCGCGCGGCCGCGGATCCTCTAGAGGCAGCAGGATCATCGGTGG
pSET152- F	GACTCTAGAGGATCCGCGGC
pSET152- R	AAGCTTCTGCAGGTCGACGGA
nemR-N-F	AGCTTGGGCTGCAGGTCGACTCTAGAAGCCAGAGGGATTCGGTCTC
nemR-N-R	TCGATATCGCGCGCGGCCGCGGATCCTCAGACCGGGCTTTCGATGT
nemR-Q-F	aaccactccacaggaggacccatatgATGCGGGGCGTTTCCCCTTC
nemR-Q-R	TGGAAAGACGACAAAACTTTGGCGCGCCTCAGACCGGGCTTTCGATGTGC
nemA1–2A2-F	tggacagggacgacagcgcc
nemA1–2A2-R	ACCGGAGCTGACGAGGTCCC
nemA2C-F	ggctcttgacgtcgatcccga
nemA2C-R	TGTCGAGGACCCTTCGGTGG
nemA3E-F	gggtggcgaggtagacggtg
nemA3E-R	CCAGATCGCCACGGTCCTCG
nemA4A3-F	ccagcagcaagcgctgctgg
nemA4A3-R	AGCCGCACACCGTCCTCGAA
nemG-F	ATGCCCGACCTTTGCGAGACC
nemG-R	CGGTCCTGTCGTCCGGGGTAC
nemF-F	CAGGACGGCCTTGGTCACGG
nemF-R	AGAGCTCATCCACGGCGTCGTC
nemA1–2-F	GTGGGTCACCGCCGATCTGC
nemA1–2-R	ACAGCCCGTCCAGATCCCACC
nemA3-F	CGAGAAGCTCGTCGAGGCGCT
nemA3-R	AGCCACCAGCCCCCACAACTC
nemA4-F	TGCTCCGCGAGTACCTCAAGCG
nemA4-R	GGGAAGTCGCCGATCGCGTC
gRNA-MOX1-up-F	gactgacactgaTAATACGACTCACTATAggagaggacgagccggtagcGTTTTAGAGCTAGAAATA
gRNA-MOX1-down-F	gactgacactgaTAATACGACTCACTATAggtcacgacgccgtggcagcGTTTTAGAGCTAGAAATA
gRNA-MOX2-up-F	gactgacactgaTAATACGACTCACTATAggcgctcggcgcggtgtgccGTTTTAGAGCTAGAAATA
gRNA-MOX2-down-F	gactgacactgaTAATACGACTCACTATAggcgtcccgcgtccccgtgcGTTTTAGAGCTAGAAATA
gRNA-R	CTCAAAAAAAGCACCGACTCGG
MOX1-up-F	agtgaattgtaatacgactcactatagggcatttaaatGAGGACGTTCACCCGTGGTTC
MOX1-up-R	CGACTCCCGAGGACAAGATGG
MOX1-down-F	CagcgccatcttgtcctcgggagtcgGTTTAAACACGTCCGAGTTGCGTGAGCG
MOX1-down-R	gcacgtgatgaaaaggacccaggtggcacACGTGCGTGTTGGAGACCAG
MOX2-up-F	agtgaattgtaatacgactcactatagggcatttaaatATCTGGGTGCCCGCCTGCTT
MOX2-up-R	GCCGTATAGCCGCCCATCA
MOX2-down-F	cagcgccatcttgtcctcgggagtcgGTTTAAACGCGTTGGTGGTGACCGTGAG
MOX2-down-R	gcacgtgatgaaaaggacccaggtggcacCGACCTCAACCGCTTCTACACC
MOX-down-F	cagcgccatcttgtcctcgggagtcgGTTTAAACGCGTTGGTGGTGACCGTGAG

[a]Restriction sites are underlined.

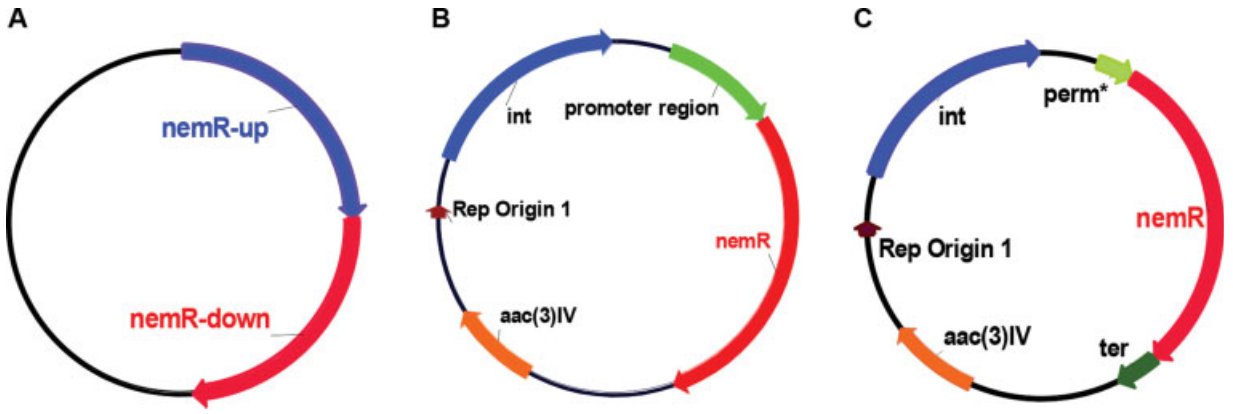

Fig. 2 Plasmid maps of pΔ*nem*R (**A**), p*nem*R (**B**), and p*nem*R-ermEp* (**C**).

Using the gDNA of MOX-101 as the template, six homologous arms, MOX1-up, MOX1-down, MOX2-up, MOX2-down, MOX-up, and MOX-down, were amplified with the primers MOX1-up-F/R, MOX1-down-F/R, MOX2-up-F/R, MOX2-down-F/R, MOX1-up-F/R, and MOX-down-F/MOX2-down-R, respectively. Then, MOX1-up and MOX1-down, MOX2-up and MOX2-down, and MOX1-up and MOX2-down were spliced together by overlapping PCR, respectively. The resulting fragments were cloned into the *Eco*RI/*Swa*I site of the plasmid pLC01 by homologous recombination to generate the capture plasmids pCL-M1, pCL-M2, and pCL-M, respectively. Capture plasmids were digested with restriction enzyme *Pme*I to obtain linearized capture plasmids.

Digested genome DNAs and linearized capture plasmids were introduced into *Saccharomyces cerevisiae* VL6–48 to clone the biosynthetic gene cluster MOX1 and MOX2. After verifying transformants, positive plasmids pCL-MOX1 and pCL-MOX2 were transferred into *Escherichia coli* EPI300 for enrichment. Positive plasmids pCL-MOX1 and pCL-MOX2 were collected by E.Z.N.A.BAC/PAC DNA (Omega Bio-Tek, China) and digested with restriction enzyme *Swa*I to obtain gene clusters MOX1 and MOX2, respectively. The obtained gene clusters and the linearized capture plasmid pCL-M were introduced into the yeast to clone the biosynthetic gene cluster MOX. After confirming the transformant, the positive plasmid pCL-MOX was transferred into *E. coli* EPI300 for enrichment. Plasmids pCL01, pCL-MOX1, pCL-MOX2, and pCL-MOX were digested with the restriction enzyme *Kpn*I to verify if the captured gene clusters were correct. The correct plasmid pCL-MOX was transferred from S17–1 into MOX-101 to generate the engineering strain MOX-101/pCL-MOX, in which the nemadectin biosynthesis gene cluster was overexpressed. As a control, the control plasmid pCL01 was transferred into MOX-101 to generate the strain MOX-101/pCL01.

Culture of *Streptomyces Cyaneogriseus*

For nemadectin production, the parental strain MOX-101 and mutant strains were first grown on solid medium (g/L): 4.0 glucose, 1.3 maltose, 4.0 yeast extract, 5.0 starch, 5.0 soybean meal, 5.0 dextrin, 1.0 KNO_3, 0.5 K_2HPO_4, 0.5 NaCl, 0.5 $MgSO_4$, 0.1 $CaCO_3$, and 20.0 agar at pH 6.8 to 7.0 and 28°C for 10 to 12 days. Then, the mycelium was inoculated into a 250-mL flask with 20 mL seed medium (g/L): 10.0 glucose, 10.0 yeast meal, 5.0 yeast extract, 15.0 soybean meal, 20.0 dextrin, 1.0 K_2HPO_4, 1.0 $MgSO_4$, and 4.0 $CaCO_3$, and the culture was grown at pH 7.0 to 7.2, 28°C, and 200 rpm. After 24 to 28 hours, 10% seed culture was transferred into a 250-mL flask with 25 mL fermentation medium (g/L): 80.0 glucose, 5.0 yeast meal, 35.0 lactose, 27.5 soybean meal, 2.5 corn meal, 1.0 $MgSO_4$, and 4.0 $CaCO_3$, and the culture was grown at pH 7.0 to 7.2, 28°C, and 200 rpm for 8 days.

Analytical Method

To analyze nemadectin yield, 1.0 mL culture broth (collected for 4, 6, and 8 days) was extracted with 3.0 mL methanol for 30 minutes and centrifuged at 10,000 × g for 5 minutes. The supernatant was analyzed by high-pressure liquid chromatography (HPLC) with a Hypersil C18 column (5 µm, 4.6 mm × 150 mm). The column was maintained at 30°C with mobile phases of methanol:water (85:15 [vol/vol]) at a flow rate of 1.0 mL/min and the product was UV-detected at 240 nm using an Agilent 1260 HPLC system.

Transcriptional Assay by Real-time Polymerase Chain Reaction Analysis

First, total RNA was isolated from the strains and used as the template to synthesize cDNA samples. Fermentation broths (10 mL) of MOX-101 and *ΔnemR* collected at day 4, 6, and 8 were centrifuged at 10,000 × g for 5 minutes, and pellets were ground into powder in liquid nitrogen. Next, an Ultrapure RNA Kit (CWBIO, China) was used for total RNA extraction according to the user manual provided by the manufacturer. The obtained RNA sample was treated with RNase-free DNase I (Takara, China) to remove the remaining gDNA. To obtain cDNA samples, reverse transcription (RT) was performed using M-MLV Reverse Transcriptase (Promega, United States).

Then, a cotranscriptional experiment utilizing RT-PCR was employed to investigate whether two nemadectin biosynthetic structural or regulatory genes spanning a short intergenic region and transcribed in the same direction were cotranscribed. Primers used to amplify the cDNA sample of MOX-101 were nemA1–2A2-F/R, nemA2C-F/R, nemA3E-F/R, and nemA4A3-F/R. gDNA and RNA samples without RT

served as the positive and negative controls, respectively, in the PCR analyses.

Then, quantitative RT-PCR (qRT-PCR) was performed by TB Green™ Premix Ex Taq™ II (Takara, China) according to the manufacturer's instructions. qRT-PCR experiments were performed to assay samples collected for 4, 6, and 8 days of fermentation. One gene was selected from each transcriptional unit for transcriptional analysis. Five primer pairs, including nemG-F/R, nemF-F/R, nemA1–2-F/R, nemA3-F/R, and nemA4-F/R, were used in the qRT-PCR tests. Transcriptional levels of the tested genes were normalized using *hrd*B (TU94_24720) as the internal control.[19] Each qRT-PCR analysis was repeated three times, and the error bar represents the standard deviation.

Results

NemR Functions as an Activator in the Biosynthesis of Nemadectin

To study the function of *nem*R in nemadectin biosynthesis, we constructed the *Δnem*R mutant strain with deletion of the *nem*R gene using MOX-101 as the parental strain (►**Fig. 3A**). Two strains were cultured in the fermentation medium, and broths were collected for 4, 6, and 8 days. The result showed that nemadectin production was significantly decreased in *Δnem*R with the deletion of the *nem*R gene. Nemadectin yield in *Δnem*R was decreased by approximately 80% in comparison with the parental strain MOX-101 (►**Fig. 3B**). To further verify the function of NemR, we performed a complementation experiment in which the complemented plasmid p*nem*R was introduced into the *Δnem*R mutant (►**Fig. 3A**). In p*nem*R, *nem*R was driven by the native promoter. The result showed that the complementation of *nem*R could restore the yield level of nemadectin to that of the parental strain (►**Fig. 3C**), revealing that the decline in nemadectin yield was mainly due to *nem*R inactivation. These results demonstrated that NemR acts as an activator for nemadectin biosynthesis in *S. cyaneogriseus*.

qRT-PCR Analysis of Transcriptional Levels of the Nemadectin Biosynthetic Gene Cluster

A transcriptional assay was performed to further study the function of the NemR protein in nemadectin production. The gene cluster for nemadectin biosynthesis contains 10 structural genes and one regulatory gene (►**Fig. 4A**). The result of the cotranscriptional experiment suggested that there were five cotranscriptional units altogether in the gene cluster of nemadectin biosynthesis, including *nem*G, *nem*F, *nem*A1–1-C, *nem*A3-D, and *nem*A4 (►**Fig. 4B**).

The transcriptional assay result indicated that *nem*R deletion strongly affects the transcription of nemadectin biosynthetic genes. As shown in ►**Fig. 4C**, compared with the parental strain MOX-101, transcriptional levels of all nemadectin biosynthetic genes in the knockout strain *Δnem*R were significantly decreased. Among these structural genes, transcriptional levels of *nem*G/F and *nem*A1–1/A1–2/A2/C/D/E/A3/A4 in *Δnem*R were only approximately 30% and less than 3% of those in MOX-101. These results suggested that *nem*R might play a positive regulatory role in nemadectin production by enhancing the expression of the nemadectin biosynthetic gene cluster.

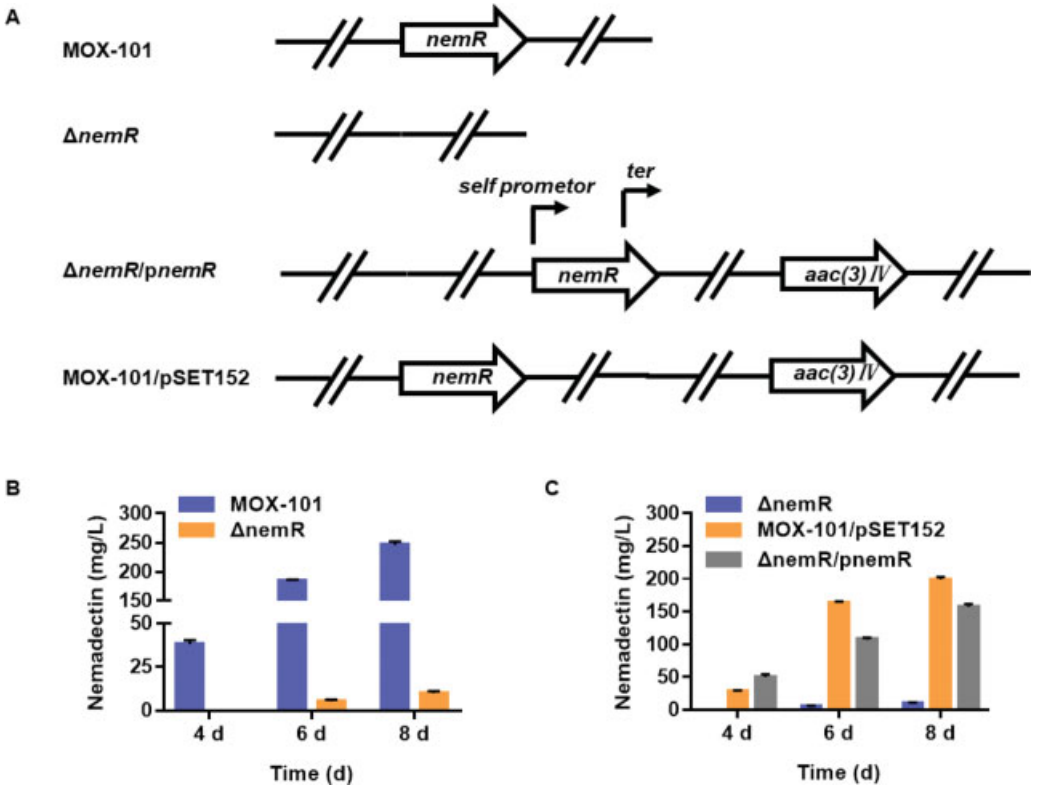

Fig. 3 Effect of *nem*R disruption on nemadectin production. (**A**) Genotypes of *S. cyaneogriseus* MOX-101 and its recombinant strains. (**B**) Nemadectin production in the parental strain MOX-101 and the mutant strain Δ*nem*R. (**C**) Nemadectin production in the mutant strains Δ*nem*R, MOX-101/pSET152, and Δ*nem*R/p*nem*R.

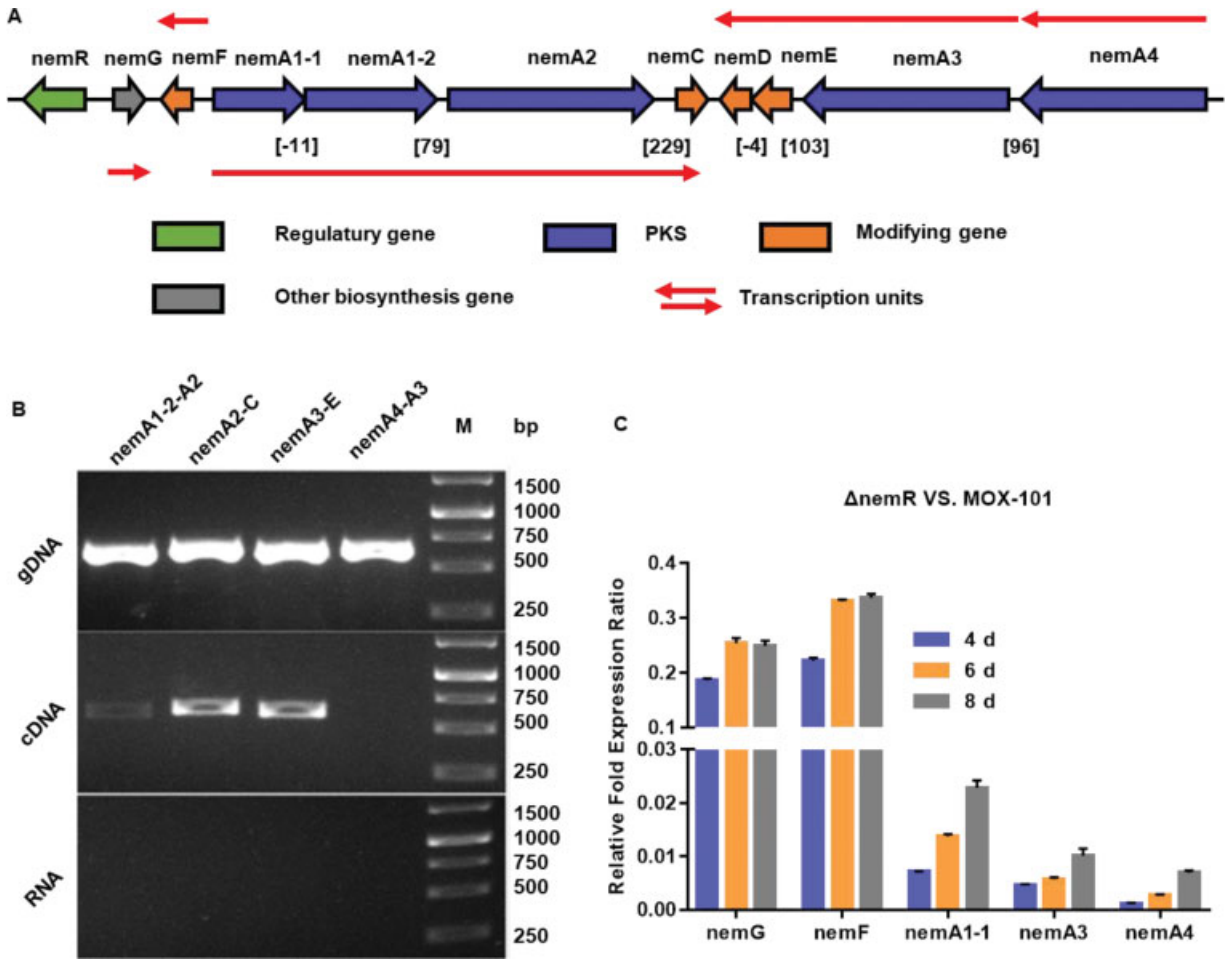

Fig. 4 Transcriptional analysis of the nemadectin biosynthetic genes upon *nem*R disruption in *S. cyaneogriseus* MOX-101. (**A**) Genetic map of the nemadectin biosynthetic gene cluster. (**B**) Cotranscriptional analysis of the nemadectin biosynthetic gene cluster. Genomic DNA (gDNA) and RNA samples without reverse transcription (RT) were used as the positive and negative controls, respectively. (**C**) Relative transcription levels between the parental strain MOX-101 and the knockout strain Δ*nem*R.

Effect of Overexpression of *nem*R on Nemadectin Production

To enhance nemadectin yield, p*nem*R under the control of the native promoter was transferred into MOX-101 to generate the overexpression strain MOX-101/p*nem*R. As a control, the control plasmid pSET152 was transferred into MOX-101 to generate MOX-101/pSET152. Three strains, MOX-101, MOX-101/p*nem*R, and MOX-101/pSET152, were cultured for 4, 6, and 8 days, and broths were collected to detect the fermentation products by HPLC analysis. The result showed that compared with the original strain MOX-101, MOX-101/p*nem*R improved nemadectin production by 56.5%, while the effect of MOX-101/pSET152 on nemadectin production was similar to that of MOX-101 (►**Fig. 5A**). These results suggested that the increase in production of MOX-101/p*nem*R was due to an extra copy of the gene *nem*R.

To confirm whether the improvement in nemadectin yield was caused by the improved expression of nemadectin biosynthetic genes, a transcriptional assay was performed by qRT-PCR analysis to determine the transcriptional levels of the genes in MOX-101 and MOX-101/p*nem*R. The result indicated that the transcription levels of the nemadectin biosynthetic genes in MOX-101/p*nem*R were higher than those in MOX-101 (►**Fig. 5B**). Among these genes, the transcription of *nem*G/F and *nem*A1–1/A3/A4 improved above 30 and 100%, respectively.

To further increase the nemadectin yield, MOX-101/p*nem*R-ermEp* was obtained by transforming p*nem*R-ermEp* into MOX-101, in which *nem*R is driven by the strong constitutive promoter ermEp*. As shown in ►**Fig. 5A**, the production of MOX-101/p*nem*R-ermEp* was 10.9% higher than that of MOX-101/p*nem*R and 73.5% higher than that of MOX-101.

Effect of Overexpression of Nemadectin Biosynthetic Gene Cluster on Nemadectin Production

The result of the transcriptional assay in MOX-101 and MOX-101/p*nem*R suggested that the improvement in nemadectin yield was caused by the increase in transcription levels of nemadectin biosynthetic genes. Then we attempted to directly overexpress the nemadectin biosynthetic gene cluster to increase its yield.

In the early stage of the experiment, several attempts were made to clone a complete nemadectin biosynthetic gene cluster, but all failed because of its large size (~90.9 kb). Therefore, two modules, MOX1 (50 kb) and MOX2 (41.7 kb), of the complete biosynthetic gene cluster were cloned respectively, and then the two modules were spliced together to

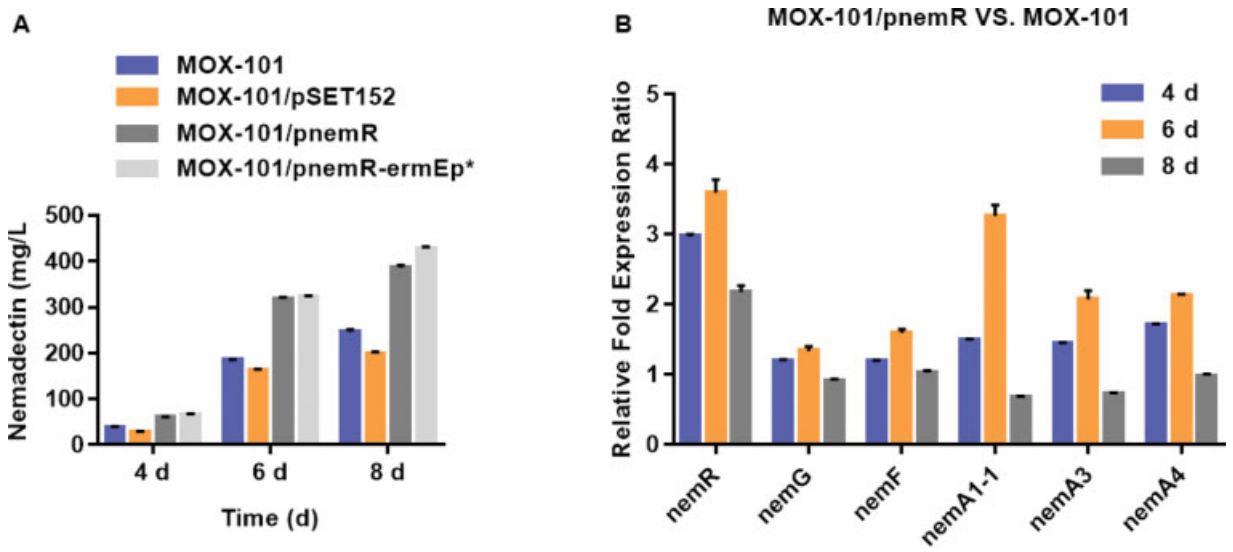

Fig. 5 Effect of *nem*R overexpression on nemadectin production. (**A**) Nemadectin production in MOX-101, MOX-101/pSET152, MOX-101/p*nem*R, and MOX-101/p*nem*R-ermEp*. (**B**) Relative transcription levels between the parental strain MOX-101 and the overexpression strain MOX-101/p*nem*R.

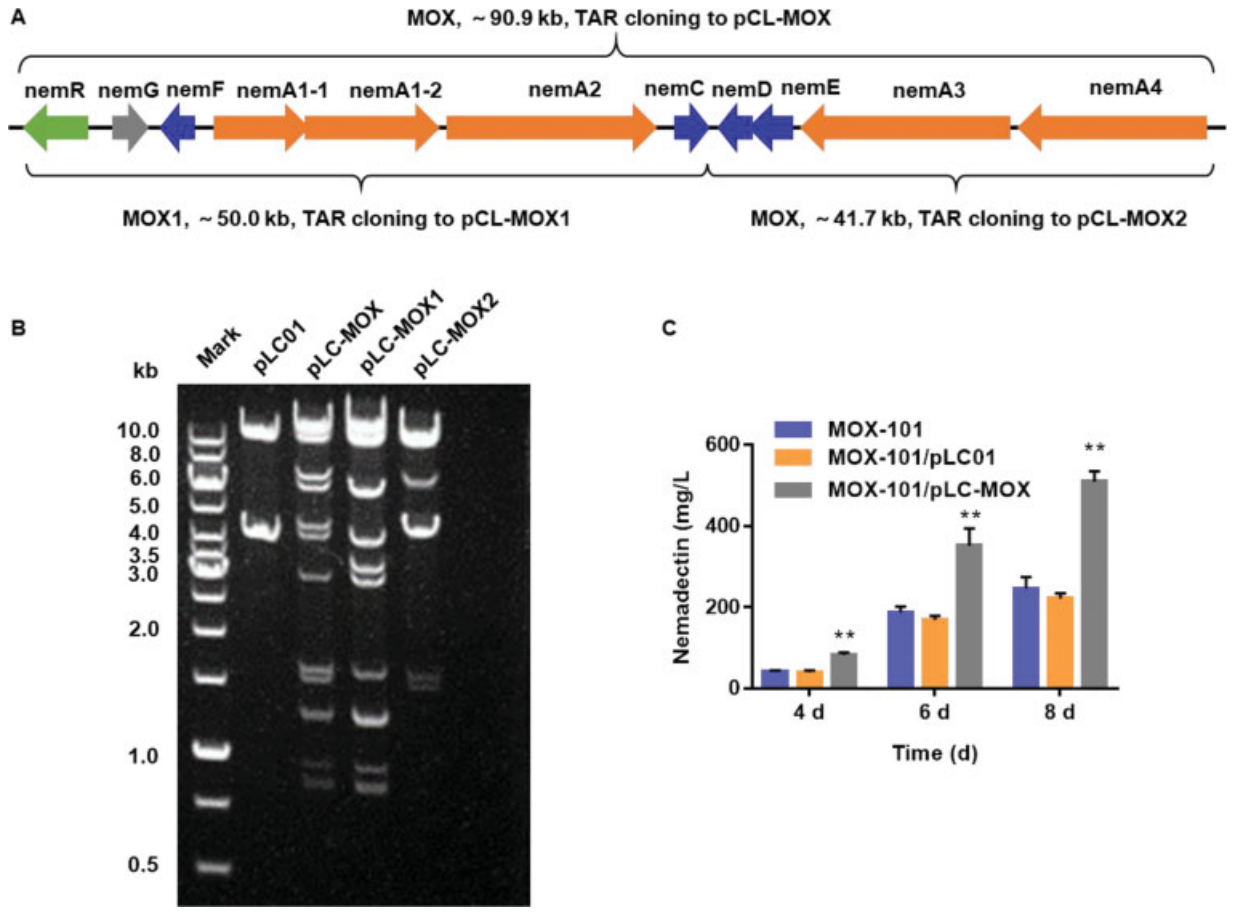

Fig. 6 Effect of biosynthetic gene cluster overexpression on nemadectin production. (**A**) Map of captured nemadectin biosynthetic gene cluster by the CRISPR-TAR method. (**B**) Restriction analysis of plasmids pCL01, pCL-MOX, pCL-MOX1, and pCL-MOX2. (**C**) Nemadectin production in MOX-101, MOX-101/pCL01, and MOX-101/pCL-MOX.

obtain the complete nemadectin biosynthetic gene cluster MOX (►**Fig. 6A**). Plasmids pCL01, pCL-MOX1, pCL-MOX2, and pCL-MOX were verified by the restriction enzyme *Kpn*I digestion, which showed that gene clusters MOX1, MOX2, and MOX were successfully cloned (►**Fig. 6B**). The plasmid pCL-MOX, containing the complete nemadectin biosynthetic gene cluster, was transferred into MOX-101 to generate the overexpression strain MOX-101/pCL-MOX. As a control, the control plasmid pCL01 was transferred into MOX-101 to generate MOX-101/pCL01. Three strains, MOX-101, MOX-101/pCL01,

and MOX-101/pCL-MOX, were cultured for 4, 6, and 8 days. The result showed that MOX-101/pCL-MOX significantly increased the production of nemadectin by 108.6% (509 mg/L) compared with the MOX-101 (►**Fig. 6C**).

Discussion

Strain improvement is very important for the industrialization of microbial medicine. Previously, random selection, such as UV mutagenesis and atmospheric and room temperature plasma mutation, and rational breeding were used to increase nemadectin yield.[11,20]

Overexpression of transcriptional regulators is an effective and commonly used method to improve antibiotic production, especially when regulators were driven by strong constitutive promoters, such as ermEp*.[21–23] In 2019, a positive transcriptional regulator, NemR, involved in nemadectin biosynthesis was reported.[15] In this study, we overexpressed *nem*R in MOX-101 under the control of both native and strong constitutive promoters. The result showed that the yield of nemadectin was considerably improved compared with that of MOX-101 owing to the increase in transcription levels of nemadectin biosynthetic genes. Therefore, we attempted to directly overexpress the nemadectin biosynthetic gene cluster to increase its yield. This is the first report that an extra copy of the complete nemadectin biosynthesis gene cluster was introduced and overexpressed in a nemadectin-producing strain and the production was more than doubled.

Overexpression of *nem*R or nemadectin biosynthesis gene cluster increased the transcriptional level of nemadectin biosynthesis-related genes and thus improved nemadectin production. This revealed that the low fermentation yield of nemadectin may be due to the low transcriptional level of nemadectin biosynthesis-related genes. These findings helped us to elucidate nemadectin biosynthesis, and provide approaches to enhance nemadectin production by modifying new positive regulatory genes or further increasing the copy number of the nemadectin biosynthetic gene cluster in MOX-101/pCL-MOX.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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