New Targets of TetR-type regulator SLCG_2919 for Controlling Lincomycin Biosynthesis in Streptomyces lincolnensis

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Abstract

The transcription factor (TF)-mediated regulatory network controlling lincomycin production in *Streptomyces lincolnensis* is yet to be fully elucidated despite several types of associated TFs having been reported. SLCG_2919, a tetracycline repressor (TetR)-type regulator, was the first TF to be characterized outside the lincomycin biosynthetic cluster to directly suppress the lincomycin biosynthesis in *S. lincolnensis*. In this study, improved genomic systematic evolution of ligands by exponential enrichment (gSELEX), an *in-vitro* technique, was adopted to capture additional SLCG_2919-targeted sequences harbouring the promoter regions of *SLCG_6675*, *SLCG_4123-4124*, *SLCG_6579,* and *SLCG_0139-0140.* These results were confirmed by electrophoretic mobility shift assays (EMSAs). RT-qPCR showed that the corresponding target genes *SLCG_6675* (anthranilate synthase), *SLCG_0139* (LysR family transcriptional regulator), *SLCG_0140* (beta-lactamase), *SLCG_6579* (cytochrome P450), *SLCG_4123* (bifunctional DNA primase/polymerase), and *SLCG_4124* (magnesium or magnesium-dependent protein phosphatase) in Δ*SLCGL_2919* were differentially increased by 3.3-, 4.2-, 3.2-, 2.5-, 4.6-, and 2.2-fold relative to those in the parental strain *S. lincolnensis* LCGL. Furthermore, the individual inactivation of these target genes in LCGL reduced the lincomycin yield to varying degrees. This investigation expands on the known DNA targets of SLCG_2919 to control lincomycin production, and lays the foundation for improving industrial lincomycin yields via genetic engineering of this regulatory network.

Introduction

Actinomycetes are a group of unicellular filamentous bacteria, which are an important source of antibiotic drugs and encode a wide variety of transcription factors (TFs) that finely control the biosynthesis of secondary metabolites (Liu et al. 2013). Typically, these TFs include global or pleiotropic regulators and cluster-situated regulators (CSRs) which exert diverse regulatory functions (van der Heul et al. 2018). Although 70% of the known natural antibiotics, such as lincomycin, spinosad, and avermectins are produced by actinomycetes and have important application values in the fields of medicine and agriculture (Palazzotto et al. 2019), these secondary metabolites still exhibit low industrial fermentation yields. Rewiring the regulatory network of TFs in actinomycetes has manifested great potential for industrial overproduction of antibiotics (Liu et al. 2021). Therefore, the definition of these complicated regulatory mechanisms will help overcome the bottleneck of low outputs of secondary metabolites.

Lincomycin, a lincosamide antibiotic produced by *S. lincolnensis*, is generally effective against gram-positive bacteria (Spížek and Řezanka 2017). It is widely used in clinics because of its favourable side effect profile and strong ability to penetrate tissues and cells (Spížek and Řezanka 2017). The lincomycin biosynthetic (*lmb*) gene cluster, spanning over 35 kb of DNA, contains twenty-five structural genes, three resistance genes, and one CSR gene *lmbU* (Koberská et al. 2008). In recent years, great progress has been made in research on the lincomycin biosynthetic pathway (Wang et al. 2020b), while investigation of the regulatory mechanism of lincomycin biosynthesis has been relatively slow (Koberska et al. 2021; Lin et al. 2020). In addition to the CSR LmbU (Lin et al. 2020), several regulatory TFs outside the *lmb* cluster,

such as BldD, AdpA, and SLCG_Lrp have been confirmed as having central roles in the control of lincomycin production. BldD is a positive regulator with direct control over lincomycin biosynthesis in *S. lincolnensis* (Li et al. 2019). AdpA has been implicated as being central to a cascade regulation of lincomycin biosynthesis by $AdpA_{lin}$, LmbU, and BldA (Kang et al. 2019). We found that an Lrp family regulator SLCG_Lrp in *S. lincolnensis* could promote lincomycin biosynthesis by directly activating *lmb* genes (Xu et al. 2020). A comparative genomics approach revealed that large fragment deletion and gene mutation, especially that of regulatory genes, are crucial for lincomycin overproduction (Wang et al. 2020a). These studies not only highlight the complexity of lincomycin biosynthetic regulation but also seem to provide a viable way to upgrade the industrial yield of lincomycin by engineering the regulatory system of *S. lincolnensis*.

The tetracycline repressor (TetR) family transcriptional regulator (TFR) plays an important role in the secondary metabolism of actinomycetes (Cuthbertson and Nodwell 2013). Various TFRs inside or outside antibiotic biosynthetic clusters in actinomycetes regulate the biosynthesis of secondary metabolites by binding to the promoter regions of their target genes (Wu et al. 2019; Wu et al. 2021; Xu et al. 2019). We first identified the TFR SLCG_2919 in *S. lincolnensis* and demonstrated that it could modulate lincomycin biosynthesis by repressing the majority of the genes within *lmb* cluster (Xu et al. 2019). However, the SLCG_2919-mediated regulatory network's role during during lincomycin biosynthesis remains to be elucidated.

Genomic systematic evolution of ligands by exponential enrichment (gSELEX) is a method derived from SELEX in which DNA fragments from a genomic library interact with a protein are screened out through successive rounds of binding, partitioning, and amplification (Darmostuk et al. 2015). We previously improved the gSELEX method and used it to capture DNA targets of BldD in *Saccharopolyspora erythraea* (Wu et al. 2015). In this study, using this method, we identified four additional SLCG_2919-targeted sequences. Furthermore, the aforementioned target genes were individually inactivated in LCGL and were confirmed to be associated with lincomycin production. This project lays the foundation for understanding the regulatory network of lincomycin biosynthesis in *S. lincolnensis*.

Materials And Methods

Strains, plasmids, and cultivation conditions

All strains and plasmids are listed in Table 1. *Escherichia coli* strains were cultured at 37°C in Luria Bertani (LB) medium and supplemented with antibiotics as required (Sambrook and Russell 2012). *S. lincolnensis* strains were cultured at 30°C with shaking at 220 rpm in liquid TSBY medium for genomic DNA extraction (Xu et al. 2018). *S. lincolnensis* strains were grown on MGM medium for spore production (Xu et al. 2019).

Improved Genomic Selex Method (Gselex)

The gSELEX workflow was performed according to the method described by Wu *et al* (Wu et al. 2015). Briefly, the genomic DNA of *S. lincolnensis* was digested with *Sau*3AI to generate DNA fragments of suitable size (< 1 kb). Then, 2 μg digested DNA fragments were mixed with 2 μM purified SLCG_2919 for 30 min at 20°C in a 20 μl binding buffer (10 mM Tris, pH 8), 60 mM KCl, 50 mM EDTA, 5 mM $MgCl_2$, 10 mM DTT, and 10% *v/v* glycerol). Potential SLCG_2919-binding DNA fragments were ligated using adapter primers (Table S1) and amplified by PCR. The enriched DNA fragments were cloned into the pGEM-T vector for sequencing. BLAST was used to locate these sequences in the *S. lincolnensis* genome.

Electrophoretic Mobility Shift Assays (Emsas)

EMSAs were performed as previously described (Hellman and Fried 2007). The promoter regions of new targets of SLCG_2919 (*SLCG_6675, SLCG_4123–4124, SLCG_6579, SLCG_0139–0140*) were obtained via PCR with their respective primers (Table S1). The binding reaction system consisted of 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 50 mM EDTA, 60 mM KCl, 10 mM DTT, 10% glycerol, 100 ng labelled probes and different concentrations of purified His_6-tagged SLCG_2919 in a total volume of 20 μl. After incubation of the mixture at 30°C for 15 min, the samples were separated on 6% native PAGE gels in ice-cold 1 × TAE buffer at 50 mA for approximately 50 min.

Rt-qpcr Assay

The relative transcriptional levels of *SLCG_6675*, *SLCG_4123*, *SLCG_4124*, *SLCG_6579*, *SLCG_0139* and *SLCG_0140* were analysed by RT-qPCR with the specific primers listed in Table S1. An RNA extraction/purification kit (Transgen) was used to isolate total RNA from *S. lincolnensis* LCGL and its derivatives after 24 hours of growth in liquid fermentation medium and quantified using a microplate reader (BioTek). Isolated RNA (500 ng) was treated with DNase I (Vazyme), and reverse transcription was performed using a cDNA synthesis kit (Vazyme). RT-qPCR was exerted on the Applied Biosystems QuantStudio 6 Flex system with Maxima™ SYBR Green/ROX qPCR Master Mix (Vazyme). The *rpoD* gene in *S. lincolnensis* acted as an internal control, and relative transcription was quantified using the comparative cycle threshold method (Livak and Schmittgen 2001).

Construction of gene deletion mutants in S. lincolnensis

To construct the *SLCG_6675* deletion mutant Δ*SLCGL_6675*, a 1.4-kb *Hin*dIII/*Xba*I fragment of the left flanking region was amplified using primer pairs (Table S1), and a 1.5-kb *Eco*RI/*Kpn*I fragment of the right flanking region was PCR-amplified with primer pairs 6675-P1/P2 and 6675-P3/P4 (Table S1). These two fragments were ligated at the corresponding sites of pUCTSR (Wu et al. 2016) to generate the plasmid pUCTSRΔ*6675*. Then, a 4.3 kb DNA fragment was digested with *Eco*RI/*Hin*dIII from pUCTSRΔ*6675*, and ligated into the same sites of pKC1139, generating pKC1139Δ*6675*. Using PEG-mediated protoplast transformation, pKC1139Δ*6675* was introduced into LCGL. Through chromosomic homologous recombination, a 720-nt fragment within *SLCG_6675* was replaced by the thiostrepton

resistance gene (*tsr*) in LCGL. The desired Δ*SLCGL_6675* mutant was confirmed using PCR with the primers 6675-P5 and 6675-P6 (Table S1).

Using the same methods, we constructed Δ*SLCGL_4123*, Δ*SLCGL_4124*, Δ*SLCGL_6579*, Δ*SLCGL_0139* and Δ*SLCGL_0140* in LCGL.

Fermentation And Lincomycin A (Lin-a) Determination

Flask fermentation of *S. lincolnensis* strains was performed as described in our previous study (Xu et al. 2019). Briefly, spores of *S. lincolnensis* and its derivatives were inoculated into 30 ml of seed medium at 30°C on an orbital shaker at 240 rpm for 2 days. Then, 2 ml of the seed culture was transferred into 30 ml industrial fermentation medium and was incubated at 30°C with 240 rpm for 7 days. Lin-A production was measured as described previously (Xu et al. 2019).

Statistical analysis

All acquired data are shown as mean ± SD and were analyszed using Student's t-test. The differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

gSELEX-based exploiting of potentially new targets of SLCG_2919

To establish the SLCG_2919-mediated regulatory network, we employed the previously improved gSELEX method to identify target sequences of SLCG_2919. Genomic DNA of *S. lincolnensis* LCGL was initially digested with *Sau*3AI to generate < 1 kb DNA fragments (Fig. S1A). After two rounds of SELEX, DNA fragments potentially binding to SLCG_2919 were cloned into the T vector for sequencing (Fig. S1B). When the SLCG_2919 concentration was 2 μM, four independent clones were obtained and sequenced (Table S2). DNA segments corresponding to the promoter regions of *SLCG_6675*, *SLCG_4123–4124*, *SLCG_6579*, and *SLCG_0139–0140* were captured at least twice. According to the genome annotation of *S. lincolnensis*, *SLCG_6675*, *SLCG_0139*, *SLCG_0140*, *SLCG_6579*, *SLCG_4123*, and *SLCG_4124* encode anthranilate synthase, LysR family transcriptional regulator, beta-lactamase, cytochrome P450, bifunctional DNA primase/polymerase, and magnesium or magnesium-dependent protein phosphatase, respectively. In summary, these results imply that SLCG_2919 participates in the control of primary metabolism, transcriptional regulation, oxidoreduction, and anabolism for lincomycin biosynthesis.

Slcg_2919 Binds Specifically To Four Captured Dna Fragments

To identify the four DNA fragments potentially interacting with SLCG_2919, we expressed His_6-tagged SLCG_2919 in *E. coli* BL21 (DE3), as previously described (Xu et al. 2019), and tested its DNA-binding affinity for the promoter regions of *SLCG_6675*, *SLCG_4123–4124*, *SLCG_0139–0140*, and *SLCG_6579*. Because the length of $P_{4123-4124}$ is 711 nt, we designed two DNA fragments $P_{4123-4124-1}$ (356 nt) and $P_{4123-4124-2}$ (355 nt), without overlapping sequences. Results from the EMSAs showed notable shifts when different amounts of His_6-SLCG_2919 were added. Then, 50-fold unlabelled probes or poly dIdC were individually added to the reaction system to evaluate the binding specificities. We found that these labelled probes were able to pull down shifted bands, whereas poly dIdC were not (Fig. 1A). This indicated that SLCG_2919 binds specifically to the above DNA fragments.

Using the defined AT-rich binding site of SLCG_2919 (Xu et al. 2019), we scanned the AT-rich motif in the genome of *S. lincolnensis* LC-G using PREDetector (http://www.montefiore.ulg.ac.be/~hiard/PreDetector/PreDetector.php). Five DNA fragments were predicted within the promoter regions of the target genes (Fig. 1B). In addition, we used the motif-finding program MEME (http://meme-suite.org/) to evaluate the predicted sequences of targeted genes and a conserved SLCG_2919-binding motif (atTcgT, a: T, C; t: T, G; c: C, G; g: A, C) was identified (Fig. 1B).

SLCG_2919 **disruption increases the transcription of its target genes**

To confirm whether SLCG_2919 transcriptionally regulates the above-mentioned target genes, we used RT-qPCR to compare the transcription of these genes between LCGL and Δ*SLCGL_2919*. The results showed that the transcriptional levels of *SLCG_6675*, *SLCG_0139*, *SLCG_0140*, *SLCG_6579*, *SLCG_4123*, and *SLCG_4124* increased by 3.3-, 4.2-, 3.2-, 2.5-, 4.6-, and 2.2-fold, respectively, in Δ*SLCGL_2919* compared to those in LCGL (Fig. 2). It could be concluded that SLCG_2919 acts as a negative regulator to modulate transcriptional expression of the six new targeted genes.

New Targets Of Slcg_2919 Positively Correlate With Lincomycin Yield

To investigate the relevance of the aforementioned target genes to lincomycin production, *SLCG_6675, SLCG_0139, SLCG_0140, SLCG_6579, SLCG_4123, and SLCG_4124* were individually disrupted with *tsr* replacement in LCGL, and the corresponding mutants were obtained and confirmed by PCR analyses (Fig. 3A and B). As shown in Fig. 3C, the mutants Δ*SLCGL_6675*, Δ*SLCGL_0139*, Δ*SLCGL_0140*, Δ*SLCGL_6579*, Δ*SLCGL_4123, and* Δ*SLCGL_4124* displayed 24%, 20%, 10%, 26%, 24%, and 26% reductions, respectively, in Lin-A yield compared with the parental strain LCGL. Thus, our findings indicated that the six new target genes of SLCG_2919 had a positive effect on lincomycin production.

Discussion

TFRs, which are widely present in prokaryotes, are generally involved in highly regulated biosynthesis such as that of multidrug efflux pumps, antibiotic biosynthesis, and osmotic stress response proteins (Cuthbertson and Nodwell 2013). We previously reported that SLCG_2919 was associated with lincomycin production by directly repressing most genes within the *lmb* cluster (Xu et al. 2019). However, knowledge of the SLCG_2919-mediated regulatory network in lincomycin biosynthesis remains limited. In the present study, we utilized the previously improved gSELEX technique, combined with EMSA, RT-qPCR, and gene inactivation, to facilitate identification of new targets of SLCG_2919 in *S. lincolnensis*.

gSELEX is an *in vitro* strategy for the direct capture of DNA-binding TFs; however, it is not very efficient when applied to identify targets from the high-GC actinomycete genome (Wu et al. 2015). We previously improved this method by circumventing genomic library construction and enriching TF targets with PCR in each cycle of SELEX (Wu et al. 2015). In this study, improved gSELEX was utilized to screen the target sequences of SLCG_2919. RT-qPCR and EMSA results showed that SLCG_2919 negatively regulated all the targeted genes, including *SLCG_6675*, *SLCG_0139*, *SLCG_0140*, *SLCG_6579*, *SLCG_4123*, and *SLCG_4124*. We found that the targets of SLCG_2919 have an AT-rich binding site, similar to the AT-rich motif previously defined by DNase I footprinting assays and EMSAs (Xu et al. 2019). However, there were no intergenic regions amenable to interacting with this motif among the targets of SLCG_2919 isolated in this study from the *lmb* cluster. We suspect that this might be due to restriction enzyme digestion or the experimental conditions. Further experiments are required to optimize this methodology and identify these intergenic regions for further clarification.

The six identified gene targets were demonstrated to be positively correlated with lincomycin production in that their knockout resulted in a substantial reduction in Lin-A yield. SLCG_6675, encoding anthranilate synthase, can transform chorismate into anthranilic acid (Chen et al. 2020). SLCG_4123, a bifunctional DNA primase/polymerase, consumes excess ATP for DNA synthesis. These two enzymes might play a significant role in the primary metabolism of *S. lincolnensis*. SLCG_0140 encodes a beta-lactamase, and its inactivation in *S. lincolnensis* might lower antibiotic resistance, possibly resulting in adverse effects on lincomycin biosynthesis. SLCG_6579, which encodes cytochrome P450, plays a key role in the reductive activation of molecular oxygen, and is possibly involved in the biosynthesis of lincomycin (Novotna et al. 2013). SLCG_4124, a manganese- or magnesium-dependent protein phosphatase, can transfer ATP or GTP phosphate groups to amino acid residues. The LysR family transcriptional regulator FkbR1 is a positive regulator of ascomycin production (Song et al. 2017). In our study, deletion of *SLCG_0139*, encoding a LysR family transcriptional regulator, also decreased lincomycin yield in *S. lincolnensis*. Together with previously determined targets, we have proposed a general framework of SLCG_2919-mediated regulatory network (Fig. 4). SLCG_2919 may participate in primary metabolism, transcriptional regulation, oxidoreduction, and anabolism, illustrating the importance of TFR in the control of the complex regulatory network of lincomycin biosynthesis.

Table 1
Bacterial strains and plasmids used in this study

Strains and plasmids	Description	Reference
E. coli		
DH5α	F *recA lacZ*M15	Novagen
S. lincolnensis		
LC-G	CGMCC7.209, a lincomymycin producer	Xinyu Pharmaceutical Co., Ltd.
LCGL	integrated attB$^{\Phi C31}$site located in *SLCG_7011*	(Xu et al. 2019)
Δ*SLCGL_4123*	LCGL derivative with *SLCG_4123* deleted	This study
Δ*SLCGL_4124*	LCGL derivative with *SLCG_4124* deleted	This study
Δ*SLCGL_6579*	LCGL derivative with *SLCG_6579* deleted	This study
Δ*SLCGL_6675*	LCGL derivative with *SLCG_6675* deleted	This study
Δ*SLCGL_0139*	LCGL derivative with *SLCG_0139* deleted	This study
Δ*SLCGL_0140*	LCGL derivative with *SLCG_0140* deleted	This study
Plasmids		
pUCTSR	pUC18 derivative containing a 1.36-kb fragment of a thiostrepton resistance gene in *Bam*HI/*Sma*I sites	(Wu et al. 2014)

Strains and plasmids	Description	Reference
pUCTSRΔ*4123*	pUCTSR derivative containing two 1.6-kb fragments, the upstream and downstream regions of *SLCG_4123*	This study
pUCTSRΔ*4124*	pUCTSR derivative containing two 2.0-kb fragments, the upstream and downstream regions of *SLCG_4124*	This study
pUCTSRΔ*6579*	pUCTSR derivative containing two 1.5-kb fragments, the upstream and downstream regions of *SLCG_6579*	This study
pUCTSRΔ*6675*	pUCTSR derivative containing two 1.5-kb fragments, the upstream and downstream regions of *SLCG_6675*	This study
pKC1139	ori (pSG5), *aac(3)IV*, *lacZ*	(Bierman et al. 1992)
pKC1139-Δ*4123*	pKC1139 derivative for *SLCG_4123*deletion	This study
pKC1139-Δ*4124*	pKC1139 derivative for *SLCG_4124*deletion	This study
pKC1139-Δ*6579*	pKC1139 derivative for *SLCG_6579* deletion	This study
pKC1139-Δ*6675*	pKC1139 derivative for *SLCG_6675* deletion	This study
pKC1139-Δ*0139*	pKC1139 derivative for *SLCG_0139* deletion	This study
pKC1139-Δ*0140*	pKC1139 derivative for *SLCG_0140* deletion	This study
pET28a	T7 promoter, His-tag, *kan*	Novagen
pET28a-2919	pET28a-derived plasmid carrying *SLCG_2919*	This study

Declarations

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Conflicts of interest

There are no conflicts of interest to declare.

Author contributions

B.Z., H.W., and Y.X. conceived and supervised the study. Y.X., J.Y., B.L., and X.L. designed and performed the main experiments and data analyses. Y.G., Q.Z., and S.C. participated in this work. Y.X. wrote the drafted manuscript. B.Z., and H.W. edited the manuscript. All authors reviewed the manuscript.

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Figures

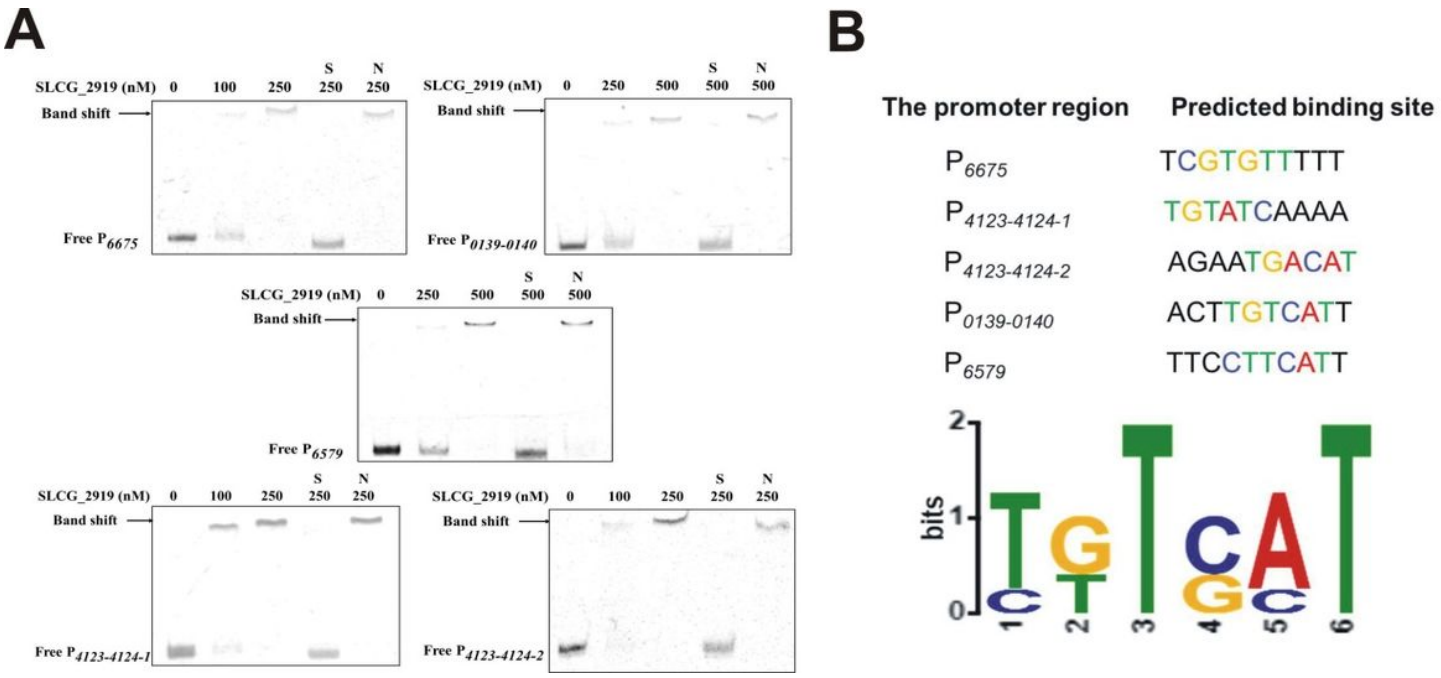

Figure 1

Confirmation of SLCG_2919 binding to its captured sequences. A EMSAs of His_6-SLCG_2919 with promoter regions of *SLCG_6675*, *SLCG_0139-0140*, *SLCG_6579*, and *SLCG_4123-4124*. Each lane contained 100 ng DNA probes. S, unlabeled specific probe (50-fold) was added; N, nonspecific probe poly dIdC (50-fold) was added. **B** SLCG_2919-binding motif within the five target sequences. The standard code of the Weblogo server is shown at the bottom using online MEME software (http://meme.nbcr.net/meme/).

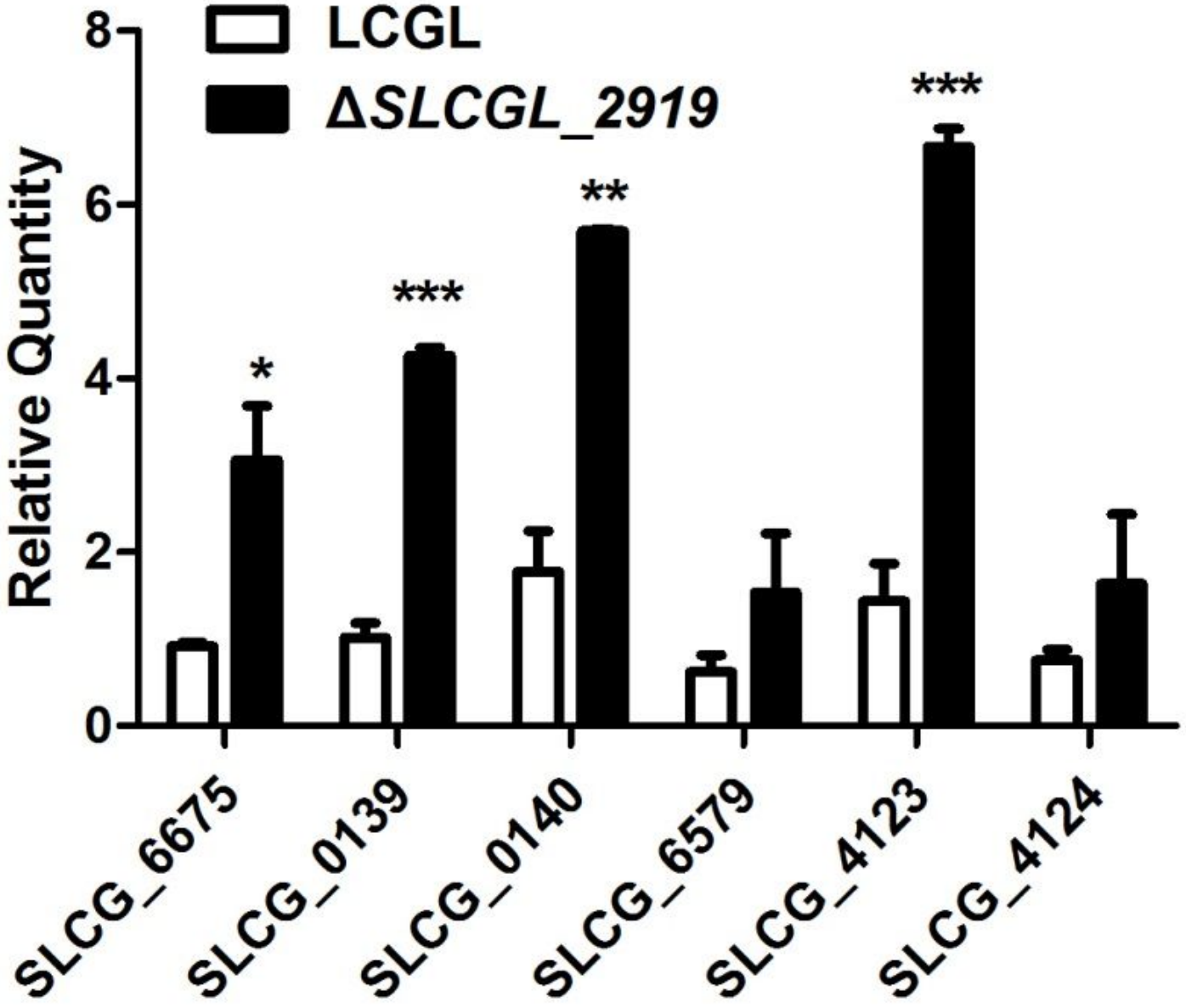

Figure 2

RT-qPCR analyses of *SLCG_6675*, *SLCG_0139*, *SLCG_0140*, *SLCG_6579*, *SLCG_4123*, and *SLCG_4124* in LCGL and Δ*SLCGL_2919* cultured for 24h in fermentation medium. Mean values of three replicates were shown, with the standard deviation indicated by error bars.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

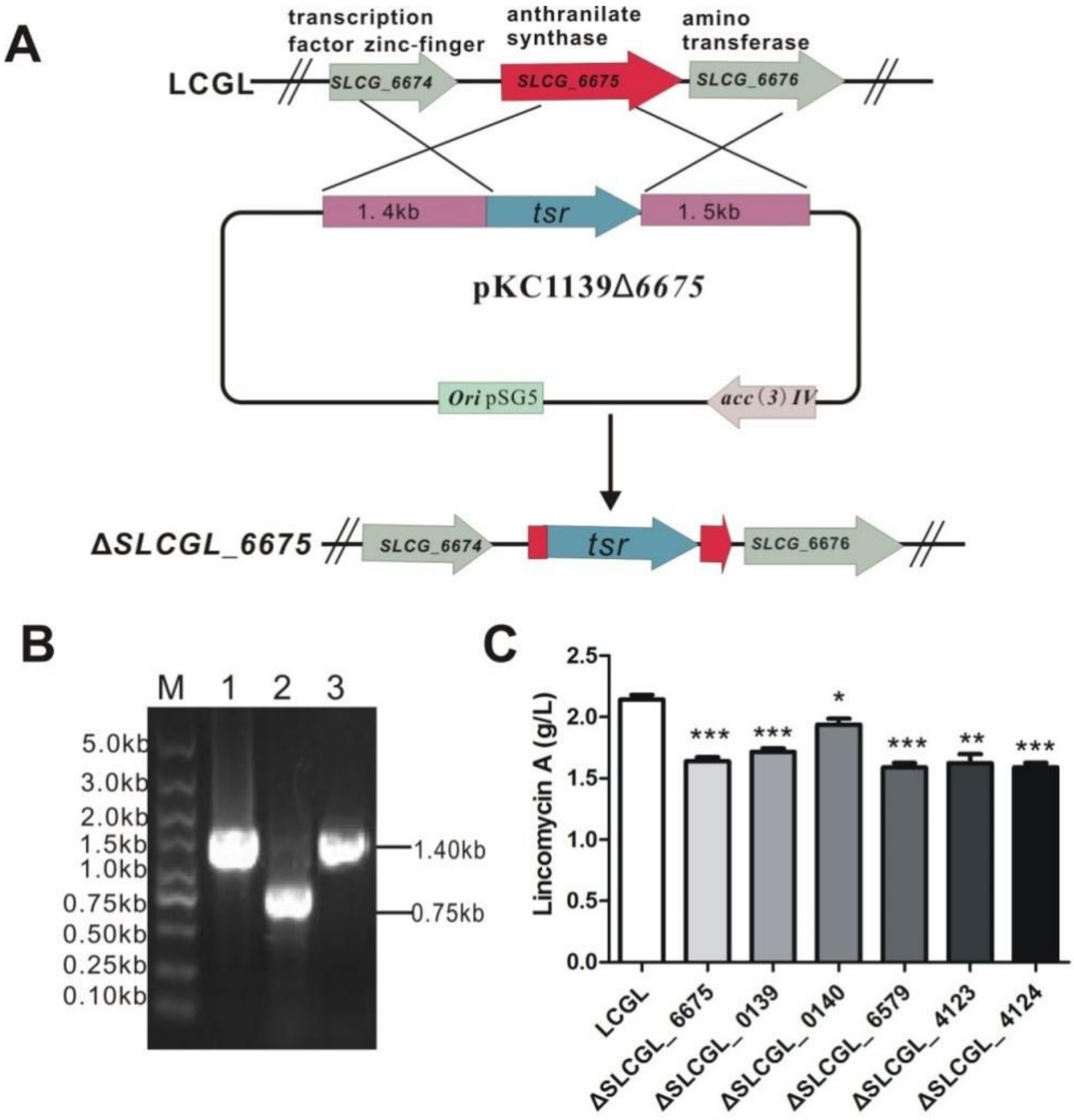

Figure 3

Effect of SLCG_2919-target genes on lincomycin production in *S. lincolnensis* LCGL. A Schematic deletion of *SLCG_6675* by homologous recombination in LCGL. **B** PCR confirmation of the *SLCG_6675* deletion mutant by the primers 6675-P5 and 6675-P6. Lanes: M. 5000-bp DNA ladder; 1. the positive control, 1,400 bp amplified from pKC1139Δ*6675*; 2. the negative control, 750 bp amplified from LCGL; 3. the sample, 1, 400 bp amplified from mutant Δ*SLCGL_6675*. **C** Lin-A production of LCGL and its derived mutants. Mean

values of three replicates were shown, with the standard deviation indicated by error bars.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

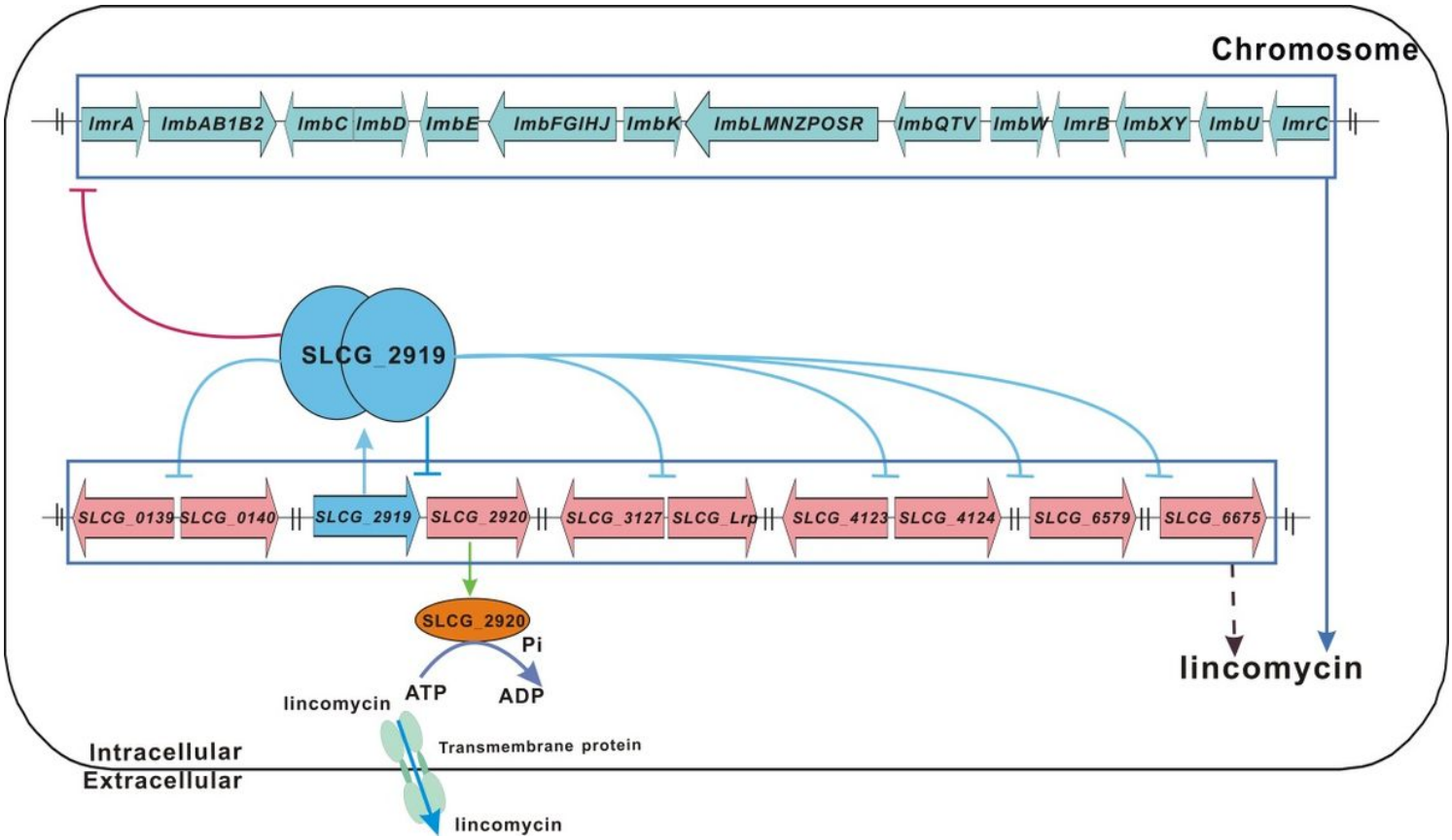

Figure 4

Regulatory network of SLCG_2919 for modulating lincomycin biosynthesis in *S. lincolnensis*. Flat-headed arrows indicate repression. Thick red line represents SLCG_2919 controlling all promoters within *lmb* cluster.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SLCG2919SupplementaryMaterialAntonievanLeeuwenhoek.docx