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Original Research Article

Coupled strategy based on regulator manipulation and medium optimization empowers the biosynthetic overproduction of lincomycin

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ABSTRACT

The biosynthesis of bioactive secondary metabolites, specifically antibiotics, is of great scientific and economic importance. The control of antibiotic production typically involves different processes and molecular mechanism. Despite numerous efforts to improve antibiotic yields, joint engineering strategies for combining genetic manipulation with fermentation optimization remain finite. Lincomycin A (Lin-A), a lincosamide antibiotic, is industrially fermented by *Streptomyces lincolnensis*. Herein, the leucine-responsive regulatory protein (Lrp)-type regulator SLCG_4846 was confirmed to directly inhibit the lincomycin biosynthesis, whereas indirectly controlled the transcription of *SLCG_2919*, the first reported repressor in *S. lincolnensis*. Inactivation of *SLCG_4846* in the high-yield *S. lincolnensis* LA219X (LA219XΔ*4846*) increases the Lin-A production and deletion of *SLCG_2919* in LA219XΔ*4846* exhibits superimposed yield increment. Given the effect of the double deletion on cellular primary metabolism of *S. lincolnensis*, Plackett-Burman design, steepest ascent and response surface methodologies were utilized and employed to optimize the seed medium of this double mutant in shake flask, and Lin-A yield using optimal seed medium was significantly increased over the control. Above strategies were performed in a 15-L fermenter. The maximal yield of Lin-A in LA219XΔ*4846-2919* reached 6.56 g/L at 216 h, 55.1 % higher than that in LA219X at the parental cultivation (4.23 g/L). This study not only showcases the potential of this strategy to boost lincomycin production, but also could empower the development of high-performance actinomycetes for other antibiotics.

1. Introduction

Antibiotics, which are bioactive secondary metabolites produced by the fermentation of actinomycetes, are extensively used in pharmacology, agriculture, and other fields [1]. Meanwhile, the biosynthesis of antibiotics is complex and depends on various factors, including intracellular gene expression, regulation of extracellular medium components, and process control [2,3]. Over the past few decades, most actinomycetes used for the industrial-scale production of antibiotics have been obtained via random mutagenesis programs [4,5]. In recent years, the modification of factors affecting antibiotic production and the optimization of fermentation process have been widely used to improve the yield of antibiotics [3,5,6]. Response surface methodology can avoid the drawbacks of classical methods and is an empirical technique for modeling and optimizing fermentation processes [7]. Recombinant DNA technologies are also efficient tools for increasing antibiotic yields in actinomycetes [8]. In *Saccharopolyspora erythraea* ZL1004, the P450 hydroxylase gene *eryK* and O-methyltransferase gene *eryG* were co-overexpressed for titer improvement of erythromycin [9]. Subsequently, fermentation process of the genetically engineered *Sac. erythraea* was optimized to improve the titer [10,11]. Therefore, combining genetic manipulation with fermentation engineering can enhance the biosynthetic yield of mutant strains, which is necessary for the industrial overproduction of antibiotics.

The expression of gene clusters for antibiotic biosynthesis in actinomycetes typically occurs in the early stationary phase, followed by a

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transition phase involving complex metabolic alterations [12]. During this process, numerous transcriptional regulators control antibiotic biosynthesis by responding to extracellular and intracellular signals [13–15]. Changes in the quantity and activity of these regulators lead to variations in nutrient uptake and utilization [16,17]. Thus, the deliberate engineering of regulators in actinomycetes is an effective strategy to improve antibiotic production. Engineering multiple regulatory elements to comprehensively adjust gene expression could boost antibiotic titers [18–21]. However, the original fermentation conditions could not fully tap the potential of the engineering strains, resulting in the inability to further increase the antibiotic yield. Fermentation optimization may be a practical approach to solve this problem. To date, a joint engineering strategy for coupling regulator manipulation with fermentation optimization has not been reported.

Lincomycin is clinically used to treat bacterial infections in patients who cannot use penicillin, cephalosporins, or macrolide antibiotics. Lincomycin A (Lin-A), comprising an α-methylthiolincosamide and N-methylated 4-propyl-L-proline, is a major fermentation product of the actinomycete *Streptomyces lincolnensis* [22]. Owing to the global market of hundreds of tons per year, improving the yield of lincomycin is of significance [23]. Random mutagenesis and fermentation optimization have been frequently used to increase lincomycin production [24,25], and considerable efforts have been recently devoted to understand lincomycin biosynthesis and its regulation [22,26–29]. Notably, several transcription factors (TFs) in *S. lincolnensis* have been discovered and used to improve lincomycin [19,30–32]. However, understanding the regulatory landscape of lincomycin biosynthesis is limited. In particular, no studies have been published by coupling TF-based genetic manipulation with fermentation optimization for lincomycin titer improvement.

In this study, a leucine-responsive regulatory protein (Lrp)-type TF, SLCG_4846, was identified to directly repress lincomycin biosynthesis, whereas indirectly control the transcription of SLCG_2919, which was the first reported negative TF in *S. lincolnensis* [30]. The double deletion of *SLCG_4846* and *SLCG_2919* in a high-yield *S. lincolnensis* resulted in the significant increase in lincomycin yield. The Plackett-Burman, response surface designs and steepest ascent method were used to optimize the seed medium for this double mutant in shake flasks. The experiments were performed in a fermenter, which could significantly boost industrial lincomycin production.

2. Materials and methods

2.1. Strains, plasmids, and cultivation conditions

All bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* strains were cultured at 37 °C in Luria-Bertani (LB) medium. *E. coli* DH5α was used for molecular cloning, and *E. coli* BL21 (DE3) for protein expression. *S. lincolnensis* and its derivatives were cultured at 30 °C with shaking at 220 rpm in a liquid trypticase soy broth with yeast extract (TSBY) (3% TSB, 0.5% yeast extract, and 10.3% sucrose) for genomic DNA extraction. *S. lincolnensis* protoplast preparation used a liquid supplemental medium (0.4% TSB, 0.4% yeast extract, 1% glucose, 0.2 g/L KH_2PO_4, 0.05 g/L $MgSO_4$, and 0.4 g/L K_2HPO_4) [33].

2.2. Gene manipulation in S. lincolnensis

Using the genome of *S. lincolnensis* LCGL as a template, 1796 bp upstream and 1819 bp downstream were amplified with the primer pairs 4846-P1/4846-P2 and 4846-P3/4846-P4 (Table S2), respectively. The upstream and downstream fragments were cleaved by *Hind*III/*Xba*I and *Xba*I/*Eco*RI and ligated into the pKC1139 plasmid treated with *Hind*III/*Eco*RI, thereby obtaining pKC1139-Δ*4846*. The pKC1139-Δ*4846* plasmid was then transformed into the LCGL protoplast to obtain the desired Δ*SLCGL_4846* mutant, which was confirmed by the primer pair 4846-P5/4846-P6 (Table S2).

To obtain the complemented strain, the *SLCG_4846* fragment was amplified using the primers 4846-P7/4846-P8 (Table S2). The *SLCG_4846* fragment was treated with *Nde*I/*Xba*I and cloned into plasmid pIB139 digested with the same enzymes. The obtained pIB139-*4846* plasmid was transferred into the protoplast of Δ*SLCGL_4846* to obtain the complemented strain Δ*SLCGL_4846*/pIB139-*4846*, which was identified by primers Apr-P1/Apr-P2 (Table S2).

2.3. Fermentation and lincomycin determination

The flask fermentation of *S. lincolnensis* and determination of Lin-A production were performed as previously described [33]. For the 15-L bioreactor fermentation, *S. lincolnensis* LA219X and its derivatives were grown on MGM (2% soluble starch, 0.001% $FeSO_4$, 0.1% KNO_3, 0.5% soybean flour, 0.05% NaCl, 0.05% K_2HPO_4, 0.05% $MgSO_4$, and 2% agar) for sporulation. A 1-mL aliquot of spore suspension (approximately $1x10^7$ CFU/mL) was inoculated into a 250-mL flask containing 30 mL primary inoculum medium (2% soluble starch, 1% glucose, 1% soybean flour, 3% corn steep liquor, 0.15% $(NH_4)_2SO_4$, and 0.5% $CaCO_3$) for cultivating at 30 °C with shaking at 240 rpm for 2 d. The primary inoculum culture (10% v/v) was transferred to a 250-mL secondary medium (2% soluble starch, 2.8% glucose, 1% soybean flour, 1.5% corn steep liquor, 0.35% $NaNO_3$, 0.6% NaCl, 0.25% $(NH_4)_2SO_4$, 0.03% KH_2PO_4, and 1% $CaCO_3$) at 30 °C with shaking at 240 rpm for 2 d. All seed cultures were transferred to an 8-L fermentation medium (0.45% soluble starch, 1.75% glucose, 1.54% soybean flour, 1.54% corn steep liquor, 0.48% $NaNO_3$, 0.43% NH_4NO_3, 0.85% NaCl, 0.43% $(NH_4)_2SO_4$, 0.03% KH_2PO_4, and 0.77% $CaCO_3$) in a 15-L fermenter (Bailun, Shanghai, China) at 30 °C with shaking at 400 rpm for 9 d. Lin-A was extracted from the fermentation culture every 24 h and quantified using high-performance liquid chromatography (HPLC, Waters, USA) [19].

2.4. Determination of fermentation parameters

Fermentation broth (10 mL) was used as the sample, which was removed using centrifugation (4000×*g*, 10 min), and the packed mycelium volume (PMV) was considered the percentage of the precipitate volume in 10 mL of fermentation broth [24]. The Fehling method was used to measure reducing sugar concentrations [23], and the supernatants of the samples were analyzed for nitrogen levels via formaldehyde titration [10].

2.5. Electrophoretic mobility shift assays

Using LCGL genomic DNA as a template, the *SLCG_4846* gene was amplified with primers 4846-P7 and 4846-P8 (Table S2), which introduced *Nde*I/*Hind*III restriction sites. Amplified DNA fragments were cloned into pET28a to obtain pET28a-*4846*. This plasmid was then transformed into *E. coli* BL21(DE3) cells for the induction of protein expression using IPTG. A Ni^{2+}-NTA spin column (Bio-Rad) was employed to purify His_6-tagged SLCG_4846 and SLCG_2919 using a previously described method [15]. The promoter regions of the DNA fragments were amplified from the LCGL genomic DNA using the primer pairs listed in Table S2.

The electrophoretic mobility shift assays (EMSAs) were performed according to previously described methods [14]. DNA probes were mixed with purified SLCG_4846 and SLCG_2919 in binding buffer, then the mixtures were incubated at 30 °C for 20 min. Following incubation, the reactants were separated on 6% native polyacrylamide gel electrophoresis gels with 1 × Tris-acetate-EDTA buffer as the running buffer at 60 mA for 40–50 min. Competing assays were performed using a 50-fold excess of unlabeled P_{4846} or a 50-fold excess of nonspecific probe poly (dI-dC).

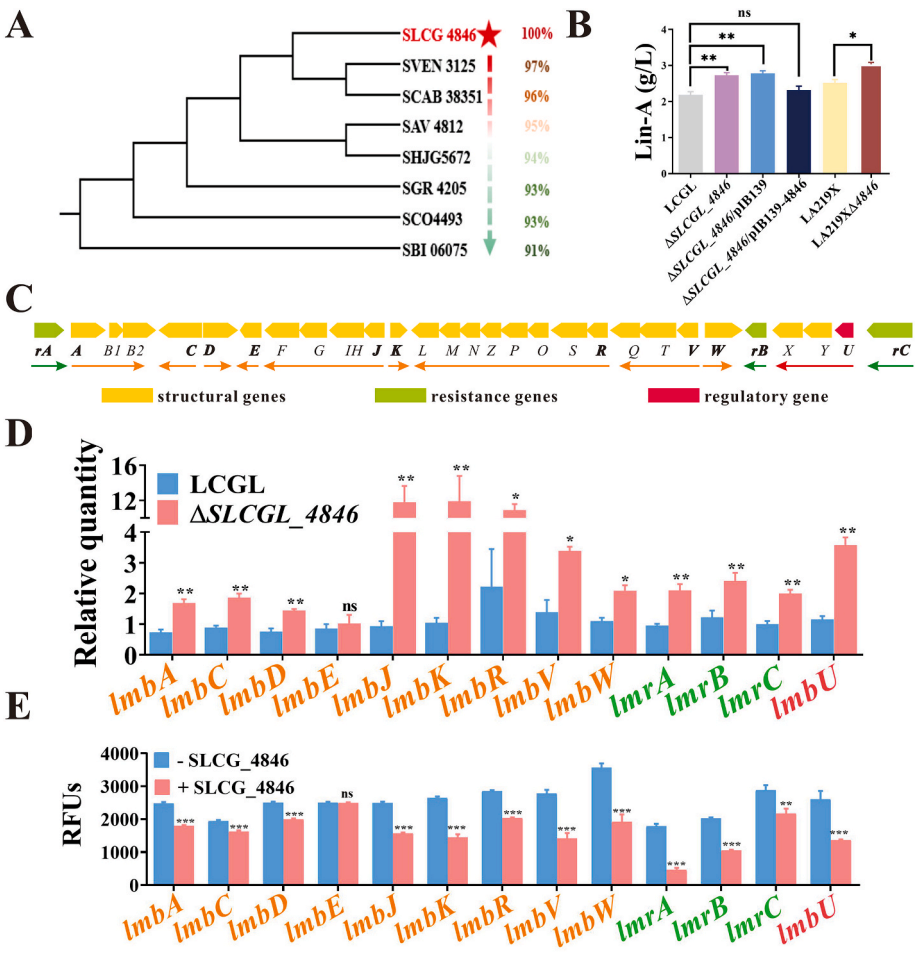

Fig. 1. Effects of *SLCG_4846* deletion on lincomycin biosynthesis in *S. lincolnensis*.
(A) Neighbor-joining distance tree constructed from the SLCG_4846 amino acid sequence from the genome sequences of antibiotic-producing actinomycetes using MEGA6. Percentages represent the identities between Lrp homologs and SLCG_4846. (B) Lin-A production of *S. lincolnensis* LCGL, LA219X, and their derivatives. (C) Genetic organization of *lin* clusters in *S. lincolnensis* LCGL. The arrows represent 13 transcription units, and the first gene of each unit is marked in bold font. (D) Real-time quantitative polymerase chain reaction (RT-qPCR) analyses of *lmrA, lmbA, lmbC, lmbD, lmbE, lmbJ, lmbK, lmbR, lmbV, lmbW, lmrB, lmbU*, and *lmrC* in LCGL and Δ*SLCGL_4846* cultured in the fermentation medium. (E) Detection of the regulatory relationship of SLCG_4846, and promoters of the *lmb* cluster using egfp reporter plasmids in *E. coli*. The mean values of three replicates are shown, with the standard deviation indicated by error bars. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

2.6. Quantitative real-time PCR analysis

Total RNAs in LCGL, LA219X and their derivative mutants cultured for 24 h were extracted using an RNA extraction/purification kit (Transgen). First, 1 μg RNA was inverted into cDNA using the HiScript II Q RT SuperMix for quantitative polymerase chain reaction (qPCR, +gDNA Wiper) (Vazyme). Real-time q-PCR (RT-qPCR) was performed on a QuantStudio TM6 Flex (Applied Biosystems) with AceQ Universal SYBR qPCR MasterMix (2X) (Vazyme). The reaction protocol consisted of 95 °C for 10 min, followed by 40 cycles of 95 °C for 19 s and 60 °C for 35 s. Each experiment was performed in triplicate. The *rpoD* gene in *S. lincolnensis* was used as an internal control, and relative transcription was quantified using a comparative cycle threshold method. The primers used for RT-qPCR are listed in Table S2.

2.7. Green fluorescent protein reporter assay

The reporter gene encoding the enhanced green fluorescent protein (eGFP) was amplified with PCR using the primer pair *egfp* F/R from pUPW-EGFP and digested with *Xba*I/*BamH*I. In addition, the promoter regions of the lincomycin biosynthetic cluster (*lin* cluster) were amplified with the primer pairs U F/R, rA F/R, rB F/R, rC F/R, A F/R, E F/R, R F/R, C F/R, D F/R, J F/R, K F/R, V F/R, W F/R, and 2919 F/R (Table S2) and digested with *Hind*III/*Xba*I. The two DNA fragments (promoter and gene encoding EGFP) were inserted into the *BamH*I/*Hind*III digested sites of pKC1139, creating plasmids pKC-UE, pKC-rAE, pKC-rBE, pKC-rCE, pKC-AE, pKC-EE, pKC-RE, pKC-CE, pKC-DE, pKC-JE, pKC-KE, pKC-VE, pKC-WE, and pKC-2919E. *SLCG_4846* and $P_{aac(3)IV}$ were inserted into the abovementioned plasmids to generate pKC-4846-UE, pKC-4846-rAE, pKC-4846-rBE, pKC-4846-rCE, pKC-4846-AE, pKC-4846-EE, pKC-4846-RE, pKC-4846-CE, pKC-4846-DE, pKC-4846-JE, pKC-4846-KE, pKC-4846-VE, pKC-4846-WE and pKC-4846-2919E, respectively. These plasmids were then successively transformed into *E. coli* DH5α, and the fluorescence values were detected (excitation at 485 nm; emission at 510 nm, Molecular Devices) to estimate the interactions of SLCG_4846 and its targets.

2.8. RNA sequencing

Total RNA was isolated using TransZol Up (Transgen, China) from cultures of *S. lincolnensis* LA219X and LA219XΔ*4846-2919* grown in a fermentation medium after 24 h. RNA quality and quantity were examined using a microplate reader (BioTek, USA) and confirmed using electrophoresis. RNA samples of LA219X and LA219XΔ*4846-2919* were used for RNA sequencing, and library construction and sequencing were performed using Illumina novaseq 6000. The data obtained from the

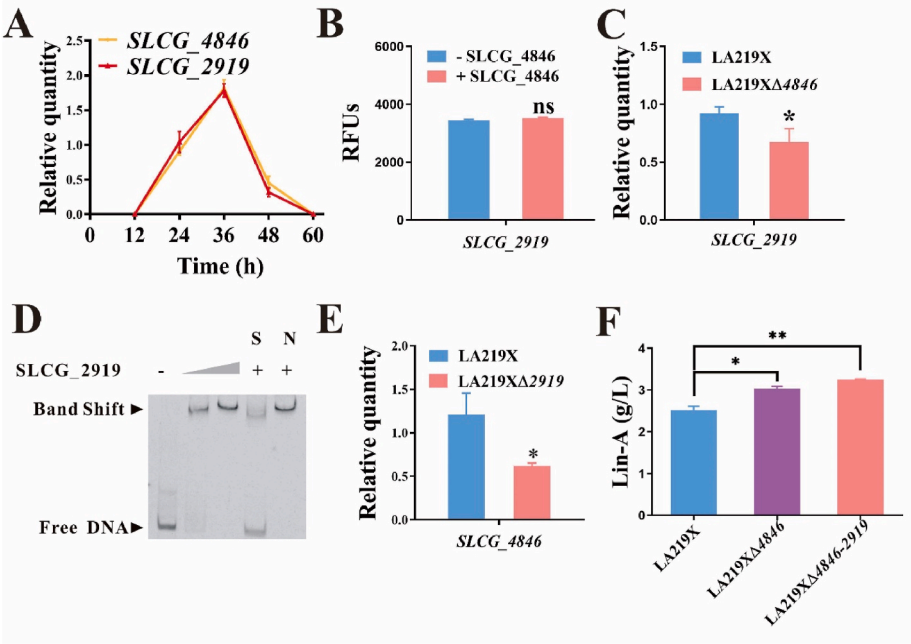

Fig. 2. Regulatory relationship between *SLCG_2919* and *SLCG_4846*. (A) Temporal profiles of *SLCG_4846 and SLCG_2919* transcripts in LA219X. (B) Detection of the regulatory relationship of SLCG_4846 and P_{2919} using *egfp* reporter plasmids in *E. coli*. (C) RT-qPCR analysis of *SLCG_2919* in LA219X and LA219XΔ4846. (D) Electrophoretic mobility shift assays (EMSAs) of His_6-tagged SLCG_2919 with P_{4846}. Each lane contains 150 ng DNA probes. S: unlabeled specific probe (50-fold); N: nonspecific probe poly dIdC (50-fold). (E) Relative transcriptional level of *SLCG_4846* in LA219X and LA219XΔ*2919*. (F) Lin-A production of LA219X, LA219XΔ*4846*, and LA219XΔ*4846-2919*. The mean values of three replicates are shown with standard deviations (SDs). Error bars indicate SD for the three biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$.

sequencing of the Illumina novaseq 6000 were called raw reads or raw data, and the raw reads were subjected to quality control to determine if a resequencing step was required. After filtering the raw reads, clean reads were aligned with the reference sequence. The original sequence data have been submitted to the NCBI Sequence Read Archive PRJNA1015539 database under accession number SUB13834218. For gene expression analysis, matched reads were calculated and normalized to probability using the NOISeq package. The significance of the differential expression of genes was defined using the bioinformatics service according to the combination of the $|\log_2(\text{FoldChange})$. FC $| \geq 1$ and *p*-value ≤ 0.05. Finally, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using $Blast_2GO$.

2.9. Plackett-Burman design

The Plackett-Burman design (PBD) was used to select significant parameters for the most important medium components for Lin-A production. This design was applied to a combination of seed medium components (i.e., corn starch, soybean powder, corn steep liquor, $(NH4)_2SO_4$, glucose, and $CaCO_3$) and fermentation parameters (i.e., seeding age, rotation speed, and inoculum amount). A 12-run PBD was used to evaluate the factors of each combination. Each variable was examined at two levels: −1 for the low level and +1 for the high level.

2.10. Statistical experimental designs

Based on the results of the PBD, the most influential parameters on Lin-A biosynthesis were selected for further optimization using the response surface method. The experiments were performed with two parameters and two levels for each parameter, and two blocks were used to assess potential heterogeneity during the experiment. Soybean powder and corn steep liquor were selected as independent variables.

2.11. Statistical analysis

All acquired data are shown as mean ± SD and analyzed using the Student's *t*-test or ANOVA. Differences were considered statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Direct repression of lincomycin biosynthesis by the new Lrp SLCG_4846

Because Lrp is widely present in prokaryotes and involved in antibiotic synthesis in actinomycetes [15,19,34], several Lrps associated with lincomycin yield in *S. lincolnensis*, including SLCG_Lrp [19], and the novel SLCG_4846, have been identified. SLCG_4846 exhibits high similarity to homologous proteins from *Streptomyces*, such as SCO4493 from *S. coelicolor* A3(2) (93% identity), SAV_4812 from *S. avermitillis* MA-4680 (95% identity), and SGR_4205 from *S. griseus* IFO13350 (93% identity), inferring that it has a conserved function (Fig. 1A). Using double-crossover recombination, the Δ*SLCGL_4846* mutant was first constructed in *S. lincolnensis* LCGL (Fig. S1). Culture extracts of LCGL and Δ*SLCGL_4846* were analyzed using HPLC. The results showed that the Lin-A yield of Δ*SLCGL_4846* (2.73 g/L) was higher than that of LCGL (2.18 g/L), and complementation of *SLCG_4846* in Δ*SLCGL_4846* restored the yield to its initial level (Fig. 1B). Furthermore, *SLCG_4846* was deleted in the high-yield *S. lincolnensis* LA219X, and the Lin-A yield of LA219XΔ*4846* was 20.4% higher than that of LA219X (Fig. 1B). These results demonstrate that SLCG_4846 negatively controls lincomycin production in *S. lincolnensis*.

The *lin* cluster contained 13 putative operons, the first of which were *lmbU*, *lmrA*, *lmrB*, *lmrC*, *lmbA*, *lmbC*, *lmbD*, *lmbJ*, *lmbK*, *lmbV,* and *lmbW* (Fig. 1C). To investigate the regulatory mechanism of SLCG_4846, RT-qPCR was used to compare the transcription of these genes between LCGL and Δ*SLCG_4846*. Results showed that, except *lmbE* in Δ*SLCG_4846,* the genes in Δ*SLCG_4846* were differentially increased

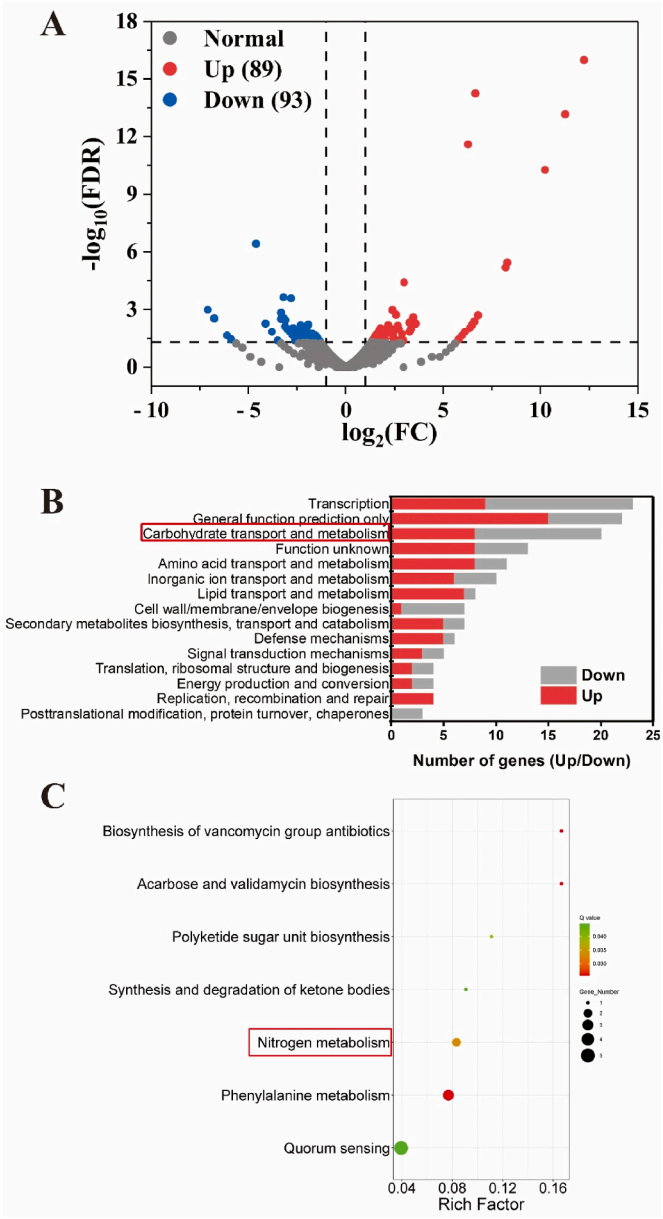

Fig. 3. Transcriptome analysis of LA219XΔ*4846-2919*. (A) Analysis of the differentially expressed genes (DEGs). Fold change shows the ratio of expression levels (LA219XΔ*4846-2919*/LA219X), $\log_2$ (fold change) represents the value of differential folds, and $\log_{10}$ (significance) shows the levels of significance. A total of 182 DEGs were identified. (B) Gene Ontology (GO) enrichment analysis, and the functional categorization of upregulated and downregulated genes. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment. The y-axis indicates the pathway name, and the x-axis indicates the enriched factor for each pathway. Rich ratio = term candidate gene num/term gene num. The bubble size indicates the number of genes. The color bar indicates the corrected Q value.

compared with those in LCGL (Fig. 1D), indicating that SLCG_4846 negatively controlled lincomycin biosynthesis by transcriptionally repressing the *lin* cluster. The His_6-tagged SLCG_4846 in *E. coli* BL21 (DE3) was expressed (Fig. S2) and its affinity to the promoters of the *lin* cluster was examined using EMSAs. However, this experiment failed because of the low activity of SLCG_4846, despite considerable efforts. Therefore, biosensor systems were constructed to examine these regulatory relationships (Fig. S3). Taking *lmbU* as an example, plasmid pKC-UE containing *egfp* under P_{4846} was successively constructed and transformed into *E. coli* DH5α, and the obtained strain showed excellent fluorescence signals. When the segment $P_{aac(3)IV}$-SLCG_4846 was ligated into pKC-UE and transformed into DH5α, the fluorescence signal was sharply downgraded (Fig. 1E). A similar phenomenon was observed for the other genes of the *lin* cluster, expect *lmbE* (Fig. 1E). These data demonstrated that SLCG_4846 directly repressed the expression of most genes within the *lin* cluster.

3.2. Enhanced lincomycin yield by double deletion of SLCG_2919 and SLCG_4846

The TetR-type regulator SLCG_2919, the first reported repressor, negatively regulates lincomycin biosynthesis by inhibiting expression of the *lin* cluster [30]. Therefore, we first investigated the regulatory relationship between SLCG_4846 and SLCG_2919. The transcripts of *SLCG_4846* and *SLCG_2919* appeared in the early stage, peaked at 36 h, and subsequently decreased to extremely low levels (Fig. 2A). The eGFP reporter assay showed that the addition of SLCG_4846 did not affect bioluminescence (Fig. 2B), and the transcriptional level of *SLCGL_2919* in LA219XΔ*4846* grown for 24 h decreased compared with that in LA219X (Fig. 2C). An EMSA showed that SLCG_2919 bound specifically to the promoter region of *SLCG_4846* (Fig. 2D), and RT-qPCR results indicated that the transcriptional level of *SLCG_4846* in LA219XΔ*2919* was 48.9% lower than that in LA219X (Fig. 2E). These data indicate that SLCG_4846 and SLCG_2919, directly or indirectly, reciprocally promoted the other's expression at the transcriptional level.

Furthermore, *SLCG_2919* was inactivated in LA219XΔ*4846* (Fig. S4), generating the double mutant LA219XΔ*4846-2919*. Fermentation analysis showed that the double deletion of *SLCG_4846* and *SLCG_2919* resulted in an increase in the Lin-A yield by 29.1% (Fig. 2F). Therefore, a high lincomycin-producing strain was obtained by manipulating key transcriptional regulators, suggesting the potential for improving industrial lincomycin manufacturing.

3.3. Transcriptome analysis of this double mutant LA219XΔ4846-2919

To explore the effects of the double deletion of *SLCG_4846* and *SLCG_2919* on cellular metabolism, RNA sequencing was employed to perform a comparative transcriptome analysis between LA219X and LA219XΔ*4846-2919*. With the standards of a $|\log_2(FC)| \geq 1$ and *p*-value ≤ 0.05, 182 differentially expressed genes (DEGs) were identified (Fig. 3A). Among these DEGs, expression levels of 93 were downregulated and those of 89 were upregulated (Table S3), and the transcripts of some lincomycin biosynthetic genes were significantly upregulated in LA219XΔ*4846-2919* in comparison to those in LA219X. Twenty DEGs were associated with carbohydrate transport and metabolism, inferring that the lack of *SLCG_2919* and *SLCG_4846* affected carbon uptake and assimilation (Fig. 3B). Several nitrogen metabolic genes were also significantly regulated (Fig. 3C). These data suggested that the inactivation of *SLCG_4846* and *SLCG_2919* altered cellular primary metabolism, including carbon and nitrogen metabolism, to affect the biosynthesis of lincomycin.

3.4. Time-course fermentation profile of LA219XΔ4846-2919 in shake flask and fermenter

To deep examine the metabolic performance of the double mutant, the time-course fermentation of LA219X and LA219XΔ*4846-2919* was first exerted in the shake-flask culture. The cell growth of LA219XΔ*4846-2919* increased at the late stage of fermentation compared with the control, which was similar to the daily Lin-A yield (Fig. 4A, B). The maximum Lin-A yield of LA219XΔ*4846-2919* (3.135 g/L at 7 d) increased by 22.3% with respect to that of LA219X (2.563 g/L at 7 d). By monitoring the consumption of carbon and nitrogen throughout the fermentation, we found that LA219XΔ*4846-2919* had no significant influence on consumption of reducing sugar, but displayed higher cost of amino nitrogen than LA219X (Fig. 4C and D).

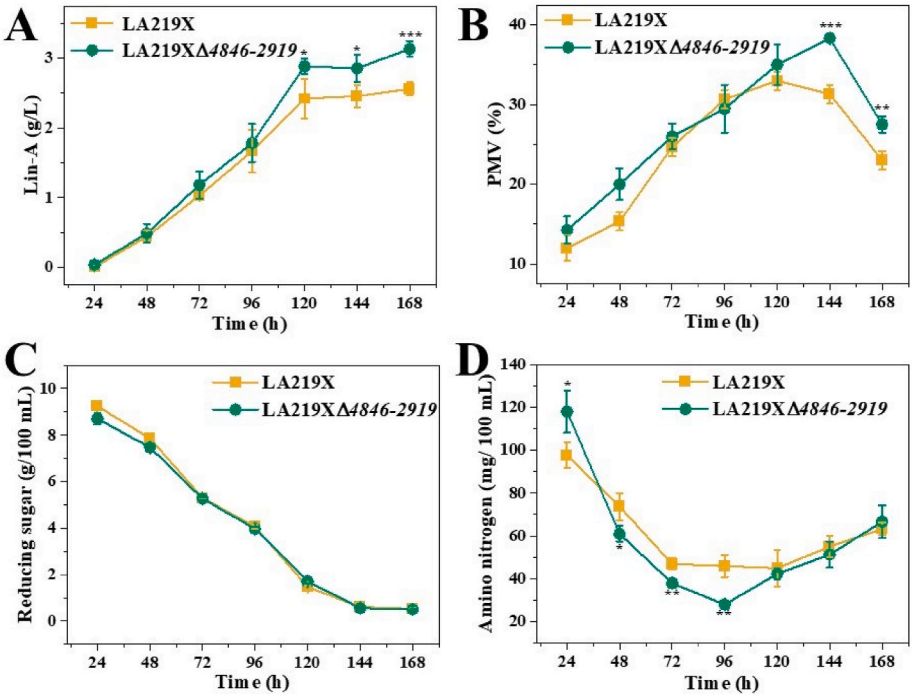

Fig. 4. Time-course profile of *S. lincolnensis* LA219X and LA219XΔ*4846-2919* grown during shake-flask fermentation. (A) Production of Lin-A. (B) Packed mycelium volume (PMV). (C) Reducing sugar concentration. (D) Amino nitrogen concentration. The mean values of three replicates are shown with the standard deviation indicated by error bars. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

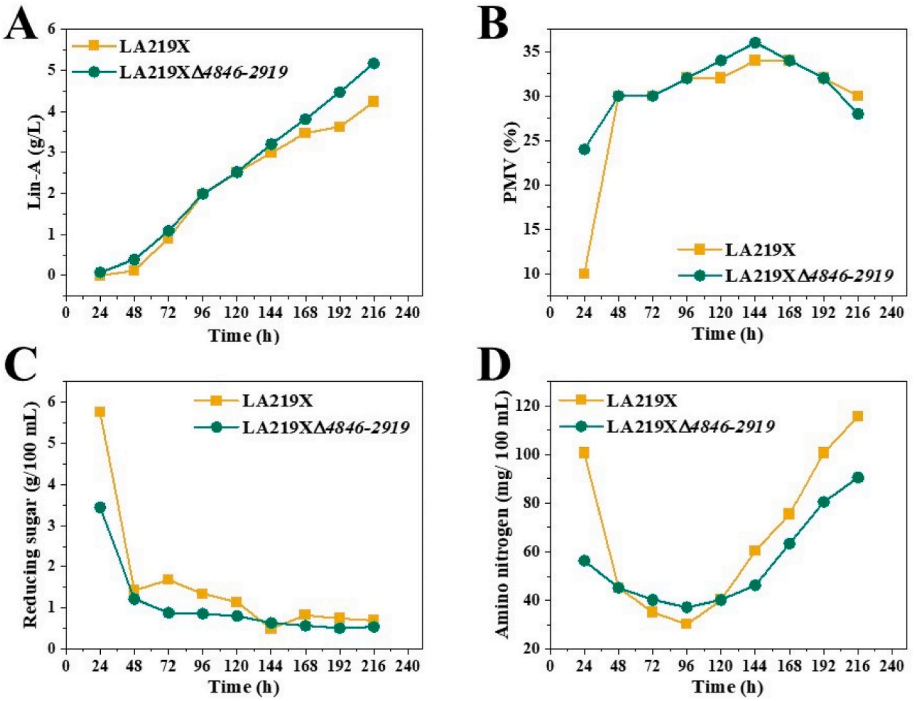

Fig. 5. Time-course profile of *S. lincolnensis* LA219X and LA219XΔ*4846-2919* grown during 15-L fermentation. (A) Production of Lin-A. (B) PMV. (C) Reducing sugar concentration. (D) Amino nitrogen concentration.

Furthermore, the time-course culture of this mutant was performed in a 15-L fermenter, and LA219XΔ*4846-2919* exhibited a 22.2% higher Lin-A yield, with increased production from 4.23 to 5.17 g/L at 216 h (Fig. 5A). This variation in daily biomass coincided with the shake-flask fermentation results (Fig. 5B). Notably, LA219XΔ*4846-2919* accelerated the consumption of nutrient sources, including amino nitrogen and reducing sugar (Fig. 5C and D). Therefore, LA219XΔ*4846-2919* maintained the fermentation characteristics of the high-performance strain.

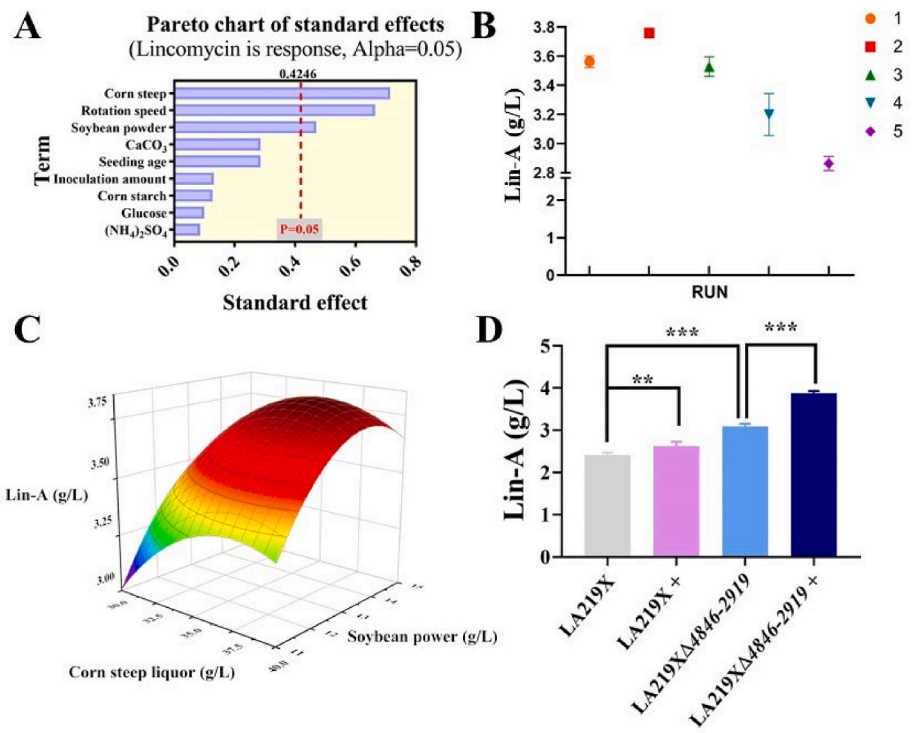

Fig. 6. Effects of the optimized seed medium on lincomycin yield. (A) Pareto chart of nine-factor standard effects on Lin-A production. (B) Lin-A yield of the steepest climbing experiment. (C) Response surface plot of the interaction of corn steep liquor and soybean powder on the yield of Lin-A. (D) Lin-A yield of the two strains during shake-flask fermentation with optimized medium (+). The mean values of three replicates are shown with the standard deviation indicated by error bars. *$p <$ 0.05, **$p < 0.01$.

3.5. Optimized seed medium for LA219XΔ4846-2919 to improve lincomycin yield

To achieve maximal level of lincomycin yield, it is of great importance to optimize the medium. Given that composition of the seed medium both in shake flask and fermenter is similar, we chose to, in the present study, optimize the seed medium of LA219XΔ*4846-2919* through response surface methodology. Nine factors, including corn

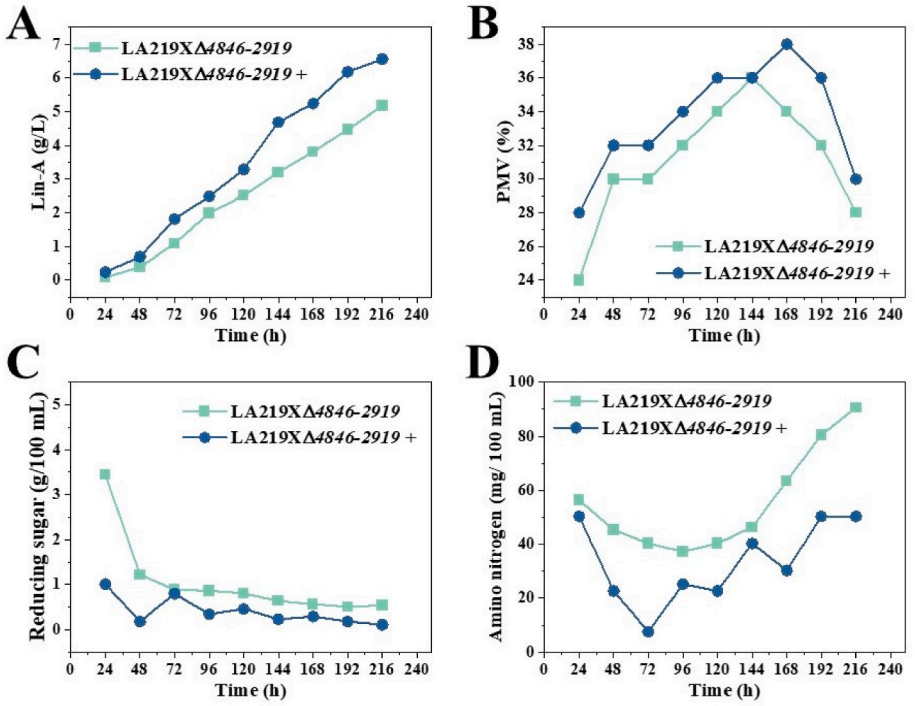

Fig. 7. Time curve fermentation of LA219XΔ*4846-2919* with optimized and non-optimized seed medium in a 15-L bioreactor. (A) Production of Lin-A. (B) PMV. (C) Reducing sugar concentration. (D) Amino nitrogen concentration. +: optimized medium.

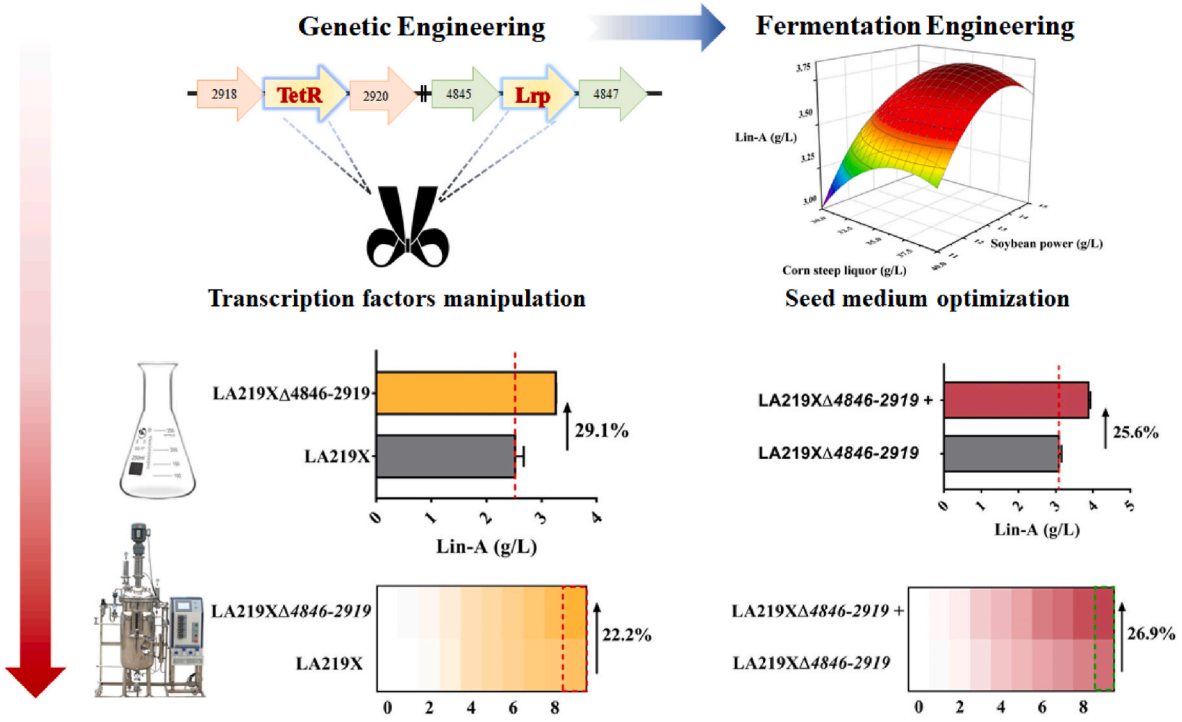

Fig. 8. Schematic diagram to improve lincomycin yield.

starch, glucose, soybean powder, corn steep liquor, $(NH_4)_2SO_4$, $CaCO_3$, rotation speed, inoculation amount, and seeding age, were first investigated using PBD. The effects of these factors on the response and their significance are listed in Table S4. The highest yield (3.51 g/L) of LA219XΔ*4846-2919* was achieved from run No. 5 (Table S5). Pareto chart and analysis of variance (ANOVA) of PBD directly showed that the important factors determining lincomycin yield were rotation speed, corn steep liquor, and soybean powder (Fig. 6A, Table S6).

Next, the steepest climbing experiment was used to explore the higher production of lincomycin, and the center point of the PBD was considered as the origin of the path. Results showed that optimal parameters were 12.2 g/L soybean powder and 35 g/L corn steep liquor (Fig. 6B–Table S7), which were near the region of maximum response and chosen for further optimization. Then, the statistical design of central composite model (CCD) was employed to adjust the soybean powder and corn steep liquor concentrations (Tables S8 and S9). Based on the CCD results, multivariate regression analysis was conducted using Design-Expert 10.0. The resulting regression equation was obtained using the equation:

$$Y = -14.21 + 0.5669D + 1.152C - 0.007135D \times D - 0.03791C \times C - 0.00364D \times C$$

where Y is the predicted value (Lin-A), and C and D are the concentrations of soybean powder and corn steep liquor, respectively. The response surface plots showed that the optimal variables for achieving the maximum Lin-A yield were 36.35 g/L corn steep liquor and 13.44 g/L soybean powder (Fig. 6C).

Finally, a validation experiment was performed to confirm above-mentioned predicted results. Resulted showed that the Lin-A yield of LA219XΔ*4846-2919* in the optimized condition was 3.875 g/L, which was approximately 25.6% higher than that in non-optimized condition (Fig. 6D), suggesting that experimental and predicted values (3.825 g/L) were in good agreement. This result confirmed the predicted values and effectiveness of the model, indicating that the optimized seed medium favored the biosynthesis of lincomycin.

3.6. Increased yield of LA219XΔ4846-2919 with optimized seed medium in fermenter

The optimized medium was further tested in a 15-L batch bioreactor, and the results showed that PMV and Lin-A production increased compared to that in the initial medium (Fig. 7A and B). The maximum Lin-A yield attained 6.56 g/L at 216 h, which was 26.9 % higher than the control (5.17 g/L). Moreover, the reducing sugar and amino nitrogen concentrations at optimal seed cultivation were lower than those of the control, indicating that more carbon and nitrogen were utilized in the fermentation stage with inoculated optimal seed cultivation compared to the control (Fig. 7C and D). This data indicated that these variations in nutrients during the fermentation affected the primary metabolism of *S. lincolnensis* for contributing to the biosynthesis of lincomycin.

4. Discussion

Biosynthesis is a sophisticated process that utilizes microorganisms to obtain an abundance of commercial products. The application of strains to enhance the titer during industrial production is critical for such efforts [35]. Strain modification and fermentation optimization are common strategies for improving the biosynthesis of secondary metabolites [36]. In antibiotic-producing actinomyces, it is difficult to further scale up the production of antibiotics owing to the monolithic strategy. In this report, we developed a strategy to jointly integrate genetic manipulation and fermentation optimization in *S. lincolnensis*, leading to a marked improvement in lincomycin production.

Lrp, widely present in prokaryotes, participates in the biosynthesis of antibiotics [15,19,34]. We previously confirmed that SLCG_Lrp directly stimulates the biosynthesis of lincomycin [19]. Meanwhile, SLCG_Lrp was found to be directly repressed by the TetR-type regulator SLCG_2919 [19]. However, little is known about the remaining Lrps in *S. lincolnensis*. Herein, a novel Lrp protein from *S. lincolnensis*, SLCG_4846, was identified and proven to directly repress the expression of the *lin* cluster except *lmbE*, further regulate the lincomycin biosynthesis. In addition, SLCG_4846 and SLCG_2919 regulated each other at the transcriptional level, in which SLCG_4846 indirectly promoted the

expression of *SLCG_2919* and SLCG_2919 directly controlled the expression of *SLCG_4846*. Consequently, these findings exhibit a sophisticated regulatory network for lincomycin biosynthesis. In addition, the relationship between Lrp and other regulators need to be further exploited.

The multiplex and rational engineering of transcriptional regulators in actinomycetes is an ideal strategy to upgrade the titer of antibiotics [18,37,38]. In our previous study, deletion of TetR-type regulatory genes *SACE_3986* and *SACE_3446* in *Sac. erythraea* increased the erythromycin yield [21]. Deletion of negative regulatory genes *cebR* and *txtR* in *S. albidoflavus* J1074 results in an improvement in thaxtomins yield [39]. Herein, double deletion of *SLCG_4846* and *SLCG_2919* resulted in a significant increase in the Lin-A yield. Therefore, this work reveals the potential for broader industrial applications in improving other secondary metabolites.

Microbial fermentation is the basis to boost the yields of a range of secondary metabolites with economic importance [7,40,41]. During the fermentation process, seed conditions, including the optimum age and physiological state, are regarded as the key to the biosynthetic overproduction of antibiotics [10]. Nevertheless, the seed at the optimum age and physiological state is often ignored, which could induce failure at the fermentation stage. In this work, we utilized a Plackett-Burman design, the steepest ascent method, and response surface design to optimize the seed medium of LA219XΔ*4846-2919*. The maximum yield of 3.875 g/L of Lin-A was achieved with LA219XΔ*4846-2919* in flask, which was approximately 25.6% higher than that when using the engineering strain with un-optimized seed medium. When cultivated in a 15-L bioreactor, Lin-A yield of LA219XΔ*4846-2919* reached 6.56 g/L. In summary, our results have provided a viable approach for improving the titers of most antibiotics in actinomycetes.

5. Conclusions

In this study, a strategy that combined genetic and fermentation engineering was used to increase lincomycin production (Fig. 8). Genetic engineering focused on knocking out two repressors, SLCG_4846, which is involved in lincomycin biosynthesis by controlling the *lin* cluster, and SLCG_2919, the first reported negative regulator in *S. lincolnensis*, to increase lincomycin yield. Furthermore, fermentation engineering was investigated to optimize the seed medium using a rational response surface method, which resulted in a further increase in lincomycin yield, whether in shake flasks or fermenters. Therefore, this strategy will be beneficial in boosting the development of high-performance actinomycetes for other antibiotics.

CRediT authorship contribution statement

Hang Wu and Buchang Zhang conceived and designed the study. Xinlu Cai, Wanlian Xu, Yang Zheng, Sendi Wu, Rundong Zhao, Nian Wang, Yaqian Tang and Meilan Ke performed the experiments. Xinlu Cai and Hang Wu analyzed the data and wrote the manuscript. Hang Wu and Buchang Zhang modified the manuscript. Qianjin Kang and Linquan Bai gave feedbacks on the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.01.004.

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