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RESEARCH PAPER

Decreased Lincomycin B Content by Regulating Osmotic Pressure in Fermentation of *Streptomyces lincolnensis*

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Abstract Lincomycin, a clinically important antibiotic against Gram-positive bacteria, is a sulfur-containing metabolite produced by *Streptomyces lincolnensis*. The main problem in the fermentation is the high content of lincomycin B, which is a by-product of insufficient methylation by S-adenosylmethionine (SAM) dependent methyltransferase. In this study, the content of lincomycin B was decreased from 4.04% to 0.75% and from 8.20% to 5.78% by the addition of NaCl as an osmotic regulator in flask and 15 L bioreactor fermentation, respectively. By analysis of the sulfur-containing metabolites (cysteine, homocysteine, SAM, and ergothioneine) and high-throughput RNA sequencing, the mechanism of osmotic regulation on lincomycin fermentation was studied. The results showed that the synthesis of methyl donor (SAM) and sulfur donor of lincomycin (ergothioneine and mycothiol) were increased at the metabolite level and transcription level under osmotic stimulation; the transcription of genes involved in sulfur assimilation (*tauA*, *ssuA1B1C1*) and lincomycin biosynthesis were also up-regulated significantly. This study provides a cost-effective method to reduce lincomycin B and increase the production of lincomycin A. The mechanisms of osmotic pressure regulation on lincomycin fermentation was also elucidated, which may provide more genetically modified targets for overproduction of lincomycin and other sulfur containing secondary metabolites.

Keywords: *Streptomyces lincolnensis*, lincomycin, osmotic pressure, sulfur metabolite, transcriptome analysis

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1. Introduction

Lincomycin A, produced by *Streptomyces lincolnensis*, is a chemically important lincosamide antibiotic and has potent antibacterial activity against many Gram-positive and a few Gram-negative microorganisms such as *Mycoplasma* [1]. During the fermentation process, lincomycin A is produced along with the by-product, lincomycin B, which has lower bioactivity but higher toxicity than lincomycin A [2]. The difference between lincomycin A and B lies on the side of proline moiety, where lincomycin A has a propyl-proline moiety while lincomycin B has an ethyl-proline [3]. Due to its lower bioactivity and higher toxicity, the content of lincomycin B is an important parameter to evaluate the product quality during the fermentation process. According to Chinese Pharmacopoeia, the content of lincomycin B shall not exceed 5% [4]. Higher content of lincomycin B in the fermentation broth required more separation and purification steps to remove lincomycin B, which would increase the production cost. Thus, it is essential to reduce the level of lincomycin B in the fermentation broth.

Lincomycin A is composed of an amino acid moiety (propyl-L-proline, PPL) and a C_8 backbone glycosyl moiety (methythiolincosaminide, MTL) [5]. The two moieties are biosynthesized separately and condensed to form N-demethyl-lincomycin, which can then be converted to lincomycin A by methylation [3]. Lincomycin is also a unique sulfur-containing secondary metabolite. The sulfur is incorporated into lincomycin A via the metabolic coupling of two small-molecule thiols, ergothioneine (EGT) and mycothiol (MSH). The EGT mediates the activation, transfer, and modification of C_8 glycosyl moiety, while the MSH provides sulfur element for lincomycin through thiol exchange [6]. EGT and MSH are two major low-molecular-weight thiols synthesized by actinomycetes to protect against oxidative

stress and maintain the redox balance of thiol-disulfide [7]. Microarray analysis revealed that biosynthesis of MSH and cysteine (Cys) was increased by osmotic induction in *Streptomyces coelicolor* to provide anti-osmotic and anti-oxidative substrate of MSH and replenish Fe–S clusters [7]. Despite MSH, EGT played a key role in protecting cell against stresses as well [8].

In actinomycetes, sulfur exists in the forms of sulfur-containing amino acids Cys and methionine (Met) as well as cellular cofactors, including biotin, coenzyme A, S-adenosylmethionine (SAM), iron-sulfur clusters, and low-molecular weight thiols [9]. Among them, Cys is the precursor of Met, MSH, and EGT [10,11]), while SAM is synthesized from Met and ATP through Met adenosyltransferase, serving as a methyl donor in both the primary and secondary metabolism [12].

Improvement of lincomycin A production in *S. lincolnensis* has been investigated in many studies by strain screening [13,14], fermentation engineering [15-17] or genetic manipulation [18,19]. The above studies mainly focused on the optimization of medium composition, process conditions or screening strains for more production of lincomycin A. However, few pieces of research have been performed on reducing the content of lincomycin B in fermentation broth. Previous research has demonstrated that LmbW was involved in the propyl-proline biosynthesis of lincomycin A using SAM as the methyl donor, and co-overexpression of *lmbW* and *metK* could increase the lincomycin production and decrease the content of lincomycin B [18]. There were also reports suggesting that environmental stress, such as osmotic stress, could cause major changes in the metabolism of microorganisms. Increasing the osmotic pressure of fermentation broth could promote the production of erythritol in yeast [20,21] and L-lactic acid in *Lactobacillus casei* [22]. So far, no one has studied the influence of environmental stress on lincomycin production in Streptomyces.

In this article, to reduce the unfavorable content of lincomycin by-product, lincomycin B, increasing NaCl concentration in the culture medium was found effective. Hence, osmotic regulation on intracellular metabolites biosynthesis and gene expression by performing RNA sequencing (RNA-seq) was further studied to reveal the mechanism of osmotic regulation on lincomycin production.

2. Materials and Methods

2.1. Strain and media

S. lincolnensis ZLW0306 was used as fermentation strain throughout this work. The seed medium was as follows: 10 g/L glucose, 20 g/L starch, 10 g/L soybean meal, 30 g/L corn steep liquor, 1.5 g/L $(NH_4)_2SO_4$, and 5 g/L $CaCO_3$ (pH 7.0). The fermentation medium in flask contained: 100 g/L glucose, 25 g/L soybean meal, 2 g/L corn steep liquor, 8 g/L $(NH_4)_2SO_4$, 8 g/L $NaNO_3$, 0.3 g/L KH_2PO_4 and 8 g/L $CaCO_3$ (pH 7.0). The fermentation medium in bioreactor contained: 30 g/L glucose, 5 g/L starch, 15 g/L corn steep liquor, 20 g/L soybean meal, 4.8 g/L $NaNO_3$, 4.3 g/L $(NH_4)_2SO_4$, 0.5 g/L K_2HPO_4, 6 g/L $CaCO_3$ (pH 8.6). The feeding medium contained: 300 g/L glucose, 20 g/L $(NH_4)_2SO_4$, 60 g/L soybean meal, 90 g/L corn steep liquor and 1.5 g/L $CaCO_3$ (pH 7.0).

2.2. Culture condition

One milliliter glycerol stock of *S. lincolnensis* was inoculated into 25 mL seed medium and grown at 30°C in a 250-mL flask with 220 rpm for 48 h. Then, 2.5 mL seed culture was inoculated into 25 mL fermentation medium. The fermentation process was conducted for 7 days at 30°C. Batch culture was conducted in a 15 L bioreactor (Guoqiang Bioengineering Equipment Co., Ltd) with an initial working volume of 10 L. The inoculation volume was 10% (v/v) of the medium volume. The fermentation temperature and agitation speed were maintained at 30°C and 450 rpm, respectively, and the aeration rate was 1.0 vvm. The feeding medium were fed periodically to maintain the supply of C- and N-sources in the fermentation process. Samples were taken at 8 h intervals for off-line analysis.

2.3. Analytical methods

The culture samples taken at different times were centrifuged at 4°C, 10,000 rpm for 10 min to collect the mycelia and the supernatant. The precipitated mycelia were washed three times and dried to constant weight at 105°C to measure the dry cell weight (DCW) [23].

The pH, lincomycin titer, osmotic pressure, concentration of reducing sugars and ammonia nitrogen were determined after collecting the supernatant. All assays were determined in triplicate. The osmotic pressure was determined with a freezing point osmometer (Yida Instrument Co., Ltd). The reducing sugar concentration and ammonia nitrogen concentration were determined by the Fehling method and formaldehyde titration method, respectively [23]. The lincomycin A titer was assayed by high-performance liquid chromatography (HPLC) (Agilent 1260; Agilent Technologies Co., Ltd) using a Diamonsil plus C18 column (250 × 4.6 mm, 5 μm; Dikma Corporation). The column temperature was maintained at 30°C, and UV detection was set at 214 nm. A mobile phase containing 50 mmol/L ammonium acetate: methanol (3:2, v/v) was used at a flow rate of 0.4 mL/min [17].

2.4. Determination of intracellular thiols concentration

The culture samples were centrifuged at 10,000 rpm for 10

min at 4°C to collect the mycelia. The precipitated mycelia were immediately frozen in liquid nitrogen and stored at -80°C until extraction.

The intracellular thiols were extracted by a modification of a previously reported method [24]. In brief, 200 mg frozen cells were transferred to 1.5 mL microcentrifuge cubes. A 1-mL quantity of warm (60°C) 50% (W/V) aqueous acetonitrile containing 20 mmol/L HEPES (pH 8.0) and 2 mmol/L monobromobimane was added to each tube, and the samples were sonicated by 200 W until no obvious mycelia suspended. Extracts were maintained in a 60°C water bath for 15 min in the dark and then added 50 μL 5 mol/L methanesulfonic acid. The tube was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were transferred to a new tube and stored at -80°C until analysis. Negative control and standard sample were prepared based on Fahey's reported method [25].

HPLC was performed using a Shimadzu Prominence system that included a fluorescence detector (RF-20A). The HPLC system was equipped with a Diamonsil plus C18 column (250 × 4.6 mm, 5 μm; Dikma Corporation), and the flow rate was maintained at 1.2 mL/min. The gradient program from solvent A (40 mmol/L ammonium acetate, pH 3.5) to solvent B (methanol) was 15% B in 5 min, 15% to 25% in 10 min, 25% to 75% in 15 min, 75% to 100% in 2 min, 100% to 15% in 3 min, and 15% B in 10 min.

2.5. Determination of intracellular SAM concentration

Intracellular SAM was extracted and detected as described [19]. In brief, 200 mg frozen cells were transferred to 1.5 mL microcentrifuge cubes and extracted with 1 mL of 1 mol/L formic acid at 4°C for 2 h. The samples were centrifuged at 10,000 rpm for 10 min at 4°C to collect the supernatant for determination. The SAM concentration was assayed by HPLC using a Diamonsil plus C18 column (250 × 4.6 mm, 5 μm; Dikma Corporation). The column temperature was maintained at 25°C, and UV detection was set at 280 nm. A mobile phase containing 100 mmol/L phosphate buffer (pH 7.0): methanol (4:1, v/v) was used at a flow rate of 0.5 mL/min.

2.6. RNA-seq and gene expression analysis

Cell samples were collected at 41 h (initial production stage) and 89 h (production stage) to investigate the changes of transcriptome profile. The culture samples were centrifuged at 10,000 rpm for 10 min at 4°C to collect the mycelia and immediately frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation. Total RNA was isolated using an RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech Co., Ltd.). All RNA preparations were treated with RNase-free DNase (TaKaRa) to eliminate genomic DNA contamination. The purity, integrity and concentration of RNA was determined using a Bioanalyzer 2100 (Agilent Technologies Co., Ltd). A cDNA library was constructed and sequenced by Illumina HiSeq 2500 platform (Personal Gene Technology Co., LTD) using the standard Illumina RNA-Seq protocol.

The raw sequencing reads were trimmed for low-quality ends by removing adapter sequences and ambiguous nucleotides, using the software Cutadapt 1.14 (Personal Gene Technology Co., LTD). All sequencing reads with quality scores less than 20 were excluded. The remaining high-quality reads were used in subsequent alignments.

The filtered RNA-Seq reads were aligned to the *S. lincolnensis* NRRL 2936 reference genome sequence (Accession Number CP016438) using Bowtie29 [26]. The fragments per kilo bases per million fragments was calculated as expression values for each gene. The fold change and p value was used to identify differentially expressed genes (DEGs). In this study, the DEGs between each of two samples were screened according to $\log_2|\text{fold change}| > 1$ and $p < 0.05$.

2.7. Statistical analysis

All experiments were performed in triplicates. The statistical significance of all data was determined by p value obtained through Student's *t*-test. Differences with a p value < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Effect of osmotic pressure regulation on lincomycin production in flasks

Most microorganisms have their own preferred osmotic pressure suitable for growth. In this research, 5, 10, 15, 25, and 50 g/L NaCl were added at the beginning of fermentation in shaking flasks to examine the effect of osmotic pressure on growth and lincomycin A production, and the fermentation without NaCl addition was used as a control. The osmotic pressure was measured correspondingly to the NaCl concentration, which ranged from 0.87 to 2.49 Osm/kg in flasks (Table 1). Results showed that improving the osmotic pressure by adding NaCl could significantly influence the microorganism growth. In the range of 0.87 to 1.18 Osm/kg, there are little impacts on the growth, but the cell growth was greatly inhibited when the osmotic pressure exceeded 1.35 Osm/kg ($p < 0.01$). Considering the lincomycin production (Fig. 1), adding 10 g/L NaCl, with the osmotic pressure of 1.18 Osm/kg, could promote the production of lincomycin A and decrease the content of lincomycin B. The content of lincomycin B was decreased from 4.04% to 0.75%, and lincomycin A yield reached 0.96 mg/g DCW

Table 1. Initial osmotic pressure of different NaCl addition and the effect on the growth and lincomycin production in shaking flask fermentation

NaCl addition (g/L)	0	5	10	15	25	50
Osmotic pressure (Osm/kg)	0.86 ± 0.01	1.03 ± 0.02	1.18 ± 0.02	1.36 ± 0.02	1.72 ± 0.05	2.50 ± 0.01
DCW (g/L)	6.09 ± 0.12	5.72 ± 0.27	4.66 ± 0.13	4.14 ± 0.18	2.82 ± 0.15	1.81 ± 0.40
p value	/	0.0417	0.0001	0.0000	0.0000	0.0000
Lincomycin A (mg/g DCW)	0.58 ± 0.04	0.73 ± 0.05	0.96 ± 0.07	1.01 ± 0.04	0.83 ± 0.06	0.32 ± 0.06
Lincomycin B (mg/g DCW)	0.024 ± 0.01	0.009 ± 0.01	0.007 ± 0.00	0.016 ± 0.00	0.016 ± 0.00	0
Lincomycin B (%)	4.04 ± 1.26	1.26 ± 0.99	0.70 ± 0.34	1.56 ± 0.25	1.86 ± 0.17	0.00 ± 0.00
p value	/	0.0199	0.0058	0.0144	0.0207	0.0026

Values are presented as mean ± standard deviation. p value was obtained by Student's *t*-test (3 parallel samples). DCW: dry cell weight.

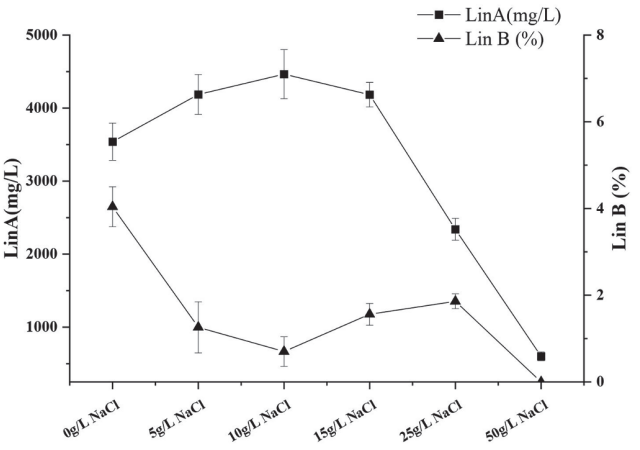

Fig. 1. The influence of NaCl concentration on the content of lincomycin A (Lin A) and lincomycin B (Lin B) in shaking flask fermentation. (■) stands for the titer of Lin A. (▲) stands for the content of Lin B.

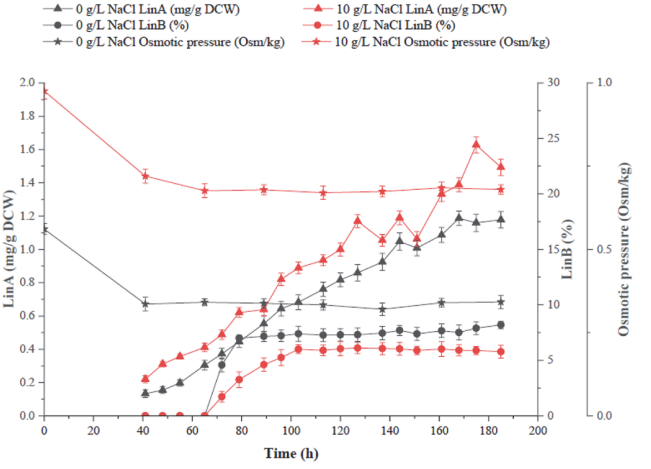

Fig. 2. Time course of Lin A (▲) and Lin B (●) changing trends in 15 L bioreactor fermentation. Black: 0 g/L (without NaCl addition). Red: 10 g/L (with NaCl addition). Each point represents the mean (n = 3) ± standard deviation. Lin A: lincomycin A, Lin B: lincomycin B, DCW: dry cell weight.

from 0.58 mg/g DCW, which was an 80% decrease in lincomycin B content and a 65% increase in lincomycin A yield compared to the control ($p < 0.01$).

3.2. Influence of osmotic pressure regulation on lincomycin production in 15 L bioreactors

To study the effect of NaCl addition on the fermentation process in bioreactors, fermentation was carried out in a 15 L bioreactor, and the fermentation process was shown (Fig. 2). The results showed that the initial osmotic pressure in the bioreactor was increased from 0.56 to 0.98 Osm/kg while adding 10 g/L NaCl to the fermentation medium. During the fermentation process, the osmotic pressure dropped rapidly to about 0.68 Osm/kg within the first 40 h because of substrate consumption. After that, the osmotic pressure became stable till ending. In contrast, the equivalent value in the control fermentation was about 0.34 Osm/kg, much lower than adding NaCl. The maximum lincomycin A yield reached 1.63 mg/g DCW with 10 g/L NaCl addition, whereas the yield was 1.18 mg/g DCW without NaCl. The content of lincomycin B with 10 g/L NaCl addition was much lower than the control before 80 h (3.36% *versus* 7.13%) and ended up with 5.78%, which decreased by 30% compared to 8.20% of the control ($p < 0.01$). The results of bioreactor fermentation showed that adding NaCl could decrease the content of lincomycin B significantly, though the promotion of lincomycin A was not prominent. Before 80 h, the synthesis rate of lincomycin A was higher with NaCl addition, an average of 0.0157 mg/g DCW /h, compared to 0.0107 mg/g DCW /h without NaCl addition.

3.3. Influence of osmotic regulation on fermentation process parameters

The trend of lincomycin fermentation parameter with or without NaCl addition in 15 L bioreactors was studied (Fig. 3). The cell concentration grew to 3.16 g/L at 41 h with NaCl addition, compared to 4.74 g/L without NaCl addition. These results indicated that addition of NaCl could influence the growth of microorganism, with a 33%

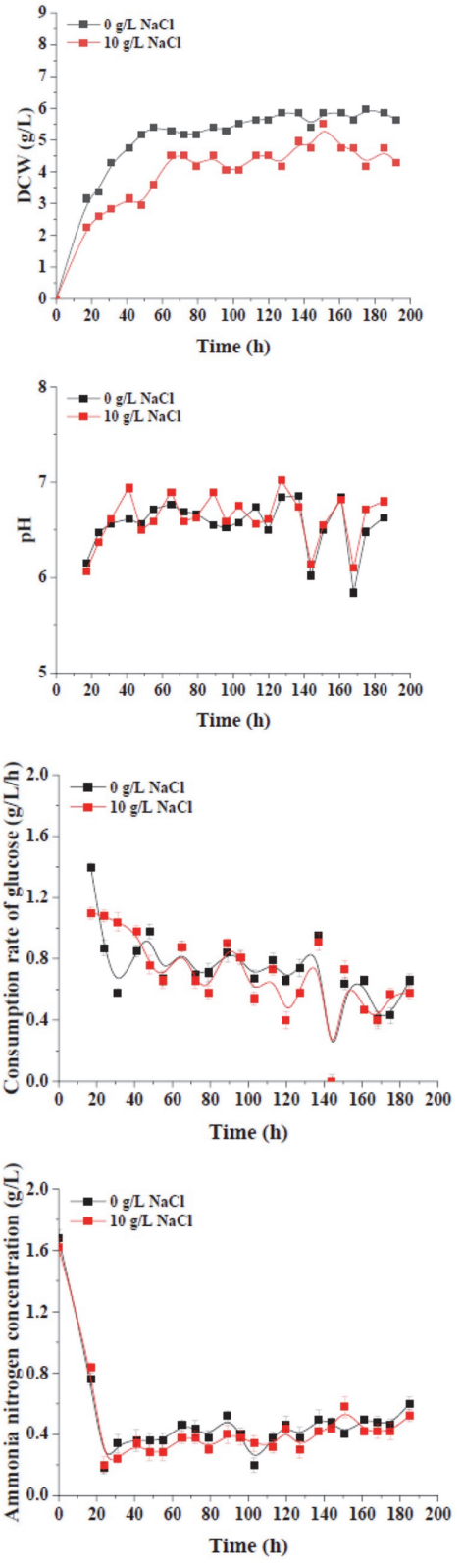

Fig. 3. Time course of metabolic parameters (DCW, pH, consumption rate of glucose, and ammonia nitrogen concentration) in 15 L bioreactor fermentation. Black: 0 g/L (without NaCl addition). Red: 10 g/L (with NaCl addition). Each point represents the mean (n = 3) ± standard deviation. DCW: dry cell weight.

reduction compared to the control ($p < 0.01$). The glucose consumption rates were similar. The changing trend of amino nitrogen concentration indicated that the addition of NaCl promoted the utilization of amino nitrogen. To maintain the amino nitrogen concentration at 0.4-0.6 g/L, a mixture of $(NH_4)_2SO_4$ and organic nitrogen feeding medium was added every 8 h. The calculated volume of feeding medium showed significant difference ($p < 0.01$) (Data not showed). Based on the data above, it can be concluded that the up-shifted osmotic pressure could inhibit cell growth, and promote amino nitrogen utilization. The pH was slightly higher with NaCl addition than the control, suggesting that osmotic pressure caused changes in cell metabolism.

3.4. Influence of osmotic pressure regulation on the intracellular accumulation of sulfur-containing metabolites

Lincomycin is a sulfur-containing substance, of which sulfur moiety is derived from EGT and MSH [14]. Lincomycin has three methyl groups, N-, C-, and S-methylation. A sufficient supply of SAM leads to the promoted methylation which causes more flux to lincomycin A rather than lincomycin B [18]. In order to study the influence of osmotic regulation on cell metabolism, especially the sulfur-containing metabolites and the mechanism of decreased lincomycin B, the changes of Cys, homocysteine (Hcys), SAM, and EGT concentrations was studied during the fermentation process (Fig. 4 and Table S1).

With NaCl addition, the concentration of Cys was similar during the growth phase (before 40 h), while in the production phase, the concentration of Cys was 0.67 μmol/g DCW, much lower than the control. At the late production phase (after 140 h), the concentration reached 1.32 μmol/g DCW, 41% higher than the control (0.94 μmol/g DCW) ($p < 0.01$), indicating less utilization or more production of Cys at late fermentation phase.

Hcys is an intermediate metabolite of Cys to Met. It was found that, after adding NaCl, the Hcys concentration increased rapidly during the growth phase and then maintained at 1.07 μmol/g DCW, almost double of the control (0.57 μmol/g DCW) (Fig. 4) ($p < 0.01$). Similar to the ratio of SAM to S-Adenosyl-L-homocysteine (SAH), the ratio of Hcys to Cys reflects the level of methylation [27]. With NaCl addition, the ratio of Hcys to Cys was higher, which suggested that high osmotic pressure could promote the transmethylation from Cys to Hcys to sustain more SAM synthesis.

SAM is the main methyl donor for many transmethylation reactions. In the process of lincomycin biosynthesis, SAM is the direct methyl donor of methylations. Sufficient SAM supply leads to increased lincomycin A production and decreased lincomycin B content by the co-overexpression of *lmbW* and *metK* [18]. In our results (Fig. 4), adding

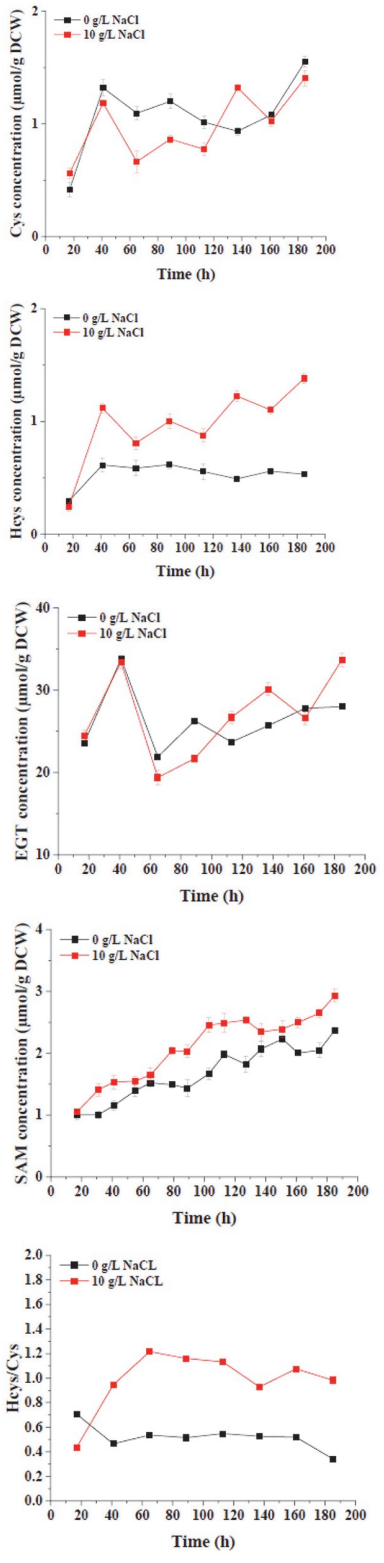

Fig. 4. Time courses of intracellular accumulation of sulfur metabolites (Cys, Hcys, EGT, SAM and the ratio of Hcys to Cys) in 15 L bioreactor fermentation. Black: 0 g/L NaCl (without NaCl addition). Red: 10 g/L NaCl (with NaCl addition). Each point represents the mean ($n = 3$) ± standard deviation. Cys: cysteine, Hcys: homocysteine, EGT: ergothioneine, SAM: S-adenosylmethionine, DCW: dry cell weight.

NaCl could also promote the production of SAM, and the intracellular SAM concentration was maintained at 1.65-2.93 μmol/g DCW during the production phase, 38% higher than that of the control (1.52-2.37 μmol/g DCW) ($p < 0.05$), which may be related to the reduction of lincomycin B production.

EGT is an intracellular small-molecule thiol that generally functions as an antioxidant, while in lincomycin biosynthesis, it can activate, transfer, and modify the C_8 scaffold. During the growth phase, the concentration of EGT was almost similar. While at the early production phase (65 h), the concentration of EGT dropped sharply to 19.40 μmol/g DCW, probably because of the enhancement of EGT utilization under higher osmotic pressure. In the middle and late phase, the amount of intracellular EGT was higher with NaCl addition, probably because the down-shifted EGT concentration stimulated the production of EGT.

3.5. Transcriptional profiling of osmotic pressure regulation

To explore the molecular mechanism of the osmotic pressure on lincomycin A production, the transcriptomes were analyzed by RNA-seq. The detailed description of RNA-seq data analysis was showed in supplementary documents. After filtering the low-quality sequences and removing the redundant reads, 41.17-48.31 MB clean reads was obtained (Table S2). The differential expression analysis was used to analyze the differences in genes of 41 h and 89 h samples with and without NaCl addition. Using the statistical criteria of >2-fold change and $p < 0.05$, a total of 184 and 428 DEGs were identified at 41 h and 89 h, respectively (Fig. S1 and Table S3).

Gene Ontology enrichment was carried out to analyze the pathways involved in the DEGs (Fig. S2). At 41 h, the most significantly regulated DEGs with NaCl addition are largely assigned with functions for redox reaction, sulfur metabolism, and stress response. Consistent with previous report [7], genes related to sulfur metabolism were moderately up-regulated at NaCl addition. The transcriptional levels of most of the lincomycin biosynthetic genes were markedly enhanced at NaCl addition. At 89 h, the most significantly regulated pathways were relatively more diverse, including sulfate assimilation, mental ion binding, intracellular part, cation binding, and carbohydrate metabolic process, *etc*.

3.6. DEGs involved in central carbon metabolism

Embden-Meyerhof-Parnas pathway (EMP) is a pathway for cells to utilize glucose, which provides abundant precursors for Hexose Monophosphate Pathway (HMP), Tricarboxylic Acid (TCA) cycle, sulfur metabolism and lincomycin biosynthesis. The results showed that some key enzymes for EMP were moderately up-regulated, such as the gene *glk*, which encodes glucokinase, was up-regulated by 3.05

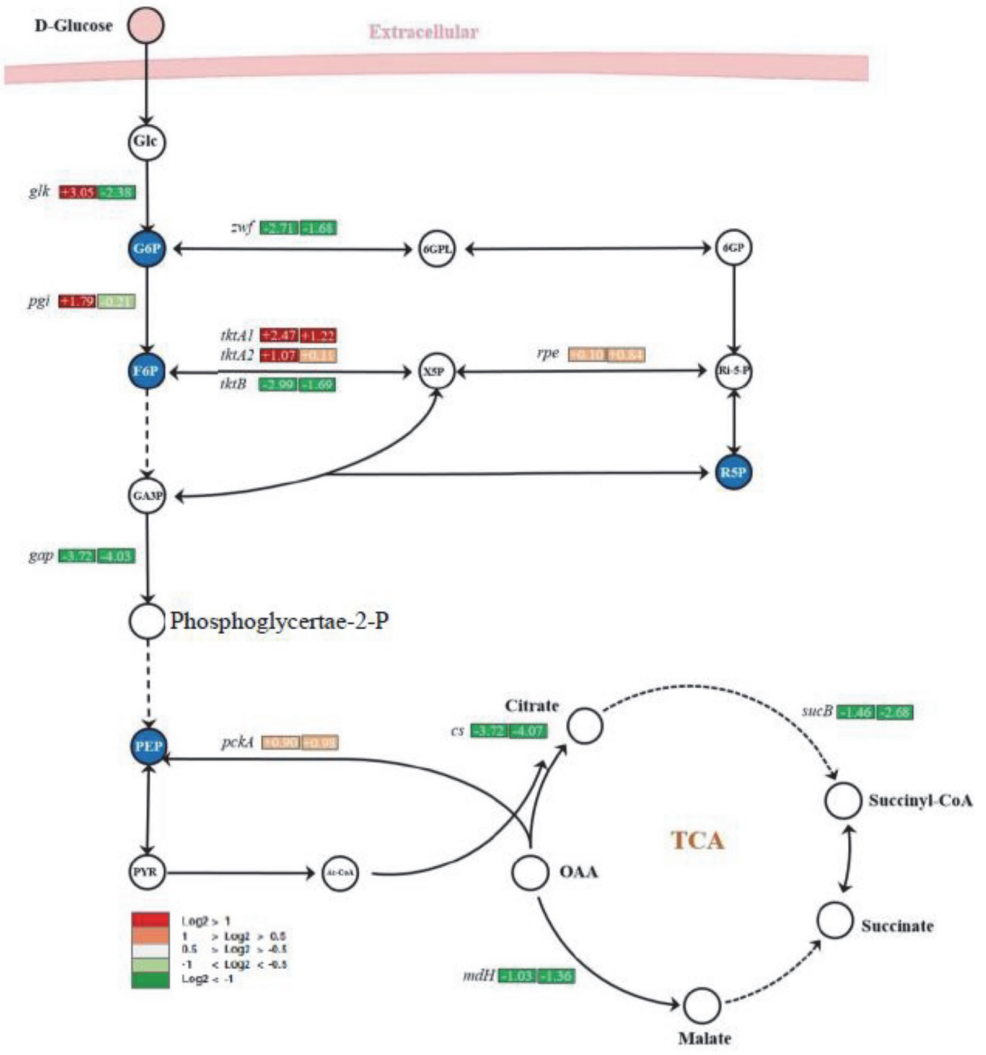

Fig. 5. Transcriptional profiling of central carbon metabolic pathways. Red represents up-regulation and green represents down-regulation. Data in left and right rectangles stand for the $\log_2$fold change (with NaCl addition *vs.* without NaCl addition) at 41 h and 89 h, respectively. Glc: glucose, G6P: Glucose-6-P, F6P: Fructose-6-P, 6GPL: glucono-1,5-lactone-6-P, 6GP: gluconate-6-P, X5P: xylulose-5-P, Ri5P: ribulose-5-P, R5P: ribose-5-P, GA3P: glyceraldehyde-3-P, PEP: phosphoenol pyruvate, PYR: pyruvate, OAA: oxaloacetate.

folds at 41 h ($p < 0.05$) (Fig. 5 and Table S4). Gene *gap*, which encodes glyceraldehyde-3-phosphate dehydrogenase (responsible for the catalyzation of glyceraldehyde-3-phosphate to synthesize 1,3-biphosphoglycerate), was down-regulated by 3.73 and 4.03 folds at 41 h and 89 h, respectively ($p < 0.01$).

HMP provides important precursors for the synthesis of lincomycin. Among them are ribose-5-P and sedoheptulose-7-P for MTL, NADPH for reduction, erythrose-4-P for tyrosine and PPL. Thus, the HMP was very important for lincomycin production. Zhuang *et al*. [17] have shown that adding calcium gluconate could improve lincomycin production. At 41 h, *tkt* orthologs genes encoding transketolase *tktA1* and *tktA2* were up-regulated by 2.47 folds and 1.07 folds, respectively, while *tktB* was down-regulated by 2.99 folds ($p < 0.01$). The gene *zwf*, which encodes glucose-6-P dehydrogenase catalyzing the conversion of glucose-6-P into gluconate-6-P, was also down-regulated by 2.71 folds and 1.68 folds at 41 h and 89 h, respectively.

TCA cycle is the source of precursors for many metabolism pathways such as amnio acids, glycerol and fatty acid. Gene *cs* encoding citrate synthase which is the rate-limiting enzyme for TCA cycle was down-regulated by 3.72 folds and 4.07 folds at 41 h and 89 h ($p < 0.01$), indicating that higher osmotic pressure could decrease TCA cycle flux.

3.7. DEGs involved in sulfur metabolism and biosynthesis pathway of MSH and EGT

The MSH, SAM, and EGT biosynthesis pathways and the

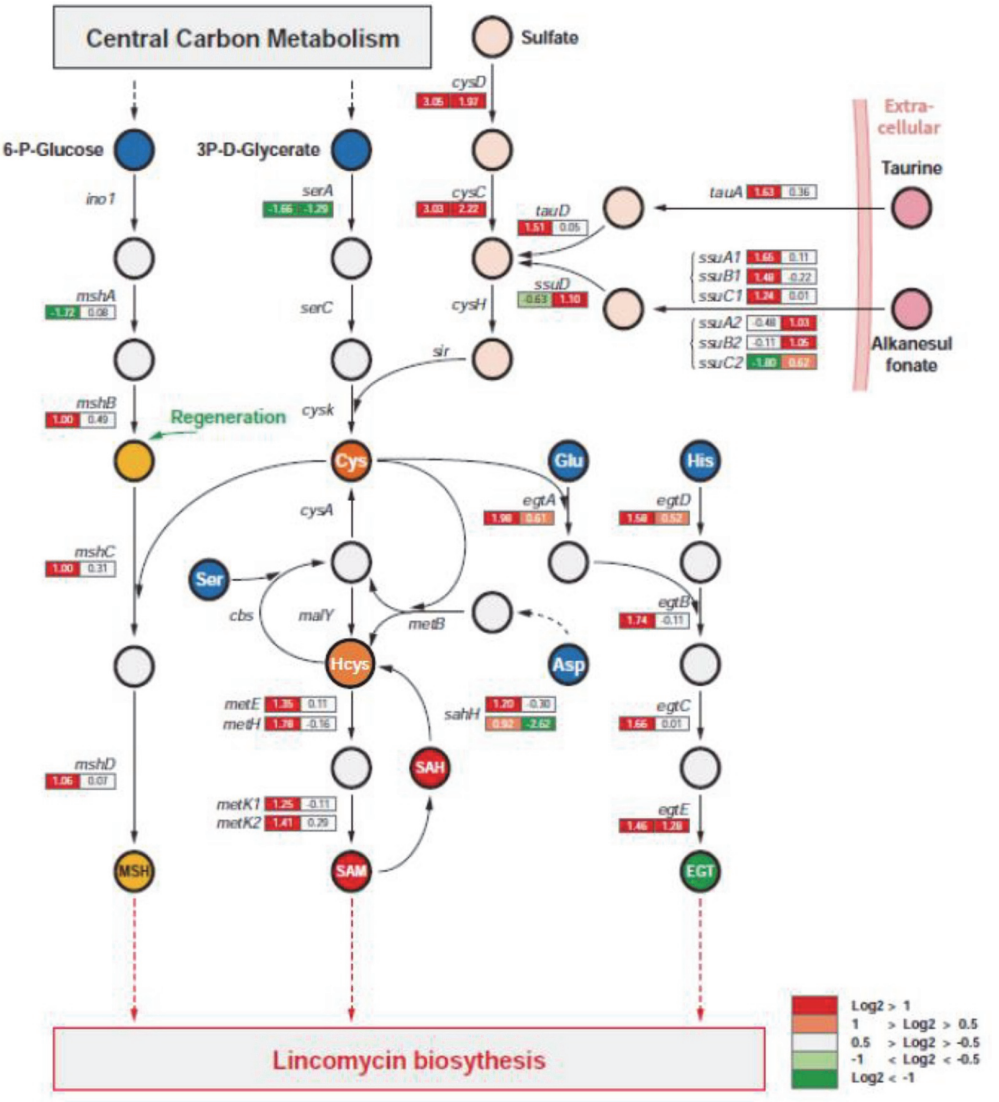

Fig. 6. Sulfur metabolism and biosynthesis pathway of MSH and EGT. Red represents up-regulation and green represents down-regulation. Data in left and right rectangles stand for the $\log_2$fold change (with NaCl addition *vs.* without NaCl addition) at 41 h and 89 h, respectively. Cys: cysteine, Glu: glutamate, His: histidine, Ser: serine, Hcys: homocysteine, Asp: aspartic acid, SAH: S-adenosyl-L-homocysteine, SAM: S-adenosylmethionine, MSH: mycothiol, EGT: ergothioneine.

expression of related genes were studied (Fig. 6 and Table S4). At 41 h, genes related to taurine and sulfonate transmembrane transport, including *tauA* and *ssuA1B1C1* were up-regulated significantly by 1.63, 1.65, 1.48, and 1.24 folds, respectively. Genes related to sulfate metabolism, including *cysD* (encoding adenosine pyrophosphatase), *cysC* (encoding adenosine 5'-phosphosulfate kinase) were up-regulated by 3.05 and 3.03 folds ($p < 0.05$), respectively with NaCl addition. This indicated that osmotic regulation could increase the transcription of genes related to the sulfur assimilation to produce more sulfur-containing metabolites.

At 41 h, the genes involved in the SAM biosynthesis pathway, such as *metE* and *metH*, encoding Hcys S-methyltransferase and Met synthase, were also up-regulated by 1.35 folds and 1.78 folds, respectively with NaCl addition. At the same time, the expression of *metK* encoding Met adenosyltransferase, which catalyzes the reaction from Met to SAM, increased by almost two folds. Interestingly, the gene encoding adenosylcysteinase SahH for recycling of SAH to SAM was up-regulated by 1.20 folds at 41 h. These data showed that the increased osmotic pressure could enhance the synthesis and recycling of SAM to promote lincomycin methylation.

MSH and EGT are important protective antioxidants in actinomycetes and S-donors for lincomycin biosynthesis. The RNA-seq data showed that the genes in MSH biosynthesis pathway, such as *mshB*, *mshC*, and *mshD*, were up-regulated. After the trans-sulfuration in lincomycin

biosynthesis, MSH turns into GlcN-Ins, which is transferred into MSH through a two-step reaction catalyzed by MshC and MshD. In the EGT biosynthesis pathway, *egtABCDE* were also up-regulated by 1.98, 1.74, 1.66, and 1.46 folds, respectively. These results indicated that increased osmotic pressure could improve the synthesis of EGT and MSH with NaCl addition, which might promote the lincomycin production at the early phase.

3.8. DEGs involved in the biosynthesis pathway of lincomycin A

The gene cluster of lincomycin biosynthesis is composed of 23 structural genes, three resistance genes (*lmrA*, *lmrB*, and *lmrC*), and two regulator genes (*lmbU* and *lmbQ*). At 41 h, almost all of the 23 genes were significantly up-regulated by 1.5 folds with NaCl addition compared to the control (Fig. 7 and Table S4). Among them, the expression

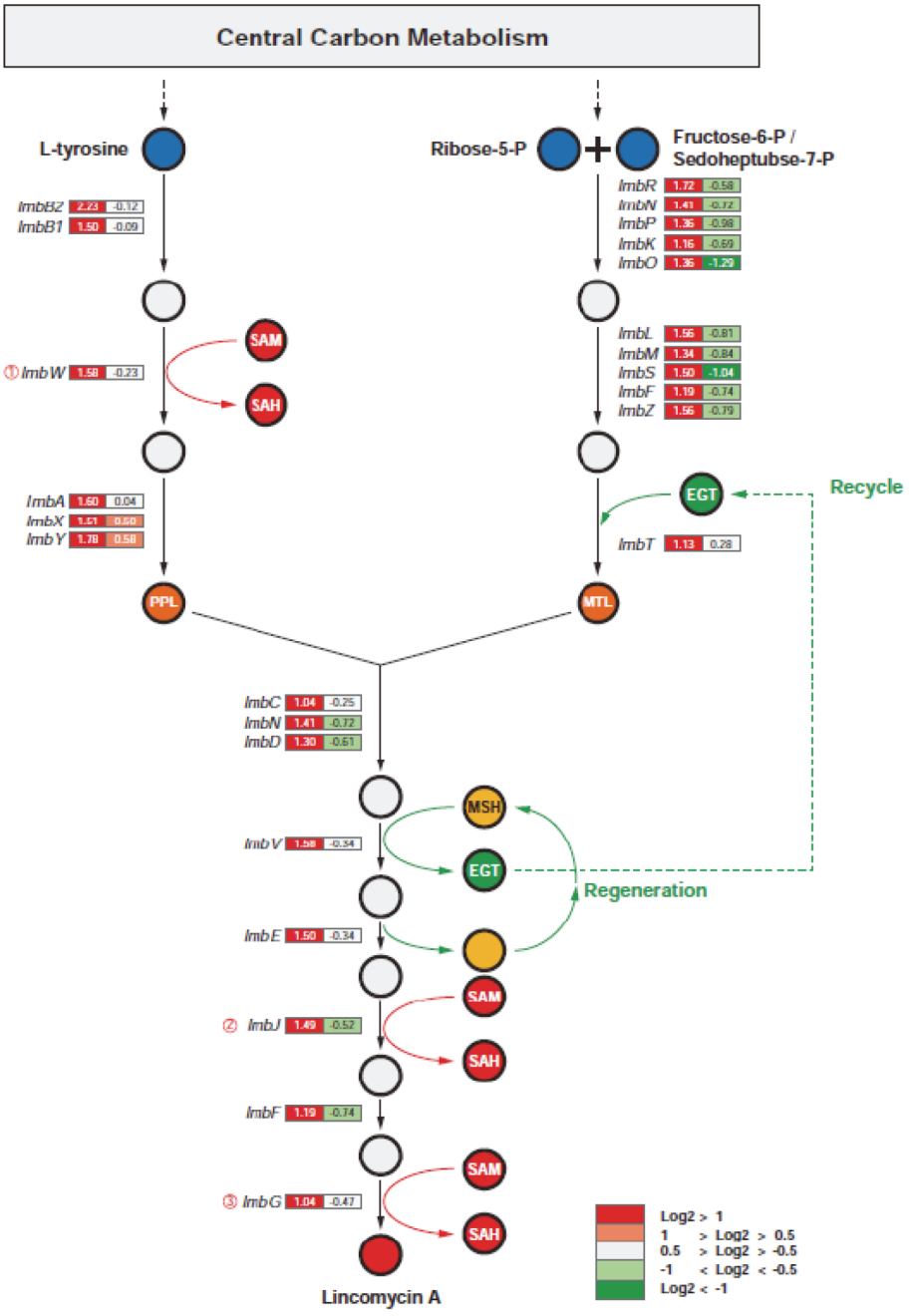

Fig. 7. Biosynthesis pathway of lincomycin A. Red represents up-regulation and green represents down-regulation. Data in left and right rectangles stand for the $\log_2$fold change (with NaCl addition *vs.* without NaCl addition) at 41 h and 89 h, respectively. SAH: S-adenosyl-L-homocysteine, SAM: S-adenosylmethionine, PPL: propyl-L-proline, MTL: methythiolincosaminide, MSH: mycothiol, EGT: ergothioneine.

of *lmbB2*, which catalyzes the first step of PPL synthesis, and *lmbR*, a transaldolase catalyzing the first step of MTL synthesis, were up-regulated by 2.23 and 1.72 folds, respectively. *lmbW*, related to C-methylation, increased by 1.58 folds. The regulator genes, *lmbU* and *lmbQ*, were up-regulated by 1.45 and 1.62 folds, respectively. This indicated that the up-regulation of the cluster could promote the methylation and thus enhance the synthesis of lincomycin.

At 89 h, the expression of resistance genes, *lmrA* (encodes a transmembrane protein relevant to lincomycin secretion) and *lmrC* (encodes the ABC protein for lincomycin output) were up-regulated by 1.13 and 1.36 folds with NaCl addition compared to the control. But most of the production-related genes were slightly down-regulated. Among them, *lmbO*, encoding guanosine transferase for the synthesis of GDP-octose, and *lmbS*, encoding NDP-hexanose amino acid transferase, were down-regulated by 1.36 folds and 1.04 folds, respectively. This might be responsible for the less-promoted production of lincomycin A in the late fermentation period with NaCl addition in the 15 L bioreactor.

4. Conclusion

It was found that the NaCl addition in flask and bioreactor fermentation could promote lincomycin A production and decrease lincomycin B content. In flask, the yield of lincomycin A could reach 0.96 mg/g DCW when adding 10 g/L NaCl into fermentation, an increase of 65% compared to 0.58 mg/g DCW without NaCl addition ($p < 0.01$). The content of lincomycin B was only 0.75%, a sharp decrease of 80% compared to 4.04% without NaCl addition ($p < 0.01$). In 15 L bioreactor fermentation, the contents of lincomycin B were consistently lower after adding 10 g/L NaCl during the whole fermentation process and ended up with a remarkable decrease from 8.20% to 5.78% compared to the control ($p < 0.01$).

The concentrations of intracellular SAM and Hcys were higher with NaCl addition, and the ratio of Hcys to Cys was persistently increased by 20% after 40 h ($p < 0.05$). The higher ratio of Hcys to Cys reflects higher level of methylation [27]. SAM is an important methyl donor for lincomycin methylation. Intracellular SAM with NaCl addition was 38% higher than the control, resulting in a promoted methylation and reduction of lincomycin B.

The level of intracellular EGT was remarkably lower with NaCl addition at the beginning of production phase, indicating that the cells consumed more EGT to produce lincomycin. In the late production phase, the EGT accumulated probably due to the adaption of cells to osmotic stimulation.

The comparison of transcriptome data at 41 h and 89 h with or without NaCl addition were studied to elucidate the influence of osmotic regulation on lincomycin synthesis. 41 h was the beginning of lincomycin production, while 89 h was in the middle period of production. According to the transcriptomics analysis, the most DEGs regulated by osmotic regulation were enriched in sulfur assimilation and metabolism, synthesis of MSH and EGT, and lincomycin biosynthesis pathways. The differential expression of carbon metabolism genes showed that the genes involved in EMP and HMP, such as *glk*, *tktA1* and *tktA2*, were up-regulated while TCA cycle was down-regulated. It was noted that the reversible decarboxylation was up-regulated, inferring the enhancement of biosynthesis in multiple pathways. The transcription of genes related to taurine (*tauA*) and sulfonate transmembrane transport (*ssuA1B1C1*) were up-regulated by 1.63, 1.65, 1.48, and 1.24 folds, respectively. Genes of sulfur metabolism (*cysD* and *cysC*) were significantly up-regulated by about 3 folds and 2 folds at 41 h and 89 h, respectively. Genes related to MSH and EGT biosynthesis, *mshBCD* and *egtABCDE* were all up-regulated at 41 h. Genes related to SAM biosynthesis, *metEHK1K2* were also up-regulated at 41 h by 1.35, 1.78, 1.25 and 1.41 folds, respectively. This indicated that osmotic regulation enhanced the sulfur assimilation and production of Cys related substances, such as SAM, MSH and EGT, especially at 41 h. All the above metabolites could positively influence the production of lincomycin A and the reduction of lincomycin B. The DEGs involved in the lincomycin synthesis cluster were also up-regulated with NaCl addition. SAM was reported to act as a signal molecule to promote the transcription of lincomycin biosynthesis genes *lmbA*, *lmbR*, and *lmbU* [19]. The up-regulated transcription of lincomycin synthesis cluster might be the results of higher SAM concentration after osmotic regulation.

EGT and MSH are low molecular weight thiols participating in a number of important biological functions as redox buffers and reducing cofactors [10]. It had been demonstrated a constructive role of EGT and MSH, in which EGT serves as a carrier via the first reversible S-glycosylation, and then transfer to MSH through a second irreversible S-glycosylation. EGT and MSH are recyclable or reproducible, thus maintaining the biosynthetic pathway to lincomycin A. The first S-conjunction was catalyzed by LmbT with an equilibrium constant Keq of 1.94 [6].

EGT is a histidine derivative synthesized from Cys, SAM, glutamate and ATP catalyzed by EgtABCDE in mycobacteria [8]. Our results showed that all the EGT biosynthesis genes were upregulated after NaCl addition, which indicated an increased production of EGT. But the EGT concentration was slightly lowed compared to the control at 65 h, which might indicate more S-conjunction for lincomycin A by LmbT.

It was found that the amount of Cys and MSH biosynthesis was induced by osmotic stress [7]. Cys could be the precursor to provide anti-oxidative substance of MSH and Fe-S cluster to resist oxidative damage after osmotic shock. In our result, those genes for synthesis of Cys, MSH, EGT and sulfur assimilation were also upregulated, which might contribute to the anti-oxidation function. Cys is the substrate to synthesize many sulfur-containing substances, such as MSH, EGT and SAM. The fluctuation of intracellular Cys concentration maybe the result of many demands for biosynthesis of these sulfur containing substance.

It was reported that elevated pool of SAM could increase the lincomycin A production and the transcription of lincomycin biosynthetic genes *lmbA*, *lmbR* and regulatory gene *lmbU* [19]. These results demonstrated that SAM may function as a transcriptional activator of lincomycin biosynthetic pathway. Another research studied the co-overexpression of *lmbW* (encoding C-methyltransferase) and *metK* (encoding S-adenosylmethionine synthetase), which showed a 35% improvement in lincomycin titer and a remarkable decrease of lincomycin B content. In our results, transcription of *metK*, *metK*, and *lmbW* were all enhanced significantly at 41 h, and the intracellular SAM concentration were higher after NaCl addition, which might be the reason of decreased lincomycin B content and increased lincomycin A yield for the higher pool of SAM and higher LmbW activity.

Although the exact regulatory mechanism of lincomycin biosynthesis is unclear, some transcription regulators have been detected. Among them, the coding gene of LmbU lies in the lincomycin biosynthetic gene cluster, and positively regulates the transcription of the most lincomycin biosynthetic genes, such as *lmbA*, *lmbC*, *lmbJ*, *lmbW*, while inhibits transcription of *lmbK* and *lmbU* [28]. It has also been reported that some global or pleiotropic regulators can directly regulate the transcription of the *lmb* genes. BldA, BldD, AdpA and SLCG_Lrp function as positive regulators for lincomycin biosynthesis [29]. A TetR family regulator SLCG_2919 represses lincomycin production [29].

A redox-sensing regulator Rex had recently been found to promote the transcription of gene *lmbU* and the structural genes *lmbA*, *lmbC*, *lmbV*, *lmbW*, while negatively regulates cell growth and positively regulates lincomycin production in *S. lincolnensis* [29]. We detected a marginal down-regulated transcription of *rex* at 89 h (0.79 folds), which might regulate the lincomycin gene cluster responded to osmotic pressure and oxidative stress.

The lincomycin biosynthetic gene cluster consists of three lincomycin resistance proteins: LmrA, LmrB and LmrC. LmrA is a lincomycin transporter to ensure sufficient self-resistance; LmrB is a 23S rRNA methyltransferase, confers high resistance to lincomycin; LmrC is an ATP-binding cassette family F ATPase. Recent results showed that LmbC has dual functions of moderate antibiotic resistance and simultaneously a ribosome-mediated attenuator to gene expression. LmrC responses to lincosamide and triggers the transcription of *lmbU* and consequently the expression of lincomycin biosynthetic gene cluster [30]. Both LmrA and LmrB reduce *lmrC* expression in response to antibiotics, which may serve as a negative feedback loop to the lincomycin-LmrC-LmbU signaling cascade of lincomycin biosynthesis [30]. The higher intracellular concentration of lincomycin A at the stationary phase might be the reason of increased transcription of *lmrA*. Our results showed that the transcription of gene *lmrA* and *lmrC* were up-regulated by 1.13 and 1.36 folds, respectively, but the most of the lincomycin biosynthetic gene cluster were slightly down-regulated, in which, *lmbO*, encoding guanosine transferase for the synthesis of GDP-octose, and *lmbS*, encoding NDP-hexanose amino acid transferase, were down-regulated by 1.36 folds and 1.04 folds, respectively. All these results showed that the regulation of lincomycin biosynthesis is complex, which is controlled by multiple regulators to maintain synchronized antibiotic production.

Overall, the increased production of lincomycin A and decreased content of lincomycin B might be contributed to the higher pool of SAM and LmbW activity to promote C-methylation, and rapid turnover of S-glycosylation with EGT and MSH by LmbT and LmbV. NaCl addition increased the osmotic pressure. It is well known that osmotic shock could result in an increase in reactive oxygen species and deformation of cell components, and thus makes them more labile to oxidative damage. To overcome the osmotic stress and oxidative damage, cells synthesized osmo-protectants such as proline, glycine betaine, trehalose, Cys and MSH to provide anti-oxidative function [7]. Our results showed the increase osmotic pressure promote the biosynthesis of SAM, MSH, Cys, and EGT, with the upregulated transcription of *lmbW*, *lmbT*, and *lmbV*. All these results could explain the increased production of lincomycin A and decreased content of lincomycin B.

A negative feedback loop to the lincomycin-LmrC-LmbU signaling cascade of lincomycin biosynthesis was discovered recently, which added additional regulation of lincomycin biosynthesis. At the stationary phase (89 h), the titer of lincomycin A had reached an equivalent concentration of flask fermentation. The higher lincomycin concentration may trigger the negative feedback loop to control the lincomycin production, so the most of lincomycin biosynthetic genes were down-regulated.

Overall, a simple and effective method was established to reduce the content of lincomycin B based on the osmotic regulation via NaCl addition. The osmotic regulatory mechanism on metabolite production and gene transcription

were also studied. The results showed that the biosynthesis of Cys, SAM, EGT and MSH were improved with osmotic regulation, more transmethylation precursors and sulfur donors were supplied during lincomycin biosynthesis to reduce the lincomycin B and increase the lincomycin A production. SAM also acts as a signal molecule that can promote the transcription of *lmbA*, *lmbR*, and *lmbU* and thus can enhance lincomycin A production. Our data indicated that under osmotic pressure, carbon metabolism, sulfur assimilation and lincomycin synthesis were enhanced, which might provide more methyl and sulfur donors for lincomycin biosynthesis.

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Author's Contributions

SL was responsible for implement of the experiments, data analysis and manuscript writing. LZ was responsible for the design and implement of the experiments. SG was responsible for the funding, experiment design and revision of the manuscript. All authors read and approved the final manuscript.

Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

Data Availability

All data and materials are available in the main text and additional file.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-023-0007-6) contains supplementary material, which is available to authorized users.

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