Improvement of Nemadectin Production by Overexpressing the Regulatory Gene nemR 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 nemR and is involved in nemadectin biosynthesis in S. cyaneogriseus. In this report, gene disruption and complementation experiments showed that nemR plays a positive role in the biosynthesis of nemadectin. The transcription level of nemadectin biosynthetic genes in the nemR knockout strain was significantly decreased compared with those in the wild-type strain MOX-101. However, overexpression of nemR 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
biosynthetic gene cluster of nemadectin has been identified, and its complete sequence is also available, the study on genetic modification to nemadectin-producing strain remains largely unknown. In 2019, the function of the gene nemR was characterized, which is a positive regulatory gene encoding a LAL family transcriptional regulator within the nemadectin biosynthesis gene cluster of the strain NMWT1. The low fermentation yield of nemadectin resulted in the high production cost of moxidectin. In this study, the LAL family regulatory gene nemR, which serves as an activator for nemadectin biosynthesis, was confirmed and was employed to improve nemadectin production by overexpression of nemR 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 ΔnemR mutant with deletion of nemR is as follows. A 1.5-kb fragment upstream of the nemR start codon and a 1.5-kb fragment downstream of the nemR 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 nemR-deleted plasmid pΔnemR using the seamless ligation method (ClonExpress II One Step Cloning kit) (Fig. 2A). The plasmid pΔnemR 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 nemR was named ΔnemR.

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

The gene nemR was amplified from MOX-101 gDNA using the primers nemR-Q-F/R and cloned into the NdeI/AciI sites of pSET152 to generate the overexpression plasmid pnemR-ermEp*, in which nemR is driven by the strong constitutive promoter ermEp* (Fig. 2C). Next, pnemR bearing the native promoter and pnemR-ermEp* were transferred into MOX-101 to obtain overexpression strains MOX-101/pnemR and MOX-101/pnemR-ermEp*.
Construction of Nemadectin Biosynthetic Gene Cluster Overexpression Strain

Cloning nemadectin biosynthetic gene cluster was conducted following the CRISPR-TAR method reported previously. 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-M4 were constructed to capture MOX1, MOX2, and MOX gene clusters, respectively.

Table 1 Strains and plasmids used in this study

| Strains or plasmids | Relevant characteristic | Reference or source |
|---------------------|-------------------------|---------------------|
| **Strains**         |                         |                     |
| E. coli            |                         |                     |
| DH5 α              | F-, φ80lacZΔM15, Δ(lacZYA-argF) U169, deoR, recA1, endA1, hsdR17 (rk+, mk−), phoA, supE44, λ−, thi-1, gyrA96, relA1 | CWBIO               |
| S17-1              | supE44, ΔlacU169 (FlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, par phage lysogenic | Gibco-BRL           |
| EPI300             | F-, mcrA (mrr-hsdRMS-mcrBC) Δ80dclazΔ31ΔlacX74 recA1 endA1 araD139 Δ (ara, leu)7897 galU galK λ− rpsL (StrR) sup6 trp6 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      |
| ∆nemR/pnemR        | MOX-101 with deleted nemR | This study          |
| ∆nemR/pnemR        | ∆nemR mutant with pnemR | This study          |
| MOX-101/pSET152    | MOX-101 strain with pSET152 | This study         |
| MOX-101/pnemR      | MOX-101 strain with pnemR | This study          |
| MOX-101/pnemR ermEp* | MOX-101 strain with pnemR 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 supF | 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                   |
| pΔnemR             | pSET152 with the upstream and downstream DNA fragments of nemR | This study          |
| pnemR              | pSET152 with the expression of nemR under the control of native promoter | This study          |
| pnemR 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          |
Table 2 Primers used in this study

| Primers          | Sequences (5′→3′)               |
|------------------|----------------------------------|
| nemR-up-F        | AAAGATCCGGTGCACCTGCAGAAATCTTTGCGCTCACCTACGCGCATGACCAAGG |
| nemR-up-R        | GTTCAGACCGGCGGCTTTTCTGATTAATCTCTCTCTCGACTCCCTCCCG |
| nemR-down-F      | ACGCTTCAGGATGAGCTCAAGAGCCGAGG |
| nemR-down-F      | TCGGCCGCGCGCGCGCGATCTCTGAGCACGAGGATCATCCTGGTGG |
| pSET152-F        | GACTCTAGAGTACCGCGCC |
| pSET152-R        | AGGCTTGGGGTGCAGGAGGTCCC |
| nemR-N-F         | CAGCAGTCCGCCACGTTCTTCGG |
| nemR-N-R         | TAACTCACCCGCCGCGGATCCCGATCTCG |
| nemR-Q-F         | ATGGAAAGACGACAAAAACCTTTGCGCGCTCACAGCGCGTTTGGATGTGC |
| nemA1–2A2-F      | Tggcacaggaggagccgacgcgcgg |
| nemA1–2A2-R      | ACCGGAGCTGAGGAGGTCCC |
| nemA2CF          | gggtccttgagctgcagccgac |
| nemA2CR          | TGGTCAGGACCTTCTGGGTGG |
| nemA3E-F         | GGGTCGAGGACGAGGAGGTCCC |
| nemA3E-R         | GGGTCGAGGACGAGGAGGTCCC |
| nemA4A3-F        | CAGGTCGAGGACGAGGAGGTCCC |
| nemA4A3-R        | TGGTCGAGGACGAGGAGGTCCC |
| nemG-F           | ATGGAGACCTGCTTTGGCAAGACC |
| nemG-R           | CGGGCGGCGGGGGGTAC |
| nemF-F           | CAGGACGGCCCTTGGTACAGCG |
| nemF-R           | AGGACGACCCGTCCCAGAGCCG |
| nemA1–2-F        | GTGGGCTACCCGCCGATCTCG |
| nemA1–2-R        | AGGACGACCCGTCCCAGAGCCG |
| nemA3-F          | CAGGACGGCCCTTGGTACAGCG |
| nemA4-F          | TGGTCAGGACCTTCTGGGTGG |
| nemA4-R          | CAGGACGGCCCTTGGTACAGCG |
| gRNA-MOX1-up-F   | gactgcgaagttaATACGACTACTATAGagagagagccggtagctgcGTTTTAGAGCTAGAAATA |
| gRNA-MOX1-down-F | gactgcgaagttaATACGACTACTATAGagagagagccggtagctgcGTTTTAGAGCTAGAAATA |
| gRNA-MOX2-up-F   | gactgcgaagttaATACGACTACTATAGagagagagccggtagctgcGTTTTAGAGCTAGAAATA |
| gRNA-MOX2-down-F | gactgcgaagttaATACGACTACTATAGagagagagccggtagctgcGTTTTAGAGCTAGAAATA |
| gRNA-R           | CTTCCACCCAGGCAAGCCTGGG |
| MOX1-up-F        | aagtgaattgtaatagcatactatagggcatatttaatGAGGAGCTTACCCCGTGTGTC |
| MOX1-up-R        | CAGCTCCCGGAGACACAGAGG |
| MOX1-down-F      | CagcgcgcatcttgctttctgagctgcGTTTTAACGCTTGGTACGTGAGAG |
| MOX1-down-R      | gcacgtgataagaaaggacagccggtgcctgcacAGCTTCGCTTTGAGAGAAGGAG |
| MOX2-up-F        | aagtgaattgtaatagcatactatagggcatatttaatATCCTGGTACCCCGTGTGTC |
| MOX2-up-R        | CAGCTCCCGGAGACACAGAGG |
| MOX2-down-F      | CagcgcgcatcttgctttctgagctgcGTTTTAACGCTTGGTACGTGAGAG |
| MOX2-down-R      | gcacgtgataagaaaggacagccggtgcctgcacAGCTTCGCTTTGAGAGAAGGAG |

*Restriction sites are underlined.
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 EcoRI/Swal site of the plasmid pC01 by homologous recombination to generate the capture plasmids pC1-M1, pC1-M2, and pC1-M, respectively. Capture plasmids were digested with restriction enzyme Pmel 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 pC1-MOX1 and pC1-MOX2 were transferred into Escherichia coli EPI300 for enrichment. Positive plasmids pC1-MOX1 and pC1-MOX2 were collected by E.Z.N.A.BAC/PAC DNA (Omega Bio-Tek, China) and digested with restriction enzyme Swal to obtain gene clusters MOX1 and MOX2, respectively. The obtained gene clusters and the linearized capture plasmid pC1-M were introduced into the yeast to clone the biosynthetic gene cluster MOX. After confirming the transformant, the positive plasmid pC1-MOX was transferred into E. coli EPI300 for enrichment. Plasmids pC1-MOX1, pC1-MOX2, and pC1-MOX were digested with the restriction enzyme Kpnl to verify if the captured gene clusters were correct. The correct plasmid pC1-MOX was transferred from S17–1 into MOX-101 to generate the engineering strain MOX-101/pC1-MOX, in which the nemadectin biosynthesis gene cluster was overexpressed. As a control, the control plasmid pC101 was transferred into MOX-101 to generate the strain MOX-101/pC101.

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 KNO₃, 0.5 K₂HPO₄, 0.5 NaCl, 0.5 MgSO₄, 0.1 CaCO₃, 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 K₂HPO₄, 1.0 MgSO₄, and 4.0 CaCO₃, 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 MgSO₄, and 4.0 CaCO₃, 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, nemA3–3–F/R, and nemA4–4–F/R, were used in the qRT-PCR tests. Transcriptional levels of the tested genes were normalized using hrdB (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 nemR in nemadectin biosynthesis, we constructed the \(\Delta\)nemR mutant strain with deletion of the nemR gene using MOX-101 as the parental strain (\(\text{\(\Delta\)nemR}\)). 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 \(\Delta\)nemR with the deletion of the nemR gene. Nemadectin yield in \(\Delta\)nemR was decreased by approximately 80% in comparison with the parental strain MOX-101 (\(\text{\(\Delta\)nemR}\)). To further verify the function of NemR, we performed a complementation experiment in which the complemented plasmid pnemR was introduced into the \(\Delta\)nemR mutant (\(\text{\(\Delta\)nemR}\)). In pnemR, nemR was driven by the native promoter. The result showed that the complementation of nemR could restore the yield level of nemadectin to that of the parental strain (\(\text{\(\Delta\)nemR}\)), revealing that the decline in nemadectin yield was mainly due to nemR 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 (\(\text{\(\Delta\)nemR}\)). The result of the cotranscriptional experiment suggested that there were five cotranscriptional units altogether in the gene cluster of nemadectin biosynthesis, including nemG, nemF, nemA1–1–C, nemA3–D, and nemA4 (\(\text{\(\Delta\)nemR}\)).

The transcriptional assay result indicated that nemR deletion strongly affects the transcription of nemadectin biosynthetic genes. As shown in \(\text{\(\Delta\)nemR}\), compared with the parental strain MOX-101, transcriptional levels of all nemadectin biosynthetic genes in the knockout strain \(\Delta\)nemR were significantly decreased. Among these structural genes, transcriptional levels of nemG/F and nemA1–1/1–2/A1–2/A2/C/D/E/A3/A4 in \(\Delta\)nemR were only approximately 30% and less than 3% of those in MOX-101. These results suggested that nemR might play a positive regulatory role in nemadectin production by enhancing the expression of the nemadectin biosynthetic gene cluster.

\[\text{Fig. 3} \text{ Effect of nemR 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 \(\Delta\)nemR. (C) Nemadectin production in the mutant strains \(\Delta\)nemR, MOX-101/pSET152, and \(\Delta\)nemR/pnemR.}\]
Effect of Overexpression of nemR on Nemadectin Production

To enhance nemadectin yield, p_nemR under the control of the native promoter was transferred into MOX-101 to generate the overexpression strain MOX-101/p_nemR. As a control, the control plasmid pSET152 was transferred into MOX-101 to generate MOX-101/pSET152. Three strains, MOX-101, MOX-101/p_nemR, 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_nemR 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_nemR was due to an extra copy of the gene nemR.

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_nemR. The result indicated that the transcription levels of the nemadectin biosynthetic genes in MOX-101/p_nemR were higher than those in MOX-101 (→ Fig. 5B). Among these genes, the transcription of nemG/F and nemA1–1/A3/A4 improved above 30 and 100%, respectively.

To further increase the nemadectin yield, MOX-101/p_nemR ermEp^+ was obtained by transforming p_nemR ermEp^+ into MOX-101, in which nemR is driven by the strong constitutive promoter ermEp^+. As shown in → Fig. 5A, the production of MOX-101/p_nemR ermEp^+ was 10.9% higher than that of MOX-101/p_nemR 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_nemR 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. 4 Transcriptional analysis of the nemadectin biosynthetic genes upon nemR 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 ΔnemR.
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 KpnI 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. 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. In 2019, a positive transcriptional regulator, NemR, involved in nemadectin biosynthesis was reported. In this study, we overexpressed nemR 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 nemR 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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