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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

Enhanced lincomycin production by co-overexpression of *metK1* and *metK2* in *Streptomyces lincolnensis*

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Abstract

Streptomyces lincolnensis is generally utilized for the production of lincomycin A (Lin-A), a clinically useful antibiotic to treat Gram-positive bacterial infections. Three methylation steps, catalyzed by three different *S*-adenosylmethionine (SAM)-dependent methyltransferases, are required in the biosynthesis of Lin-A, and thus highlight the significance of methyl group supply in lincomycin production. In this study, we demonstrate that externally supplemented SAM cannot be taken in by cells and therefore does not enhance Lin-A production. Furthermore, bioinformatics and in vitro enzymatic assays revealed there exist two SAM synthetase homologs, MetK1 (SLCG_1651) and MetK2 (SLCG_3830) in *S. lincolnensis* that could convert L-methionine into SAM in the presence of ATP. Even though we attempted to inactivate *metK1* and *metK2*, only *metK2* was deleted in *S. lincolnensis* LCGL, named as Δ*metK2*. Following a reduction of the intracellular SAM concentration, Δ*metK2* mutant exhibited a significant decrease of Lin-A in comparison to its parental strain. Individual overexpression of *metK1* or *metK2* in *S. lincolnensis* LCGL either elevated the amount of intracellular SAM, concomitant with 15% and 22% increase in Lin-A production, respectively. qRT-PCR assays showed that overexpression of either *metK1* or *metK2* increased the transcription of lincomycin biosynthetic genes *lmbA* and *lmbR*, and regulatory gene *lmbU*, indicating SAM may also function as a transcriptional activator. When *metK1* and *metK2* were co-expressed, Lin-A production was increased by 27% in LCGL, while by 17% in a high-yield strain LA219X.

Keywords *Streptomyces lincolnensis* · Lincomycin · *S*-adenosylmethionine (SAM) · SAM synthetase

Introduction

Lincomycin A (Lin-A) is widely used for the treatment of Gram-positive bacterial infections [26], mainly produced by the actinomycete *Streptomyces lincolnensis*. Lin-A consists of an amino sugar precursor α-methylthiolincosaminide (MTL) and an amino acid derivative *N*-methylated 4-propyl-L-proline (PPL) moiety [34]. Lin-A and its semi-synthetic derivative clindamycin belong to lincosamide family antibiotics, and clindamycin can be used for the treatment of protozoal diseases, e.g., malaria [26]. Given the high clinical importance of lincosamide antibiotics, enhancement of lincomycin production in *S. lincolnensis* has been performed by genetic manipulation, fermentation engineering or classical mutagenesis methods over the past 50 years [3, 8, 13, 33].

The lincomycin biosynthetic gene cluster in *S. lincolnensis* spans over 35 kb of DNA, containing 29 genes concerning the biosynthesis, regulation and resistance [11, 19]. In

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the process of lincomycin biosynthesis, three methylation steps are required for *C*-, *S*-, and *N*-positions, respectively [9, 25]. Previous reports demonstrated that the methyltransferase LmbJ converted *N*-demethyllincomycin into lincomycin, and LmbW was involved in the propylproline biosynthesis of Lin-A using *S*-adenosylmethionine (SAM) as a methyl donor [14, 18]. LmbG was predicted to be responsible for the *S*-methyl reaction [9, 25] (Fig. 1). These investigations suggest that methyl group supply plays a vital role in lincomycin biosynthesis in *S. lincolnensis*. A number of studies have confirmed that SAM is an important methyl donor in both primary and secondary metabolisms [2, 4], and overexpression of SAM synthetase gene *metK* or exogenous addition of SAM enhances the production of multiple types of antibiotics in actinomycetes [16, 35, 36]. SAM is also found to activate transcription factors, which in turn regulate antibiotic biosynthesis of *Streptomyces* [4, 15, 22, 32]. We have fully sequenced the genome of a lincomycin producer *S. lincolnensis* LC-G, and found that there exist two *metK* homologs, *metK1* (*SLCG_1651*) and *metK2* (*SLCG_3830*) (Accession number CP022744 in Genbank). Recently, Pang et al. [18] reported that co-overexpression of *lmbW* and *metK* increased lincomycin production and purity in the industrial strain *S. lincolnensis* SyBE2901. Sequence alignment revealed that the reported *metK* from *S. lincolnensis* SyBE2901 was identical to *metK1* from *S. lincolnensis* LC-G. Here we report the function of *metK2,* as well as *metK1,* in *S. lincolnensis*, which could be used for enhancing Lin-A production.

Materials and methods

Strains, plasmids and growth conditions

All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was cultured in Luria–Bertani (LB) medium at 37 °C, with shaking at 220 rpm, supplemented with appropriate antibiotics as required [20]. *S. lincolnensis* and its derivatives were grown at 30 °C with shaking at 220 rpm in liquid TSBY medium (3% tryptone soya broth, 0.5% yeast extract, 10.3% sucrose, with/without apramycin or thiostrepton) for DNA extraction, or on solid MGM medium (2% soluble starch, 0.5% soybean flour, 0.1% KNO_3, 0.05% NaCl, 0.05% $MgSO_4$, 0.05% K_2HPO_4, 0.001% $FeSO_4$, 2% agar, with/without apramycin or thiostrepton) for sporulation. Spores were isolated and stored in 20% glycerol at -80 °C. Liquid SM medium (0.4% yeast extract, 0.4% tryptone soya broth, 1% glucose, 0.005% $MgSO_4$, 0.02% KH_2PO_4, 0.04% K_2HPO_4) was used for *S. lincolnensis* protoplast preparation [6].

Fermentation and HPLC analysis of Lin-A

Streptomyces lincolnensis LC-G and its derivatives were grown on MGM for sporulation (with appropriate antibiotics for the recombinant strains). The spore suspension was inoculated into a 250-ml flask containing 30 ml of the seed medium (2% soluble starch, 1% glucose, 1% soybean flour, 3% cream corn, 0.15% $(NH_4)_2SO_4$, 0.4% $CaCO_3$ for culture, with/without apramycin) at 30 °C with shaking at 240 rpm for 2 days. 2 ml seed culture was transferred into 30 ml fermentation medium (10% glucose, 2% soybean flour, 0.15% cream corn, 0.8% $NaNO_3$, 0.5% NaCl, 0.6% $(NH_4)_2SO_4$, 0.03% K_2HPO_4, 0.8% $CaCO_3$, with/without apramycin). All fermentation cultures were incubated at 30 °C and 240 rpm for 7 days. After fermentation, 200 µl supernatant of fermentation broth was mixed with 800 µl ethanol, and centrifuged at 12,000 rpm for 10 min to remove the residue. Subsequently, lincomycin extracted from these liquid fermentation cultures was quantified by Waters H13CHA 394G UPLC system on Extend-C18 column (5 µm, 150×4.6 mm), which was equilibrated with 60% methyl alcohol and 40% 5 mM ammonium acetate (pH 9.0). The products were monitored at 214 nm. An isocratic program was carried out at a flow rate of 0.4 ml/min.

Exogenous addition of SAM to the culture medium

Streptomyces lincolnensis LC-G was incubated at 30 °C, 240 rpm in fermentation medium as described above. SAM

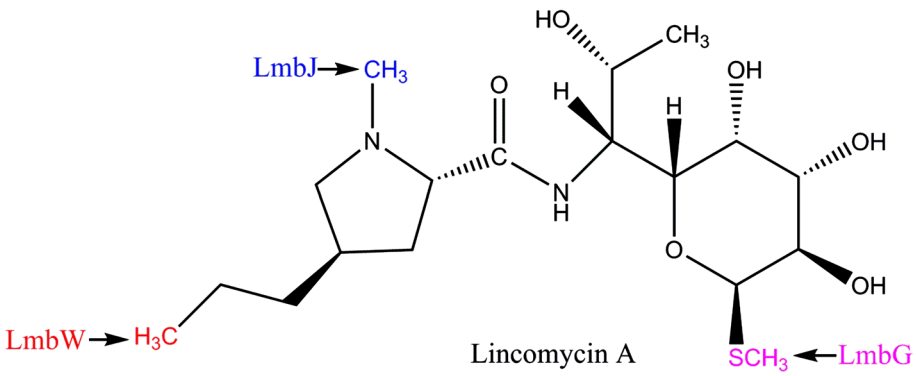

Fig. 1 Lincomycin structure and the sites methylated by LmbJ, LmbW, and LmbG, respectively

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Sources
S. lincolnensis		
LC-G	CGMCC 7.209, a lincomycin-producing strain	Xinyu Pharmaceutical Co., Ltd.
LC-G/pSOK804	LC-G carrying pSOK804	This study
LC-G/pPM927	LC-G carrying pPM927	This study
LCGL	Derived from LC-G with $attB^{\Phi C31}$site instead of *SLCG_7011*	This study
Δ*metK2*	Derived from LCGL with *metK2* deleted	This study
Δ*metK2*/pIB139	Δ*metK2* carrying pIB139	This study
Δ*metK2*/pIB139-*metK2*	Δ*metK2* carrying pIB139-*metK2*	This study
LCGL/pIB139	LCGL carrying pIB139	This study
LCGL/pIB139-*metK1*	LCGL carrying pIB139-*metK1*	This study
LCGL/pIB139-*metK2*	LCGL carrying pIB139-*metK2*	This study
LCGL/pIB139-*metK1-metK2*	LCGL carrying pIB139-*metK1-metK2*	This study
LA219	A lincomycin high-yield strain	Xinyu Pharmaceutical Co., Ltd.
LA219X	Derived from LA219 with $attB^{\Phi C31}$site instead of *SLCG_7011*	This study
LA219X/pIB139-*metK1-metK2*	LA219X carrying pIB139-*metK1-metK2*	This study
E. coli		
DH5α	F *recA lacZ*M15	Invitrogen
BL21(DE3)	F-*ompT hsd*SB ($rB^{-}mB^{-}$) *gal dcm* (DE3)	Novagen
Plasmids		
pUCTSR	pUC18 derivative	[7]
pUCTSRΔ*metK2*	pUCTSR derivative for *metK2* deletion	This study
pKC1139	*ori* (pSG5), *aac(3)IV*, *lacZ*	[1]
pKC1139-Δ*metK2*	pKC1139 derivative for *metK2* deletion	This study
pKC1139-4×$attB^{\Phi C31}$	pKC1139 derivative for $attB^{\Phi C31}$site to replace *SLCG_7011*	This study
pIB139	ΦC31 *attP-int* locus, *acc(3)IV*, *oriT*, P*ermE** promoter	[29]
pIB139-*metK1*	pIB139 derivative for expression of *metK1*	This study
pIB139-*metK2*	pIB139 derivative for expression of *metK2* pIB139 derivative for co-	This study
pIB139-*metK1-metK2*	overexpression of *metK1* and *metK2*	This study
pSOK804	*ori*, *aac(3)IV*, $attP^{VWB}$	[21]
pPM927	pSAM2 *attP-int locus*, *tsr*, *oriT*	[24]
pET28a	*kan*, P_{T7}, His-tag	Novagen
pET28a-*metK1*	pET28a derivative for expression of *metK1*	This study
pET28a-*metK2*	pET28a derivative for expression of *metK2*	This study

was added to the fermentation medium of *S. lincolnensis* LC-G after 12 h inoculation, in a final concentration of 0, 0.1, 0.5, 1 and 2 mM, respectively.

Cloning, expression and purification of MetK1 and MetK2 in *E. coli*

Two DNA fragments encoding MetK1 and MetK2 from LC-G were obtained by PCR using the primers in Table S1. The PCR products were digested with *Nde*I/*Eco*RI restriction enzymes and inserted into the corresponding sites of pET-28a (Novagen), generating N-terminal His_6-tag fusions. The constructed plasmids pET28a-MetK1 and pET28a-MetK2 were, respectively, introduced into *E. coli* BL21 (DE3), and the expression of the two proteins was induced with IPTG at a final concentration of 0.5 mM at 16 °C for 20 h. MetK1 and MetK2 His_6-tagged proteins were extracted and purified on an Ni^{2+}–NTA spin column (BIO-RAD). The concentrations of the purified proteins were quantified by BCA assays, and their purity was judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Enzymatic activity assay of MetK1 and MetK2

The activities of MetK1 and MetK2 were assayed as reported by Oh et al. [15]. Briefly, either MetK1 or MetK2 protein was incubated for 2 h at 37 °C in a reaction mixture (100 µl) containing 0.1 mM Tris/HCl (pH 8.2), 5 mM ATP, 5 mM L-methionine, 200 mM KCl and 10 mM $MgCl_2$. The controls were performed without MetK1 and MetK2, or without ATP and L-methionine. All reactions were

terminated by immediately placing the reaction vessels in an ice-water mixture. The terminated reaction mixtures were analyzed by Waters H13CHA 394G UPLC system on Extend-C18 column (5 μm, 250×4.6 mm), which was equilibrated with 100 mM phosphate buffer at pH 6.8 (A phase) and methanol (B phase) at a ratio of 80:20 (*v/v*). The products were monitored at 254 nm. An isocratic program was carried out at a flow rate of 0.5 ml/min. Furthermore, kinetic parameters were determined with the constant concentration of ATP (10 mM). The concentration of L-methionine varied between 0 and 5 mM. Velocity was plotted as a function of varied substrate concentration and the data were fitted to the Michaelis–Menten equation to calculate values of Km and Vmax [5].

Determination of intracellular/extracellular SAM concentration

Intracellular and extracellular SAM levels were determined as described by Oh et al. [15]. During the fermentation of *S. lincolnensis* LC-G and its derivatives with or without additional SAM (2, 4 and 6 days), 1 ml of the fermentation broth was centrifuged at 5,000 rpm for 10 min. The supernatant was directly applied on UPLC to determine the extracellular SAM concentration. After removal of the supernatant, the intracellular SAM was extracted with 0.5 ml of 1 M formic acid at 4 °C for 1 h. After centrifugation at 12,000 rpm for 15 min, the SAM extracted was quantified by UPLC with the same method above.

Construction of *S. lincolnensis* LCGL

In order to use the ΦC31-based integrative vector pIB139 [31], a 240-bp DNA fragment was synthesized at Sangon Biotech (Shanghai) Co., Ltd, containing four tandem ΦC31 *attB* sites [30]. The sequence was as follows: AAA*GAA TTC*CTTCTC*TCTAGA*CGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAACTAGTGGATCTCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAACTAGTGGATCTCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAACTAGTGGATCTCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAACTAGT*GGATCC*CTGGAG*AAGCTT*AAA (*Eco*RI/*Xba*I and *Bam*HI/*Hin*dIII restriction sites were shown in italics, and four *attB* sequences were underlined). With *S. lincolnensis* LC-G genomic DNA as a template, two 2.0-kb DNA fragments flanking *SLCG_7011* putatively encoding a nonribosomal peptide synthetase (NRPS) were amplified by PCR using two primer pairs attB-P1/attB-P2 and attB-P3/attB-P4 (Table S1). The PCR products and the synthetic 4×*attB*$^{\Phi C31}$ were, respectively, digested with *Eco*RI/*Kpn*I, *Xba*I/*Hin*dIII and *Xba*I/*Kpn*I, and then ligated into the corresponding sites of pKC1139, obtaining pKC1139-4×*attB*$^{\Phi C31}$. By PEG3350-mediated protoplast transformation, pKC1139-4×*attB*$^{\Phi C31}$ was introduced into LC-G. After two round homologous chromosomic recombination, the desired mutant with 4×*attB*$^{\Phi C31}$ instead of *SLCG_7011*, named as *S. lincolnensis* LCGL, was obtained and further confirmed by PCR analysis using the primers attB-P5/attB-P6 (Table S1).

In accordance with above procedures, we constructed *S. lincolnensis* LA219X containing 4×*attB*$^{\Phi C31}$ from a high-yield *S. lincolnensis* LA219.

Inactivation, complementation and overexpression of *metK1 and metK2* in *S. lincolnensis*

We tried many methods to disrupt *metK1* gene in both *S. lincolnensis* LC-G and LCGL, but failed, while it was very easy to obtain Δ*metK2* mutants.

The plasmid pKC1139-Δ*metK2*, with an internal 717-bp deletion of *metK2*, was constructed in two steps. Firstly, with the genomic DNA of LC-G as a template, two 1.5-kb fragments flanking *metK2* were, respectively, amplified by PCR using two primer pairs metK2-P1/metK2-P2 and metK2-P3/metK2-P4 (Table S1), cleaved by *Hin*dIII/*Xba*I and *Kpn*I/*Eco*RI, and ligated into the corresponding sites of pUCTSR [7], yielding pUCTSR-Δ*metK2*. Secondly, 4.5 kb DNA fragment was digested with *Eco*RI/*Hin*dIII from pUCTSR-Δ*metK2*, and then cloned into the same site of pKC1139 [1], generating pKC1139-Δ*metK2*. By PEG3350-mediated protoplast transformation, pKC1139-Δ*metK2* was introduced into LCGL. By homologous chromosomic recombination, a 717-bp fragment of the *metK2* gene was replaced by thiostrepton resistance gene (*tsr*) in *S. lincolnensis* LCGL. The desired Δ*metK2* mutant was further confirmed by PCR amplification using the primers metK2-P5 and metK2-P6 (Table S1). For the complementation of *metK2* in Δ*metK2* mutant, a 1221-bp *metK2* was amplified with the primers metK2-P7 and metK2-P8 (Table S1), cleaved with *Nde*I/*Xba*I, and ligated into the corresponding sites of pIB139, generating pIB139-*metK2*. Then, pIB139 and pIB139-*metK2* were individually introduced into Δ*metK2* mutant, and the corresponding apramycin-resistant Δ*metK2*/pIB139 and Δ*metK2*/pIB139-*metK2* were obtained and further confirmed by PCR amplification with the primers apr-P1 and apr-P2, respectively (Table S1).

Meanwhile, a 1209-bp *metK1* was amplified with the primers metK1-P1 and metK1-P2 (Table S1), digested with *Nde*I/*Xba*I, and ligated into the corresponding sites of pIB139, generating pIB139-*metK1*. pIB139-*metK1* and pIB139-*metK2* were introduced into LCGL to overexpress *metK1* and *metK2*, respectively.

For co-overexpressing *metK1* and *metK2*, the *metK2* fragment with promoter *PermE** was amplified from pIB139-*metK2* using primers metK2-P9 and metK2-P10 (Table S1),

cut with *Not*I/*Eco*RV and ligated to the corresponding site of pIB139-*metK1*, generating pIB139-*metK1-metK2*. Then the co-expression vector pIB139-*metK1-metK2* was introduced into LCGL and LA219X to generate the strains LCGL/pIB139-*metK1-metK2* and LA219X/pIB139-*metK1-metK2*, respectively.

RNA isolation and quantitative real-time PCR assay (qRT-PCR)

The relative transcriptional levels of *lmbA*, *lmbR* and *lmbU* were determined by qRT-PCR analysis. Specific primers were designed as listed in Table S1. Total RNA was isolated from *S. lincolnensis* LCGL and its derivatives after 24 h grown in fermentation liquid medium using the RNA extraction/purification kit (SBS), and the RNA concentration was determined using a microplate reader (BioTek). Isolated RNA (500 ng) was treated with DNase I (MBI Fermentas), and then reversed using a cDNA synthesis kit (MBI Fermentas). qRT-PCR was performed on the Applied Biosystems QuantStudio 6 Flex system with Maxima™ SYBR Green/ROX qPCR Master Mix (MBI Fermentas). The *rpoD* gene in *S. lincolnensis* was used as an internal control, and relative transcription was quantified using a comparative cycle threshold method [12].

Results

SAM supplementation of the culture medium cannot increase Lin-A production

Since SAM is the principal methyl donor in microbial cells, we initially examined the effect of exogenous addition of SAM on lincomycin production. Under the final concentrations of SAM from 0.1 mM to 2 mM in the culture medium, the Lin-A production of LC-G kept unchanged (Fig. 2a). We further found that the extracellular SAM concentration generally remained constant throughout LC-G fermentation (Fig. 2b). Also, the intracellular concentration of SAM did not vary depending on the amount increment of extra added SAM (Fig. 2c). These results suggest that SAM cannot be transported across the membrane of *S. lincolnensis* cells.

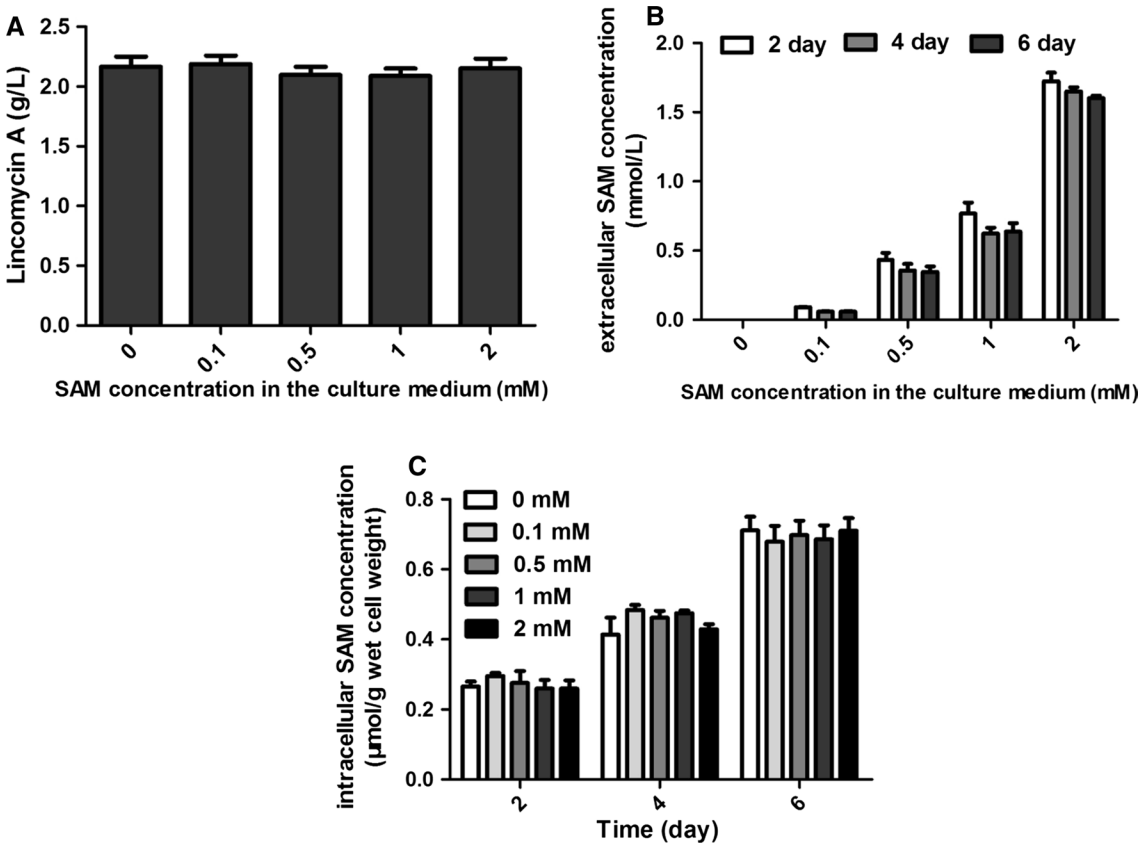

Fig. 2 Effect of exogenous SAM addition on Lin-A production in *S. lincolnensis* LC-G. **a** Lin-A production after adding SAM with different final concentrations (0, 0.1, 0.5, 1, 2) by UPLC analysis. **b** Extracellular SAM concentrations under different exogenous addition of SAM after 2, 4 and 6 days incubation. **c** Intracellular SAM concentrations under different exogenous addition of SAM after 2, 4 and 6 days incubation. Mean values of at least three independent experiments were shown, with the standard deviation indicated by error bars

Both MetK1 and MetK2 enzymatically convert L-methionine into SAM in the presence of ATP

Alignment of the genomic sequences of *S. lincolnensis* LC-G (Accession number CP022744 in Genbank) and other *Streptomyces* strains revealed there exist two SAM synthetase genes, *SLCG_1651* (*metK1*) and *SLCG_3830* (*metK2*), with high similarity to other *metK* genes, such as *SCO1476* from *S. coelicolor* A3(2) (93.5 and 81.5% identities) and *SAV_6874* from *S. avermitilis* MA4680 (93.5 and 83% identities).

To test whether MetK1 and MetK2 from LC-G have SAM synthetase activities, they were successively expressed and purified (Fig. 3a). As shown in the UPLC profiles, both MetK1 and MetK2 could convert L-methionine into SAM in the presence of ATP (Fig. 3b).

Moreover, Michaelis–Menten equation was used to determine the kinetic constants of MetK1 and MetK2, exhibiting the Km and V_{max} values of 3.42 mM and 4.12 μmol/L/min for MetK1, and 2.14 mM and 4.88 μmol/L/min for MetK2. MetK2 had a higher catalytic activity than MetK1.

Overexpression of *metK1* increases Lin-A production in *S. lincolnensis* LCGL

Given that the genome of *S. lincolnensis* LC-G lacks the typical *attB* site for ΦC31-based pIB139 vector integration, we constructed a new *S. lincolnensis* LCGL from LC-G by replacing *SLCG_7011* with $4\times attB^{\Phi C31}$. No significant differences in cell growth, morphological differentiation and lincomycin production were observed between LCGL and LC-G (Fig. S1).

Because of repeated failures in deleting *metK1* gene in LC-G and LCGL, we assume that MetK1 may be essential for LC-G growth. To investigate the role of *metK1* gene in the biosynthesis of lincomycin, plasmid pIB139-*metK1* was transformed into *S. lincolnensis* LCGL. Compared with the control LCGL/pIB139, LCGL/pIB139-*metK1* successively exhibited 1.8-, 2.5- and 1.8-fold increases in intracellular SAM concentrations during incubation periods of 2, 4 and 6 days (Fig. 4a). Consistent with the increase of intracellular SAM, LCGL/pIB139-*metK1* showed a 15% ($p<0.01$) higher level of Lin-A production on the seventh day of fermentation than LCGL/pIB139 (Fig. 4b). These results indicate that *metK1* overexpression in LCGL leads to enhancement of intracellular SAM concentration and Lin-A production.

Overexpression of *metK2* increases Lin-A production in *S. lincolnensis* LCGL

To investigate whether *metK2* was also involved in lincomycin biosynthesis, it was disrupted in *S. lincolnensis* LCGL through *tsr* replacement, and the resulted mutant Δ*metK2* was confirmed by PCR analysis (Fig. 5a, b). As shown in Fig. 5c, the intracellular SAM concentrations of Δ*metK2* were decreased by approximately 31, 42 and 50% after 2, 4 and 6 days fermentation compared to its parental strain LCGL, respectively (Fig. 5c). Correspondingly, Δ*metK2* had a 55% reduction in Lin-A yield after a 7-day fermentation, from 2.2 to 1.0 g/L ($p<0.001$) relative to its parental strain. Complementation of Δ*metK2* with pIB139-*metK2* recovered Lin-A production (Fig. 5d). Δ*metK2* showed similar growth rates in YMG liquid medium and sporulation rates on MGM agar medium to its parent strain *S. lincolnensis* LCGL,

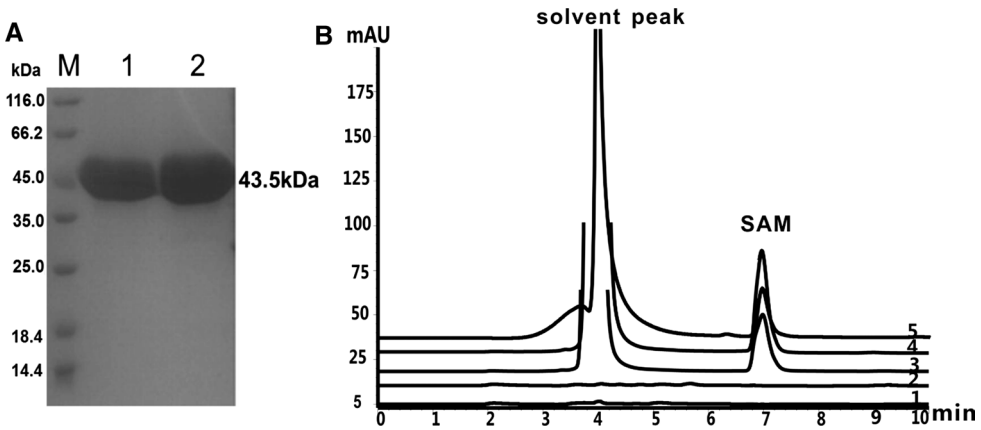

Fig. 3 Enzymatic analyses of MetK1 and MetK2. **a** Purification of His_6-tagged MetK1 and His_6-tagged MetK2. Abbreviations: M, protein molecular weight marker; 1, purified MetK1 protein; 2, purified MetK2 protein. **b** Both MetK1 and MetK2 converted ATP and L-methionine into SAM. The reaction with either MetK1 or MetK2 was performed in the mixture of ATP, L-methionine, KCl and $MgCl_2$. 1, KCl, $MgCl_2$ and MetK1 or MetK2; 2, ATP, L-methionine, KCl and $MgCl_2$; 3, ATP, L-methionine, KCl, $MgCl_2$ and MetK1; 4, ATP, L-methionine, KCl, $MgCl_2$ and MetK2; 5, standard SAM (retention time = 6.9 min)

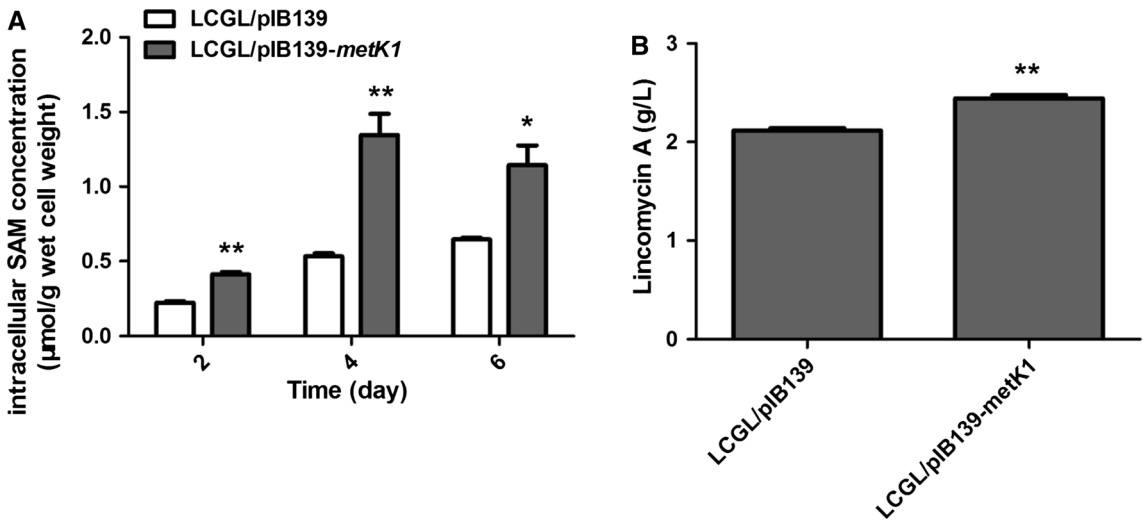

Fig. 4 Overexpression of *metK1* increases the yield of Lin-A in *S. lincolnensis* LCGL. **a** Intracellular SAM concentrations of LCGL/pIB139 and LCGL/pIB139-*metK1*. **b** Lin-A production of LCGL/pIB139 and LCGL/pIB139-*metK1*. Mean values of at least three replicates were shown, with the standard deviation indicated by error bars. $*p < 0.05$, $**p < 0.01$

indicating that MetK2 is not involved in cell growth and morphological differentiation of *S. lincolnensis* (Fig. S2).

To further probe the involvement of MetK2 in lincomycin biosynthesis, *metK2* was overexpressed in LCGL. As shown in Fig. 5c, the levels of the intracellular SAM in LCGL/pIB139-*metK2* was, respectively, increased by 2.6-, 3.0- and 2.2-fold during the incubation period of 2, 4 and 6 days in comparison with that of LCGL/pIB139 (Fig. 5c). In accordance with the increased amount of intracellular SAM, the Lin-A yield of LCGL/pIB139-*metK2* was increased by 22% ($p < 0.001$) on the seventh day compared to LCGL/pIB139 (Fig. 5d). These data further confirm that MetK2 is certainly involved in the SAM biosynthesis, further enhancing Lin-A production in *S. lincolnensis*.

MetK1 and MetK2 promote the transcription of the genes involved in Lin-A biosynthesis and regulation in *S. lincolnensis*

Since SAM was found to act as an intracellular signal molecule for regulating the antibiotic biosynthesis in *Streptomyces* independent of its role as a methyl donor [4, 36], we measured the transcripts of *lmbA* (*SLCG_0227*, a Lin-A biosynthetic gene encoding gamma-glutamyl transferase), *lmbR* (*SLCG_0245*, a Lin-A biosynthetic gene encoding transaldolase) and *lmbU* (*SLCG_0253*, a positive regulatory gene controlling Lin-A biosynthesis) by qRT-PCR. The transcriptional levels of *lmbA*, *lmbR* and *lmbU* in LCGL/pIB139-*metK1* were, respectively, increased by 1.75-, 1.83- and 2.2-fold at 24 h compared with those in LCGL/pIB139, while the transcriptional levels of *lmbA*, *lmbR* and *lmbU* in *metK2* overexpression strain raised by 5.5-, 3.7- and 6.1-fold (Fig. 6). These results demonstrate that SAM surely acts as a signal molecule to promote the transcription of lincomycin biosynthetic genes in *S. lincolnensis*. Furthermore, MetK2 makes a more significant contribution than MetK1.

Co-overexpression of *metK1* and *metK2* further improve Lin-A yield in *S. lincolnensis*

In order to further improve lincomycin production, *metK1* and *metK2* were co-overexpressed in the LCGL. The Lin-A yield of LCGL/pIB139-*metK1-metK2* was remarkably increased by 27% ($p < 0.01$) relative to that of LCGL/pIB139 (Fig. 7a).

To examine the applicability and universality of co-overexpressing *metK1* and *metK2* for enhancement of Lin-A production, the pIB139-*metK1-metK2* was introduced into a higher-yield *S. lincolnensis* strain LA219X. As expected, LA219X/pIB139-*metK1-metK2* (2.92 g/L) showed 17% ($p < 0.01$) improvement of Lin-A production relative to LA129X/pIB139 (2.5 g/L) when cultured in 30 mL industrial fermentation medium for 7 days (Fig. 7b). It is likely that co-overexpressing *metK1* and *metK2* in other industrial *S. lincolnensis* strains will be of significantly commercial value.

Discussion

Although the plasmids pPM927 and pSOK804, containing phage VWB and pSAM2 integration sites, have been widely used in the research field of actinomycetes [17, 24, 27, 28], we found that the yields of Lin-A were both lowered by

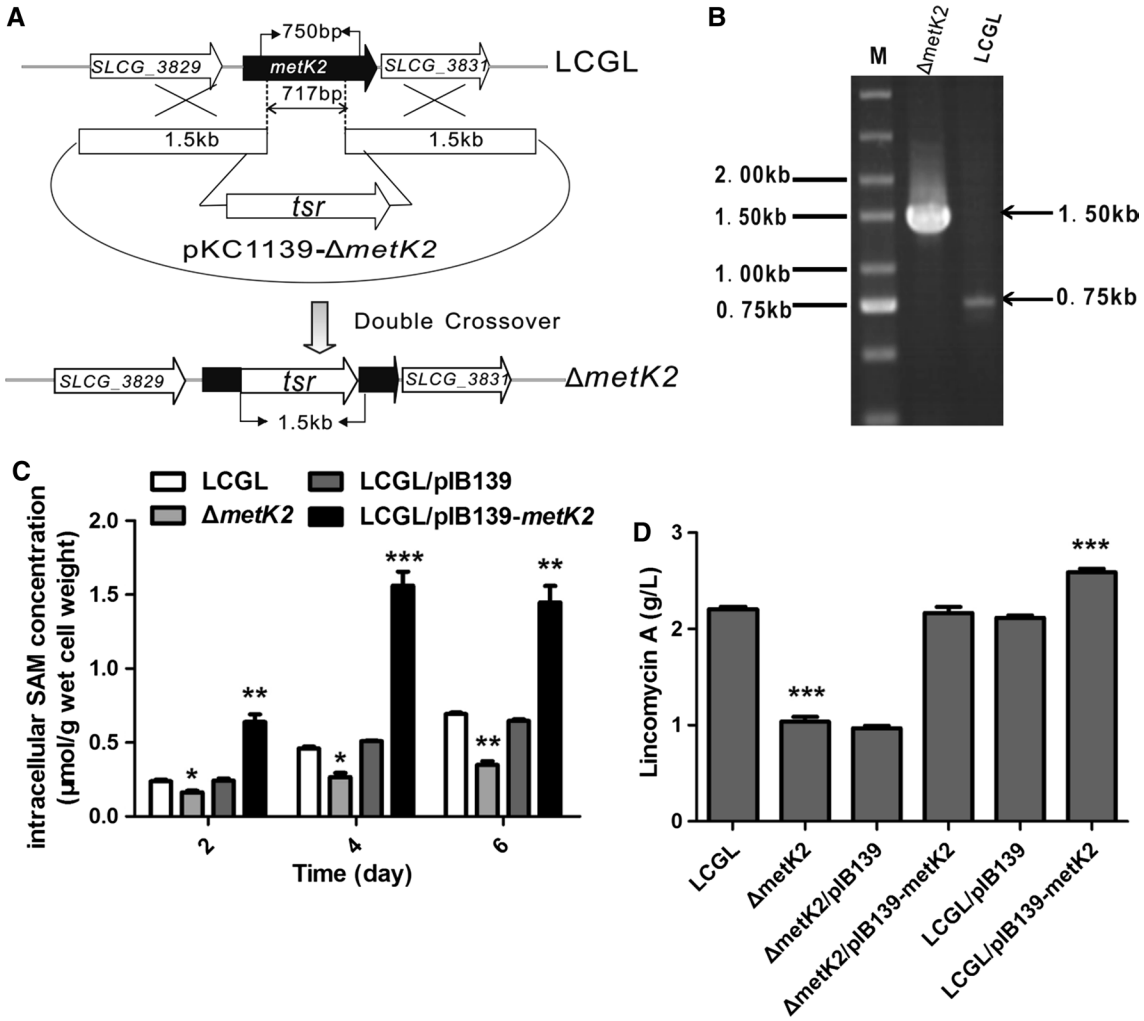

Fig. 5 Effect of inactivation and overexpression of *metK2* on Lin-A production in *S. lincolnensis* LCGL. **a** Schematic deletion of *metK2* in LCGL. **b** PCR confirmation of Δ*metK2* mutant using the primers metK2-P5 and metK2-P6. A 750-bp band was observed with LCGL, while a 1,500-bp band was detected with Δ*metK2*. **c** Intracellular SAM concentrations in LCGL, Δ*metK2*, LCGL/pIB139 and LCGL/pIB139-*metK2* cultured for 2, 4 or 6 days. **d** Lin-A production in LCGL and its derivatives cultured for 7 days. Mean values of at least three independent experiments were shown, with the standard deviation indicated by error bars. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

about 50% when they were introduced into *S. lincolnensis* LC-G (Fig. S1C), indicating that they are not suitable for engineering industrial *S. lincolnensis* strains. Due to lack of the ΦC31 *attB* site in the genome of *S. lincolnensis* LC-G, we used homologous recombination to replace *SLCG_7011,* putatively encoding a nonribosomal peptide synthetase (NRPS), with ΦC31 *attB* sites. The resulting mutant LCGL showed similar cell growth, morphological differentiation and lincomycin production relative to LC-G (Fig. S1D, E and F). When pIB139 was introduced into LCGL, there was no effect on the lincomycin production (Fig. S1D), indicating LCGL strain is suitable for plasmids with ΦC31 *attB* sites to express heterologous genes.

The rationale using *attB* sequence is that phage ΦC31 integrase catalyzes site-specific exchange to generate stable mutants [23]. For integration, the *attB* site undergoes the recombination with phage *attP* site of pSET152-derived plasmid to form hybrid sites *attL* and *attR*. Only *attL* and *attR* at the franking regions of inserted linear ΦC31-based vector by exconjugants, other *attB* sites were eliminated [30]. Taken the genomic DNA of *S. lincolnensis* strains LCGL/pIB139, LCGL/pIB139-*metK1*, LCGL/pIB139-*metK2* and LCGL/pIB139-*metK1-metK2* as templates, these DNA fragments between *attL* and *attR* were, respectively, amplified with three primer pairs (Fig.S3A, B and C). The presence of *attL* and *attR* in above recombinant *S. lincolnensis* strains was further verified by DNA sequencing (Fig.S4A and B). Thus, our current purpose of inserting the 4×*attB* cassette with a suitable size of 240 bp is only to facilitate molecular cloning, consistent with the description of using 8x *attB*

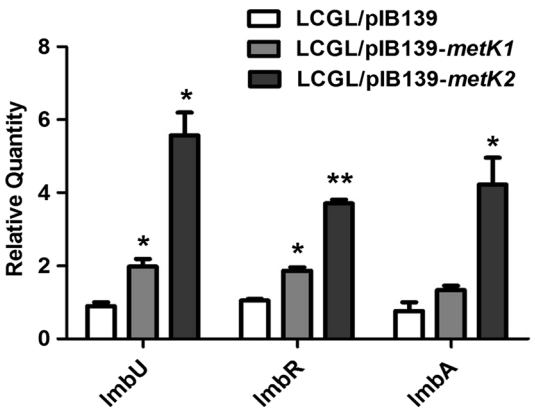

Fig. 6 Overexpression of *metK1* or *metK2* enhances the transcription of *lmbA*, *lmbR* and *lmbU* in *S. lincolnensis*. qRT-PCR was used to quantify the transcriptional levels of *lmbA*, *lmbR* and *lmbU* in LCGL/pIB139, LCGL/pIB139-*metK1* and LCGL/pIB139-*metK2* cultured for 24 h in fermentation medium. Mean values of at least three independent experiments were shown, with the standard deviation indicated by error bars. $*p < 0.05$, $**p < 0.01$

cassette in *Saccharopolyspora erythraea* for site-specific recombination [30].

SAM, acting as a major methyl donor, plays a vital role in primary and secondary metabolism [2]. Most of investigations demonstrated that addition of exogenous SAM or overexpression of SAM synthetase caused common yield improvement of antibiotics in actinomycetes [10, 36]. However, our results showed that extra SAM added to culture media could not enter *S. lincolnensis* cells. This may be due to lack of a transporter for SAM uptake in *S. lincolnensis,* which is similar to the phenomenon observed in *Streptomyces coelicolor* M512 [36].

We have completely sequenced the genome of the lincomycin-producing *S. lincolnensis* LC-G and found two homologs of SAM synthetase genes, *SLCG_1651* (*metK1*) and *SLCG_3830* (*metK2*). Consistent with other MetKs in *Streptomyces* [15], both MetK1 and MetK2 harbor a conserved ATP-binding motif and two metal-binding regions for Mg^{2+} and K^{+} (Fig. S5A). The *metK1* (*SLCG_1651*) is located among the genes encoding a primosome assembly protein (SLCG_1650), a bifunctional phosphopantothenoylcysteine decarboxylase/phosphopantothenate synthase (SLCG_1652), and an omega subunit of DNA-dependent RNA polymerase (SLCG_1653) (Fig. S5B), inferring MetK1 might be mainly involved in DNA modification and primary metabolism. That may be the reason why we failed to inactivate *metK1* in LC-G or LCGL. A similar phenomenon was also reported in *Streptomyces peucetius* var. *caesius* [15]. The *metK2* (*SLCG_3830*) is clustered in the *metK2-adoK-metH-metF-sahH* genes (*SLCG*_3830 ~ *SLCG_3834*) (Fig. S3B), which are mostly responsible for the SAM cycle [36]. Pang et al. reported that overexpression of *metK* could promote the production of Lin-A in an industrial *S. lincolnensis* SyBE2901 [18]. According to the sequence alignment, we deduced that the *metK* reported from *S. lincolnensis* SyBE2901 was identical to the *metK1* from *S. lincolnensis* LC-G. In our study, we found that overexpression of both *metK1* and *metK2* increased Lin-A production by upgrading the intracellular SAM concentration in *S. lincolnensis* LCGL, and overexpression of *metK2* had a more significant effect. Furthermore, co-overexpression of *metK1* and *metK2* dramatically enhanced the yield of Lin-A in LCGL, even in a higher lincomycin-producing *S. lincolnensis* strain. We anticipate that the strategy of co-overexpression of the

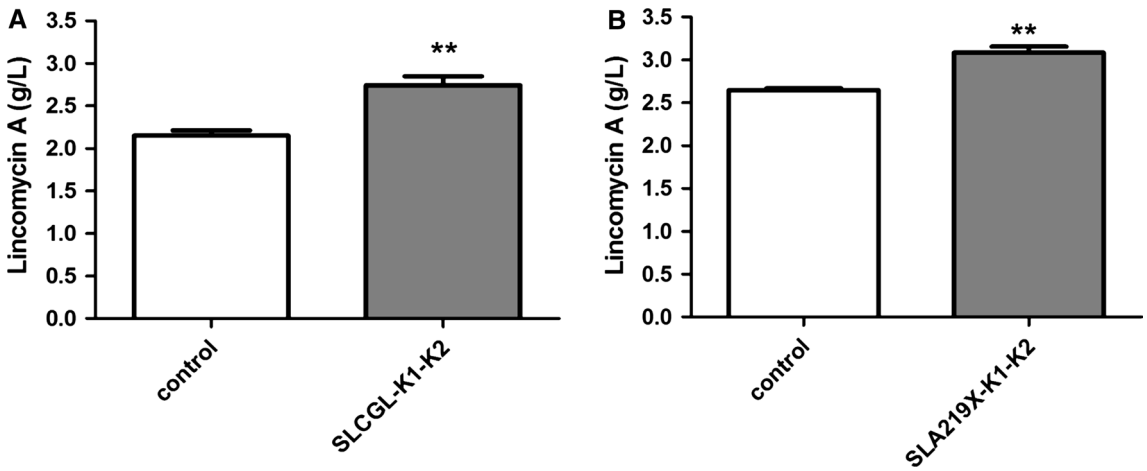

Fig. 7 Co-overexpressing of *metK1* and *metK2* improves the yield of Lin-A in *S. lincolnensis*. **a** Lin-A production of LCGL/pIB139 (control) and LCGL/pIB139-*metK1-metK2* (SLCGL-K1-K2). **b** Lin-A production of LA219X/pIB139 (control) and LA219X/pIB139-*metK1-metK2* (SLA219X-K1-K2). Mean values of at least three replicates were shown, with the standard deviation indicated by error bars. $**p < 0.01$

two SAM synthetase genes will be generally applicable for improvement of other antibiotics in industry.

Previous findings gave a hint that besides a methyl group supplier, SAM also played a role in transcriptional regulation of antibiotic biosynthesis in *Streptomyces* [15, 22, 32, 36]. Hereby, we provide new evidences that SAM is indeed a signal molecule to regulate the transcription of lincomycin biosynthetic and regulatory genes, but the mechanism remains to be explored.

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Compliance with ethical standards

conflict of interest The authors declare that they have no competing interests.

Research involving human and animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

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