Canadian Journal of Microbiology

Positive regulation of MarR-type regulator *slnO* and improving salinomycin production of *Streptomyces albus* by multiple transcriptional regulations

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2021-0342.R1
Manuscript Type:	Article
Date Submitted by the Author:	12-Dec-2021
Complete List of Authors:	Zhang, Hongrui; Zhejiang Chinese Medical University Chen, Weiwei; Zhejiang Chinese Medical University Wang, Xinyi; Zhejiang Chinese Medical University Li, Yongquan; Zhejiang University Zhu, Zhenhong; Zhejiang Chinese Medical University
Keyword:	<i>Streptomyces albus</i> , Salinomycin, MarR-family, <i>SlnO</i> , Transcriptional regulation
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

**Positive regulation of MarR-type regulator slnO and improving
salinomycin production of *Streptomyces albus* by multiple
transcriptional regulations**

Hongrui Zhang¹, Weiwei Chen¹, Xinyi Wang¹, Yongquan Li^{*2}, Zhenhong Zhu^{*1}

¹College of Life Science, Zhejiang Chinese Medical University, Hangzhou 310053, China;

² Institute of Pharmaceutical Biotechnology, School of Medicine, Zhejiang University,

Hangzhou 310058, China

***Corresponding author**

E-mail: zhenhongzhu@aliyun.com; lyq@zju.edu.cn

Draft

Abstract

The purpose of this study is to explore the function of MarR-family regulator *slnO*. In addition, the high-yield strain of salinomycin was constructed by using combined regulation strategies.

Firstly the *slnO* gene over-expression strain (GO) was constructed in *Streptomyces albus*. Compared to wild type (WT) strain, salinomycin production in GO strain was increased about 28%. Electrophoretic mobility gel shift assays (EMSAs) confirmed that SlnO protein can bind specifically to the intergenic region of *slnN-slnO*, *slnQ-slnA1* and *slnF-slnT*. qRT-PCR experiments also showed that *slnA1*, *slnF*, and *slnT1* were significantly up-regulated, while the expression level of the *slnN* gene was down-regulated in GO strain. Secondly, *slnN* gene deletion strain (slnNDM) was used as the starting strain, and the pathway specific gene *slnR* in salinomycin gene cluster was over expressed in slnNDM. The new strain was named ZJUS01. The yield of salinomycin in ZJUS01 strain was 25% and 56% higher than that in slnNDM strain and WT strain.

Above results indicate that the *slnO* gene has a positive regulation effect on the biosynthesis of salinomycin. Meanwhile, the yield of salinomycin could be greatly increased by manipulating multiple transcriptional regulations.

Keywords *Streptomyces albus*, Salinomycin, MarR-family, SlnO, Transcriptional regulation

Introduction

Streptomyces is a gram-positive filamentous soil bacterium characterized by a complex life cycle and the production of a wide variety of bioactive secondary metabolites (Andam et al. 2016). These secondary metabolites are applied in antibiotics or anti-cancer treatments and other agricultural or medical aspects. The regulation of antibiotic biosynthesis is a complex process and

involves strict regulation. The gene clusters are usually regulated by pathway-specific transcriptional activators that are physically linked to the clusters (Lu et al. 2018). Investigation the regulatory genes in the antibiotic biosynthesis cluster is important for better understanding of the antibiotic regulatory mechanism; however, it may also provide strategies for constructing industrially important strains to increase the yield of their secondary metabolites. Thus far, there are at least 20 families of transcriptional regulators in *Streptomyces*, including TetR, LuxR, LysR, AraC, GntR, SARP, ROK, MerR, and MarR. MarR (multiple antibiotic resistance regulators) family transcriptional regulators are single-component response regulators that regulate the expression of coding genes in various cells, including metabolic pathways, stress responses, virulence, and degradation or excretion of antibiotics and phenolic compounds (Deochand and Grove 2017; Gong et al. 2019). To date, most members of the MarR family are characterized by inhibition of transcription, and only a small number are transcriptional activators (Otani et al. 2016). They have winged helix-turn-helix (wHTH) DNA-binding proteins that exist as dimers and bind palindromic sequences within cognate promoters. MarRs are the fourth most abundant family of transcriptional regulators in streptomyces genome, with an average of about 50 regulatory proteins of this family per genome. Despite the richness of this family of proteins, there are few reports on experimental studies on these regulatory proteins, such as the synthesis of the sesquiterpene antibiotic pentalenolactone in *S. exfoliatus* UC5319 and *S. arene* TU469 is regulated by *PenR* and *PntR*, respectively (Zhu et al. 2013).

Salinomycin, a polyether ionophore antibiotic produced by *S. albus*, is widely used as anticoccidial drugs and growth promoters in animal husbandry. It has been used for 30 years as an anticoccidial drug and to improve nutrient absorption and feed efficiency in ruminants and swine

(Zhang et al. 2017). It has the ability to selectively chelate metal ions in a hydrophobic matrix, and then transport the ions to the cell membrane, which destroys the physiological ion gradients of the cells and eventually results in cell death (Zhu et al. 2017). In addition, researchers have discovered new uses of salinomycin. Studies have shown that salinomycin not only kills breast cancer stem cells in mice, but also inhibits them from producing new tumor cells, while also slowing the growth of existing tumors (Dewangan et al. 2017). Salinomycin can also inhibit the growth of various tumors such as liver and pancreatic cancer (Sun et al. 2017). This will provide a theoretical basis of the research of anti-tumor drugs.

The gene clusters for salinomycin biosynthesis (*sal*) have been cloned and identified from *S. albus* XM211 and DSM 41398 respectively, and the *sal* gene clusters in the two strains are completely homologous (Jiang et al. 2012). In *S. albus* XM211, the *sal* gene clusters include genes encoding a type I PKS (*slnA1-slnA9*, *slnDI*, *slnD II*), oxidative cyclization (*slnB I*, *slnB II*, *slnB III*, *slnE*, *slnC*), hydroxylation (*orf11*), regulation (*slnR*, *orf15*), export (*slnT I*, *slnT II*) and other ancillary genes (*slnF*, *slnM*) (Jiang et al. 2012). Analysis of the 104 kbp continuous sequence in *S. albus* DSM 41398 by bioinformatics revealed 33 open reading frames, of which 27 are believed to play a role in the biosynthesis of salinomycin, including nine *sal* PKS genes (*salA I-salA IX*), upstream PKS genes *salN*, *salO*, *salP*, *salQ*, downstream PKS genes *salE*, *salF*, *salD*, *salC*, *salJ*, *salX*, *salL*, etc (Yurkovich et al. 2012). In fact, *S. albus* XM211 also has *salN* and *salO* genes that are completely similar to *S. albus* DSM 41398 (herein named *slnN*, *slnO*). The *slnN* and *slnO* are located upstream of the salinomycin biosynthetic gene cluster. Bioinformatics analysis showed that *slnN* and *slnO* belongs to MarR family with the typical MarR family WHTH motif (Fig.1). *SlnN* had been confirmed that it has a significant negative regulatory effect on the biosynthesis of

salinomycin (Zhu et al, 2019). *SlnR*, as a positive pathway-specific regulatory factor in the *sal* gene cluster, the increase in its transcription level can directly improve the yield of the salinomycin production (Zhu et al. 2017). However, the regulatory role of *SlnO* involved in salinomycin biosynthesis is unclear.

The purpose of this study was to clarify the regulatory role of *slnO* gene in salinomycin biosynthesis. In addition, in order to construct salinomycin high expression strain, we overexpressed *slnR* gene in *slnN* gene knockout strains (*slnNDM*) to explore multiple transcriptional regulation to improve the yield of salinomycin.

Materials and methods

Strains, plasmids, and growth conditions

S. albus BK3 (wild-type strain) was obtained from Zhejiang Shenghua Biok Biological Company (China). It was incubated at 28 °C and grown on ISP4 agar plates (Difco™, BD) for sporulation. Liquid YEME medium (0.3 % yeast extract, 0.3 % malt extract, 0.5% tryptone, 4% glucose) was used for the growth of mycelia for the purpose of extracting DNA. The seed and fermentation medium were as described previously (Zhu et al. 2017). *E. coli* TG1 and *E. coli* BL21 (DE3) (Novagen) were used as cloning and expression hosts respectively. *E. coli* ET12567 (*dam dcm hsdS*) was used to propagate nonmethylated DNA for transformation into *S. albus*. *Bacillus subtilis* was used as the indicator strain for salinomycin bioassays. *E. coli* strains grow at 37 °C in Luria-Bertani (LB) medium. The medium is supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml), apramycin (50 µg/ml), thiostrepton (50 µg/ml) and chloramphenicol (25 µg/ml) as needed. Vector pIJ8660 (apramycin resistant), pIJ8661 (Derived from pIJ8660,

spectinomycin resistant) were used to provide the *S. albus* strong constitutive promoter ermE*_p. pGEX-2T (Novagen) was used for production of recombinant GST-tagged protein in *E. coli*.

Overexpression of the *slnO* gene in *S.albus* wt strain (BK3)

A DNA fragment encoding the predicted 160 amino acids of the *slnO* protein was generated by PCR using primers slnO8660-F/slnO8660-R (Table S1). The PCR product was digested with *NdeI/KpnI* and ligated to generate the recombinant plasmid pIJ8660-slnO. The constructed pIJ8660-slnO was transformed into ET12567/pUZ8002 and introduced by intergenic conjugation into *S. albus* wt to obtain the new strain, which generated the apramycin-resistant exconjugants. The genotype of the exconjugants was confirmed by PCR analysis and named GO strain.

Expression and purification of the recombinant GST-tagged SlnO protein

The 483-bp *slnO* coding region (160 amino acids) was amplified by PCR with primers 2TO-F and 2TO-R. The PCR fragment was digested with *BamHI/EcoRI* and inserted into expression vector pGEX2T to generate pGEX2T-slnO recombinant plasmid, which was verified by DNA sequencing. pGEX2T-slnO was transformed into *E. coli* BL21, and recombinant protein was induced with 0.1 mM IPTG for 20 h at 16°C. Cells were collected by centrifugation, and disrupted in lysis buffer (10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, 2 mM KH₂PO₄ pH 7.4) by sonication on ice. The supernatant was then recovered by centrifugation (4 °C, 13,000×g for 10 min). Soluble GST-SlnO was purified with GST binding resin, as described by the manufacturer's protocol (Novagen, USA).

Electrophoretic mobility gel shift assays

The EMSAs were performed as reported by Zhu et al. 2017. The promoter regions were amplified by PCR with their respective primers (Table S1), then cloned into a pTA2 vector and verified by

sequence analysis. The binding reaction system consisted of 1 ng labeled probes, 10 µg salmon sperm DNA mixed with 0.5, 1, 2 mM purified GST-tagged SlnO in a total volume of 10 µl. After incubation of the mixture at 30°C for 30 min, the samples were separated on 5% PAGE gels in ice-cold 0.5×TAE buffer at 50 mA for about 1 h.

RNA extraction and quantitative RT-PCR analysis

Total RNA was prepared from mycelium grown in YMG liquid medium by using a RNA extraction kit (Ailaide, China). Contaminating DNA was removed by DNaseI (Takara, Japan) treatment according to the manufacturer's instructions. The cDNA first-strand synthesis was performed using M-MLV reverse transcriptase (Takara, Japan) according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) analysis was performed by SYBR Premix Ex Taq kit (Takara, Japan) primers listed in Table S1. The *hrdB* gene, which encodes the major sigma factor in *Streptomyces*, was used as the internal positive control. Each qPCR experiment was done in triplicate using RNA samples.

Salinomycin bioassay and HPLC analysis

Taking *B. subtilis* as an indicator strain, the salinomycin produced by *S. albus* or mutant strains were determined using the disc agar diffusion method. For HPLC analysis, the fermentation broth (5 ml) was extracted with 45 ml of methanol for 30 min and centrifuged at 12,000×g for 10 min. The supernatant is filtered by organic filter membrane, and 10 µl was directly applied to a high performance liquid chromatography (HPLC) system (Aglient 1260 infinity), three duplicate fermentation cultures were used. The column was packed with C18 (angilent XDB C18 4.6 × 150 mm) and developed with acetonitrile (91 %) and Ultra pure water (9 %). The flow rate was 0.4

mL/min. Salinomycin was detected by UV absorption at 210 nm where authentic samples of salinomycin were used as internal standards.

Construction of salinomycin high-yielding strain by multiple transcriptional regulations

In previous studies, *slnR* was confirmed as a pathway specific positive regulatory gene (Zhu et al. 2017). Overexpression of *slnR* gene can increase the yield of salinomycin about 25%. In addition, *slnN* was demonstrated to be a negative regulatory gene for salinomycin biosynthesis (Zhu et al. 2019). In order to construct salinomycin high-yield strain, we intend to overexpress *slnR* gene on the basis of *slnN* gene deletion strain (*slnNDM*). The full-length *slnR* gene (2718 bp) was amplified by PCR using the primers P8661-F and P8661-R. The PCR products were digested with *NdeI/KpnI* and then inserted into the integrative vector pIJ8661 (spectinomycin resistance) to produce the recombinant vector pIJ8661-*slnR*. The recombinant plasmid was introduced by intergenic conjugation into *slnNDM*. This generated the spectinomycin -resistant exconjugants. The new genetically engineered strain was identified by PCR and named ZJUS01.

Results

Construction and identification of *slnO* gene overexpression strain

In order to explore the role of *slnO* in salinomycin biosynthesis, we constructed the *slnO* gene overexpression strain (named GO) using the integrated plasmid pIJ8660, so that the *slnO* gene was under the control of the strong promoter *ermE**p. The recombinant strain was screened on ISP4 plates with apramycin, and then verified by PCR with primers *ermE**p-F/2TO-R. PCR amplification experiment showed that about 750 bp band was amplified using the GO strain genome as the template, but no band could be amplified from the WT genome (Fig.S1). The result indicate that the strong promoter *ermE**p had been fused to the chromosome.

Overexpression of *slnO* leads to increase of salinomycin production

Using *B. subtilis* as the indicator strain, a biological indicator test was used to compare the bacteriostatic effect between the *S. albus* WT and the GO strain. The experimental results showed that the inhibition zone of GO strain was significantly larger than that of WT (Fig. S2). These findings indicate that the *slnO* gene may be positively regulate the biosynthesis of salinomycin.

In order to verify the yield of salinomycin in GO strain, shake flask fermentation was carried out between GO strain and WT strain. Salinomycin production was monitored over 9 days at every 24 hours. It was found that there was little difference in the yield of salinomycin in the first 4 days. After the fifth day, the yield of GO strain increased dramatically, reached the peak on the 7th-8th day, and the yield was about 28% higher than that of WT strain (Fig. 2). The above results clearly show that salinomycin production in *S. albus* can be increased by the overexpression of *slnO*.

SlnO protein binds specifically to the intergenic region of *slnN-slnO*, *slnQ-slnA1*, *slnF-slnT1*

To examine whether *slnO* might directly regulate the transcription of genes in salinomycin biosynthesis, we expressed recombinant soluble protein GST- SlnO in *E. coli* BL21 (DE3) and purified by GST binding affinity resin (Fig. S3). The soluble expression of GST-SlnO protein and eight biotin-labeled probes (probes 1–8) containing all possible promoter regions were used for the EMSAs (Fig. 4a). The results showed that probe 1 (290bp of the *slnN-slnO* intergenic region), probe 2 (288bp of the *slnN-slnO* intergenic region), probe3 (308bp of the *slnQ-slnA1* intergenic region), and probe 4 (248bp of the *slnF-slnT1* intergenic region) could all bind to SlnO protein, and the binding strength increased with the increase of protein concentration (Fig 4b). Nevertheless, no retarded signals occurred with probes 5, 6, 7, or 8 (Fig. S6 & S7). SlnO, as a regulator of the MarR family, can be bind to the intergenic region of the *slnN-slnO*. This finding

revealed that *slnO* could regulate the expression of the *slnN*. In addition, SlnO can also direct interaction with the *slnQ-slnA1*, *slnF-slnT1* gene, this show SlnO can improve the biosynthesis of salinomycin, at the same time, and it promotes the efflux of salinomycin.

Regulation of *slnO* on salinomycin biosynthetic genes

Protein-blast comparison analysis of the *slnO* gene found that the gene belongs to the MarR family regulatory gene, but the MarR family regulatory gene can be either positively regulated or negatively regulated. Through overexpression of this gene, bacteriostasis experiments and shake flask fermentation experiments have confirmed the increase in salinomycin production, speculating that the *slnO* gene may have a positive regulatory effect on the biosynthesis of salinomycin. To further elucidate the relationship between *slnO* and salinomycin production, we performed quantitative RT-PCR analysis using total RNAs isolated from mycelium of GO strain (Fig. 5). Among the high-expression strains of *slnO* gene, genes related to the salinomycin synthesis cluster such as *slnQ*, *slnA1*, *slnF*, and *slnT1* were significantly up-regulated, while the expression level of the *slnN* gene was down-regulated, and no clear difference was observed in transcription levels of other genes. These results indicated that *slnO* had a certain positive effect on salinomycin biosynthesis. Its mechanism of action is on the one hand to suppress the negative regulation effect of the *slnN* gene. On the other hand, it also promotes the biosynthesis of salinomycin. QRT-PCR analysis revealed that the over-expression of the *slnO* gene had an inhibitory effect on the *slnN* gene, and the inhibition of the *slnN* gene contributed to the biosynthesis of salinomycin. From the hypothetical scheme of the regulatory pathway of *slnO* (Fig. 4), we can understand the regulatory role of MarR family member SlnO in the salinomycin biosynthesis cluster.

Improvement of salinomycin production by manipulating pathway regulator

In this study, based on the knockout strain of *slnN* gene (slnNDM), taking slnNDM strain as the chassis, the *slnR* gene was overexpressed in slnDM strain. Through screening by plates containing spectinomycin, the obtained strain was named ZJUS01. The screened strains were identified by PCR, as showing in Fig. S4. The *ermE**p promoter sequence was used as the upstream primer, and a segment of the internal sequence of *slnR* gene was used as the downstream primer. The amplified band size is about 2500 bp, which is consistent with the target size, indicating that the *ermE**p promoter has been integrated into the slnDM genome.

The bacteriostatic experiment showed that the bacteriostatic circle diameter formed by ZJUS01 strain was the largest, followed by slnNDM, and the bacteriostatic circle diameter of WT was the smallest. It shows that the production of salinomycin can be further improved by overexpression of *slnR* gene with slnNDM strain (Fig. S5). Shake flask fermentation experiment also showed that the yield of ZJUS01 strain increased significantly, reached the peak at the 7th-8th day, and the yield was about 56% higher than that of the WT strain, as showed in Fig.6. The results reported that the yield of salinomycin could be greatly increased by the combination strategy of knocking out negative regulatory genes and increasing positive regulatory genes.

Discussion

In this study, we demonstrated that SlnO has a positive regulation effect on the salinomycin production in the *S. ablus*. It has been reported that overexpression of positive regulator is one way to improve strain productivity (Gong et al. 2019). Shake flask fermentation experiments revealed that the significantly enhanced production of salinomycin (up to 28%) in the genetically engineered strain GO. EMSA is widely used to study the in vitro interaction of regulators and

promoters. In this study, SlnO protein can directly act on the intergenic region of *slnN-slnO*, *slnQ-slnA1*, *slnF-slnT1*. Bioinformatics analysis and prediction showed that the binding sequence of SlnO and probes was characterized by TGAA(A/T)(G/T). These findings indicate that SlnO can affect the expression of *slnN* gene, and promote the biosynthesis of salinomycin and accelerate the efflux of salinomycin. qRT-PCR indicated that the over-expression of the *slnO* gene would cause the up-regulation of the *slnQ*, *slnA1*, *slnF*, and *slnT1* genes, while the expression level of the *slnN* gene was down-regulated. Especially, no effect on the expression levels of *slnR* gene indicates that SlnO and SlnR seem to have no synergy. SlnN, another MarR family regulator in *sal* gene cluster, had been proved a negative regulator; and SlnR has been confirmed as a pathway specific positive regulatory gene. In this study, we tried to overexpress *slnR* gene in *slnN* deletion strain (slnNDM). The purpose is to further improve the yield of salinomycin by multiple regulations. In fact, a new mutant strain ZJUS01 was constructed by highly expressing *slnR* in slnNDM strain. The yield of salinomycin in ZJUS01 strain was 56% higher than that of WT. The results show that the combined regulation strategy is better than the single regulation strategy.

MarR regulators have been identified with antibiotic and other secondary metabolite gene clusters from *Streptomyces*, but only a few regulators have been characterized in the genus, including *S. avermitilis SAV4189* (Guo et al. 2018), *S. aureofaciens F3 CtcS* (Kong et al. 2019), and so on. Actually, several regulators for salinomycin production have been identified and reported. By comparing the transcription of the salinycin biosynthesis gene *slnA3* in DSM 41398 and BK3, it was found that transcriptional regulation plays an important role in the overproduction of salinomycin (Zhang et al. 2017). SlnR binding sites were located at the *slnQ-slnA1*, *slnF-slnT1*, and *slnC-slnB3* intergenic regions (Zhu et al. 2017). Since the SlnO binding site is located in the

intergenic region between *slnQ-slnA1* and *slnF-slnT1*, it infers that both transcription factors SlnO and SlnR can act on the intergenic regions of *slnQ-slnA1* and *slnF-slnT1* genes, but there seems to be a lack of synergy between them. In summary, our findings provide a basis of the rational manipulation of *S. albus* to improve salinomycin production by engineering of *slnO*. Next, we will construct *slnO* gene deletion strains to verify whether *slnO* gene is necessary for salinomycin biosynthesis. It is worth noting that the synergistic relationship between SlnO and other regulatory factors will promote the mechanism analysis of polyether biosynthesis in *streptomyces albus*.

Funding This work was funded by National Key R & D plan, key special project of synthetic biology, reconstruction and optimization of biological system for actinomycete drug synthesis (2019YFA09005400).

Declarations

Conflicts of interest the authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

References

- Andam, C.P., Doroghazi, J.R., Campbell, A.N., Kelly, P.J., Choudoir, M.J., Buckley, and D.H. 2016. A Latitudinal Diversity Gradient in Terrestrial Bacteria of the Genus *Streptomyces*. *mBio*. 7(2): e02200-15.
- Deochand, D.K., and Grove, A. 2017. MarR family transcription factors: dynamic variations on a common scaffold. *Crit. Rev. Biochem. Mol. Biol.* 52(6): 595-613.
- Dewangan, J., Tandon, D., Srivastava, S., Verma, A.K., Yapuri, A., and Rath, S.K. 2017. Novel combination of salinomycin and resveratrol synergistically enhances the anti-proliferative and

- pro-apoptotic effects on human breast cancer cells. *Apoptosis*, 22(10):1246-1259.
- Gong, Z., Li, H., Cai, Y., Stojkoska, A., and Xie, J. 2019. Biology of MarR family transcription factors and implications for targets of antibiotics against tuberculosis. *J. cell. Physiol.* 234(11): 19237-19248.
- Guo, J., Zhang, X., Lu, X., Liu, W., Chen, Z., Li, J., Deng, L., and Wen, Y. 2018. SAV4189, a MarR-Family Regulator in *Streptomyces avermitilis*, Activates Avermectin Biosynthesis. *Front. Microbiol.* 9:1358.
- Jiang, C., Wang, H., Kang, Q., Liu, J., and Bai, L. 2012. Cloning and characterization of the polyether salinomycin biosynthesis gene cluster of *Streptomyces albus* XM211. *Appl. Environ. Microbiol.* 78(4): 994-1003.
- Kong, L., Liu, J., Zheng, X., Deng, Z., and You, D. 2019. CtcS, a MarR family regulator, regulates chlortetracycline biosynthesis. *BMC microbial.* 19(1): 279.
- Lu, W., Alanzi, A.R., Abugrain, M.E., Ito, T., and Mahmud, T. 2018. Global and pathway-specific transcriptional regulations of pactamycin biosynthesis in *Streptomyces pactum*. *Appl. Microbiol. Biotechnol.* 102(24): 10589-10601.
- Otani, H., Stogios, P.J., Xu, X., Nocek, B., Li, S.N., Savchenko, A., and Eltis, L.D. 2016. The activity of CouR, a MarR family transcriptional regulator, is modulated through a novel molecular mechanism. *Nucleic Acids Res.* 44(2): 595-607.
- Sun, J., Luo, Q., Liu, L., Yang, X., Zhu, S., and Song, G. 2017. Salinomycin attenuates liver cancer stem cell motility by enhancing cell stiffness and increasing F-actin formation via the FAK-ERK1/2 signalling pathway. *Toxicology*, 384: 1-10.
- Yurkovich, M.E., Tyrakis, P.A., Hong, H., Sun, Y., Samborsky, M., Kamiya, K., and Leadlay,

- P.F. 2012. A late-stage intermediate in salinomycin biosynthesis is revealed by specific mutation in the biosynthetic gene cluster. *Chembiochem*. 13(1): 66-71.
- Zhang, X., Lu, C., and Bai, L. 2017. Mechanism of salinomycin overproduction in *Streptomyces albus* as revealed by comparative functional genomics. *Appl. Microbiol. Biotechnol.* 101(11): 4635-4644.
- Zhu, D., Wang, Y., Zhang, M., Ikeda, H., Deng, Z., and Cane, D.E. 2013. Product-mediated regulation of pentalenolactone biosynthesis in *Streptomyces* species by the MarR/SlyA family activators PenR and PntR. *J. Bacteriol.* 195(6): 1255-66.
- Zhu, Z., Li, H., and Li Y. 2019. Regulation of *slnN* gene in salinomycin biosynthesis. *Acta Microbiologica Sinica*, 59(4): 632–641 (in Chinese) doi: 10.13343/j.cnki.wsxb.20180241.
- Zhu, Z., Li, H., Yu, P., Guo, Y., Luo, S., Chen, Z., Mao, X., Guan, W., and Li, Y. 2017. *SlnR* is a positive pathway-specific regulator for salinomycin biosynthesis in *Streptomyces albus*. *Appl. Microbiol. Biotechnol.* 101(4): 1547 -1557.

Figure legends

Fig. 1 Amino acid sequence alignment of SlnO with different protein of the MarR family. The alignment was generated using ClustalX. Black shade indicated 100% similarity, green and red shade mean $\geq 50\%$ similarity. Conservation of the winged-helix DNA binding motif was based on the NCBI protein-blast. NCBI database accession numbers of the sequences used in this analysis are as follows: ADO85569.1(PntR), ADO85585.1(PenR), KAF0174864.1(HpaR), CDQ52599.1(HosA), AAK21292.1(MarR), AEI198662.1(CtcS), CCD31887.1(SlnO).

Fig. 2 Overexpressing *slnO* improves salinomycin production. (a) Growth curves of WT and GO strains in liquid culture. (b) Time course of salinomycin production in WT, *slnO* gene overexpression strain (GO). Data are the means $\pm$ SD of three duplicate flasks. Error bars indicate the standard deviation from three paralleled flasks.

Fig. 3 Gel electrophoretic mobility shift assays (EMSAs) for determination of SlnO protein binding to the *slnN-slnO*, *slnQ-slnA1* and *slnF-slnT1* intergenic region.

a. Schematic representation of the probes used for EMSAs. The lengths and positions of the eight probes are shown.

b. EMSA results with probes 1, 2, 3, 4. Each lane contained 1 ng probe, Lane 1 to 4 contain 0, 0.5, 1, 2 μ M of purified GST-SlnO respectively.

Fig. 4 Hypothetical scheme of SlnO regulatory pathway related to salinomycin biosynthesis in *Streptomyces albus*. SlnO protein can directly act on the intergenic region of *slnN-slnO*, *slnQ-slnA1*, and *slnF-slnT1*.

Fig. 5 Analysis of transcriptional level of genes involved in biosynthesis of salinomycin between WT and GO strains. (The relative expression level of each gene in WT was set to 1).

Fig. 6 Comparison of salinomycin production in various *S.albus* strains grown in fermentation medium. (a) Growth curves of WT, slnNDM and ZJUS01 strains in liquid culture. (b) Time course of salinomycin production in WT, slnNDM and ZJUS01 strains. Data are the means $\pm$ SD of three duplicate flasks. Error bars indicate the standard deviation from three paralleled flasks.

Draft

Figure

Figure 1

Figure 2

(a)

(b)

Figure 3

Figure 4

Figure 5

Figure 6

(a)

(b)

Draft