

SlnR is a positive pathway-specific regulator for salinomycin biosynthesis in *Streptomyces albus*

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Abstract Salinomycin, a polyether antibiotic produced by *Streptomyces albus*, is widely used in animal husbandry as an anticoccidial drug and growth promoter. Situated within the salinomycin biosynthetic gene cluster, *slnR* encodes a LAL-family transcriptional regulator. The role of *slnR* in salinomycin production in *S. albus* was investigated by gene deletion, complementation, and overexpression. Gene replacement of *slnR* from *S. albus* chromosome results in almost loss of salinomycin production. Complementation of *slnR* restored salinomycin production, suggesting that SlnR is a positive regulator of salinomycin biosynthesis. Overexpression of *slnR* in *S. albus* led to about 25 % increase in salinomycin production compared to wild type. Quantitative RT-PCR analysis revealed that the expression of most *sal* structural genes was downregulated in the Δ *slnR* mutant but upregulated in the *slnR* overexpression strain. Electrophoretic mobility gel shift assays (EMSAs) also revealed that SlnR^{DBD} binds directly to the three intergenic regions of *slnQ-slnA1*, *slnF-slnT1*, and *slnC-slnB3*. The SlnR binding sites within the three intergenic regions were determined by footprinting analysis

and identified a consensus-directed repeat sequence 5'-ACCCCT-3'. These results indicated that SlnR modulated salinomycin biosynthesis as an enhancer via interaction with the promoters of *slnA1*, *slnQ*, *slnF*, *slnT1*, *slnC*, and *slnB3* and activates the transcription of most of the genes belonging to the salinomycin gene cluster but not its own transcription.

Keywords *Streptomyces albus* *slnR* · Salinomycin · Pathway-specific regulator · Gene expression and regulation

Introduction

Streptomyces are Gram-positive filamentous bacteria that can produce a wide variety of bioactive secondary metabolites. Applications for such metabolites include their uses in antimicrobial or anticancer treatments as well in other agricultural or veterinary end uses (Challis and Hopwood 2003). The biosynthesis of secondary metabolites involves strict regulation at a number of stages including those at global, pleiotropic, and pathway-specific levels (Chen et al. 2008). Pathway-specific regulators only affect the biosynthetic pathway of a single, specific antibiotic. These pathway-specific regulatory genes are usually found within the respective antibiotic biosynthesis gene clusters. In several cases, the overexpression of positive pathway-specific regulatory genes has resulted in elevated antibiotic production (Bibb 2005; Martin and Liras 2010).

The first pathway-specific regulatory proteins to be described in actinomycetes belonged to the protein family known as *Streptomyces* antibiotic regulatory proteins (SARPs) (Wietzorrek and Bibb 1997). These are characterized by the presence of an OmpR-like DNA-binding domain (Mizuno and Tanaka 1997). The SARP family includes TylS and TylT from the tylosin pathway (Bate et al. 2002), RedD of the undecylprodigiosin pathway (Narva and Feitelson 1990),

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and ActII-orf4 of the actinorhodin pathway (Arias et al. 1999), among others. Recently, a novel family of transcriptional regulators, the large ATP-binding regulators of the LuxR (LAL) family, was identified (Wilson et al. 2001). The LAL family is characterized by the unusually large size of its members 1' Walker A or Walker B motif and a conserved helix-turn-helix (HTH) DNA binding motif near the C-terminal end (Walker et al. 1982). So far, several regulators of the LAL family have been identified in *Streptomyces* antibiotic gene clusters. These include PikD from the pikromycin pathway in *S. venezuelae* (Wilson et al. 2001), RapH from the rapamycin pathway in *S. hygroscopicus* (Molnar et al. 1996; EnejKuscer et al. 2007), AveR from the avermectin pathway in *S. avermitilis* (Guo et al. 2010), and GdmRI and GdmRII from the geldanamycin pathway in *S. hygroscopicus* 17997 (He et al. 2008). The LAL family regulator can directly and indirectly control the expression of multiple genes (Chao et al. 2015), and many LAL family pathway-specific regulators play positive roles in the production of secondary metabolites (Arias et al. 1999; Mo et al. 2012). The LuxR family regulators usually contain less than 250 residues, constituting two functional domains, a signal-binding domain, and a DNA-binding domain. The C-terminal region contains the DNA-binding domain with a classical HTH domain. There are many examples of LuxR members in streptomycetes which are operon/cluster-specific regulators. For instance, PAS-LuxR protein (PimM in *S. natalensis*) binding specificity rely on the DBD domain and regulation mechanism is pretty well-studied (Santo-Aberturas et al. 2011). In addition, a subfamily of LuxR-regulators, named, LAL family, consists of members of unusual size (~900 amino acids), with an ATP/GTP-binding motif close to the N-terminal end and an HTH domain at the C-terminus. Up to now, the reports on the regulation mechanism of LAL family regulators are limited.

Salinomycin from *S. albus* is a polyether ionophore antibiotic that selectively binds K^+ ions and transports them across cell membranes, leading to cell death (Riddell 2002). It is widely used in animal husbandry as an anticoccidial drug and growth promoter (Jiang et al. 2012). Recently, salinomycin has been found to act in the countering of resistance and in the termination of cancer stem cells (Gupta et al. 2009). This provides new therapeutic strategies and opportunities for cancer treatments (Antoszczak and Huczynski 2015). The salinomycin (*sal*) biosynthetic gene cluster has been sequenced (*S. albus* XM211, GeneBank JN033543.1 and *S. albus* DSM 41398, GeneBank HE586118.1), and the coding for the putative regulatory factors involved in salinomycin biosynthesis has been seen to *slnR* in *S. albus* XM211 (Jiang et al., 2012), which has another name *salJ* in *S. albus* DSM41398 (Yurkovich et al., 2012). Actually, *salJ* and *slnR* are two completely homologous genes. *SlnR* is located downstream of the salinomycin biosynthetic gene cluster (Fig. 1a). Analysis using the NCBI BLASTP program revealed that *SlnR* is highly homologous to many

transcriptional regulators of the LAL family. In the papers that have been reported, members of this family all act as positive pathway-specific regulators to their corresponding biosynthetic clusters (Guo et al. 2010; Mo et al. 2012, Wilson et al. 2001).

Earlier sequence analysis showed that the salinomycin gene cluster in *S. albus* DSM41398 is completely homologous with *S. albus* XM211 (GeneBank JN033543.1 and HE586118.1). It reveals that the sequence of the salinomycin gene cluster is highly conserved. Further studies discovered biosynthesis pathway for salinomycin. The polyketide chain of salinomycin is synthesized by nine *sal* PKS genes (*slnA1* to *slnA9*) which are collinearly arranged in the cluster (Fig. 1a). These PKS genes are required for the biosynthesis of the salinomycin polyketide skeleton. Upstream of the PKS genes, *salN* and *salO* are two defined putative regulatory genes belonging to MarR family. The *salP* and *salQ* genes are involved in the formation of the butyrate extender unit for salinomycin biosynthesis (Yurkovich et al., 2012). Downstream of the PKS genes, the cluster is taken to include two genes encoding discrete (type II) thioesterases (*slnDI*, *slnDII*), acytochrome P450 (*slnF*), and associated ferredoxin (*slnE*); two putative export genes (*slnTI*, *slnTII*); three epoxide hydrolases/cyclases (*slnBI*, *slnBII*, *slnBIII*); an epoxidase (*slnC*); and a putative regulatory gene (*slnR*) (Jiang et al. 2012). Proposed biosynthesis pathway for salinomycin was shown in Fig. S1.

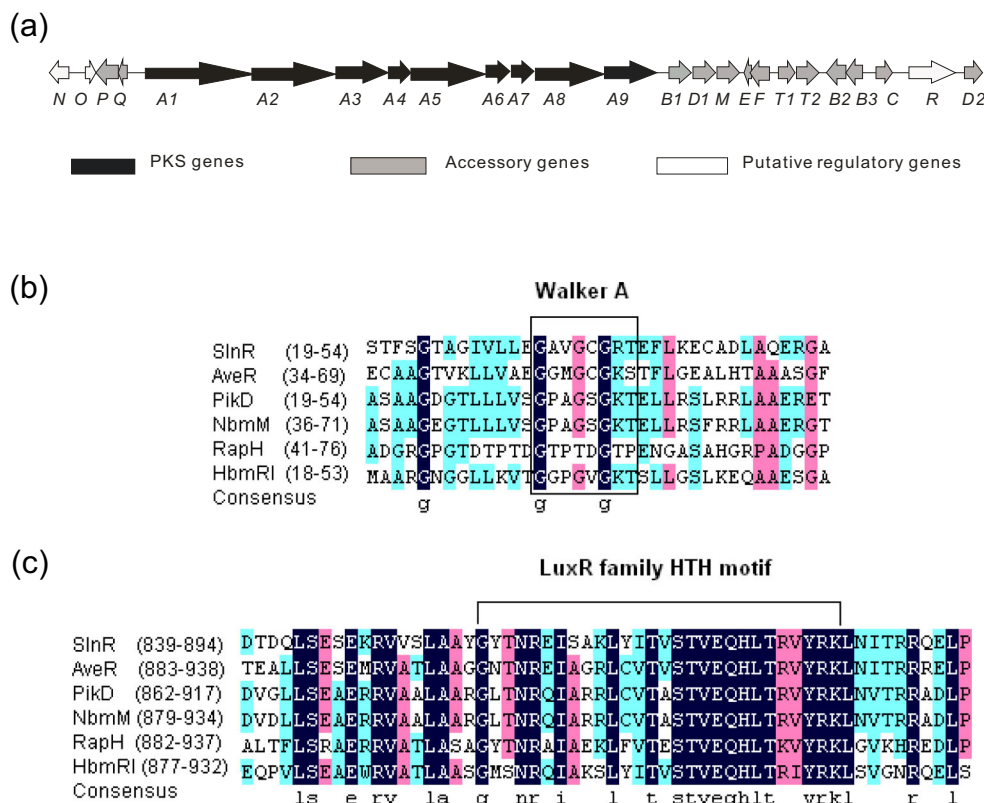
Although the biosynthesis pathway for salinomycin has been elucidated, the regulation mechanism in salinomycin production remains unknown. The aim of this study is to evaluate the general role of the *slnR* gene and to aid in the understanding of the pathway-specific regulatory mechanisms of *slnR* in *S. albus*. Our results provide evidence for *SlnR* as a pathway-specific activator for salinomycin biosynthesis. We demonstrate that *SlnR* serves as a transcriptional activator of the *sal* biosynthetic cluster, controlling up to 21 of the 25 genes within the *sal* cluster. We also show that *SlnR* regulates multiple gene expressions by directly binding to three sites of the intergenic regions of the *sal* biosynthetic cluster. Overexpression of *slnR* enhanced salinomycin production in the wild-type *S. albus* strain by approximately 25 %.

Materials and methods

Strains, plasmids, and growth conditions

S. albus (wild-type strain) was obtained from Zhejiang ShenghuaBiok Biological Company (China). It was grown at 28°C and used as a host strain for gene propagation and gene disruption. ISP4 medium (Difco™, BD) was used for sporulation, and YEME (Kieser et al. 2000) (0.3 % yeast extract, 0.3 % malt extract, 0.5 % tryptone, 4 % glucose) was used for the growth of mycelia for the purposes of extracting DNA. Tryptic soybean broth (3 % TSB) was used as seed

Fig. 1 Amino acid sequence alignment of SlnR with different proteins of the LAL family. **a** Gene organization of the salinomycin gene cluster (**b**) Walker A NTP-binding motif in the N terminal of LAL-family regulators. **c** LuxR-type HTH DNA-binding motif at the C-terminal of LAL family regulator. The NCBI database accession numbers of the sequences used in this analysis are as follows: AEZ53964.1 (SlnR), BAA84600.1 (AveR), AAC68887.1 (PikD), AAM88362.1 (NbmM), AAC38065.1 (RapH), and AAY28238.1 (HbmRI)



medium, and 0.4 % yeast extract, 1 % malt extract, and 0.4 % glucose (YMG) and 10 % soybean oil or industrial medium were used as the fermentation medium. Spores cultivated on ISP4 plates for 7–8 days were added to 250-ml flasks containing 30-ml seed medium. The cultures were then incubated for 28 h at 33 °C on a rotary shaker (220 rpm). Four milliliters of the culture was then transferred to 250-ml flasks containing 40 ml of fermentation medium. The cultures were grown for 10 days at 33 °C on a rotary shaker (245 rpm).

E. coli DH5 α and *E. coli* BL21 (DE3) (Novagen) were used as the cloning host and expression host, respectively. *E. coli* ET12567 (*dam demhds*) was used to propagate non-methylated DNA for transformation in *S. albus*. *Bacillus subtilis* was used as indicator strain for salinomycin bioassays. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium. When necessary, the media were supplemented with antibiotics (100 μ g/ml for ampicillin; 50 μ g/ml each for apramycin, kanamycin, and thiostrepton; and 25 μ g/ml for chloromycetin). pIJ8600 was used for the complementation test. pIJ8660, which contains the constitutive and strong expression promoter ermEp*, was used to construct a vector for constitutive *slnR* expression.

DNA manipulation and sequence analysis

DNA manipulation in *E. coli* and *Streptomyces* was performed according to standard procedures of Kieser et al. (2000). All

the plasmids and strains used in this study are listed in Table 1, and primers are listed in Tables S1 and S2. *S. albus* genomic DNA was isolated using the standard procedure (Liu et al. 2015). A homologous sequence database search and multiple alignment analysis were executed using BLASTP, ClustalX, and DNAMAN software. A protein secondary structure analysis was performed using Predict Protein (Rost et al. 2004).

Construction of *slnR*-deletion mutants

The construction of the *slnR::aac(3)IV-oriT* mutant was done according to Gust et al. (2003). The fosmid (7G12), harboring the *slnR* gene, was screened out from the fosmid genomic library of *S. albus* by PCR amplification using the primer pair PT1/PT2, located 928 nucleotide (nt) upstream and 817 nt downstream of the *slnR* gene, respectively. The disruption cassette *aac(3)IV-oriT* (apramycin resistance gene and oriT) was amplified using PCR with the long primer pair tar-F/tar-R (59 nt/58 nt), each of which having at the 5' end a 39-nt matching sequence adjacent to the *slnR* gene. A *HindIII*/*EcoRI* fragment carrying the apramycin cassette *aac(3)IV-oriT*, which was digested from pIJ773, served as template DNA. The mutagenized 7G12 with an *slnR* deletion was created by electrotransforming the PCR fragment containing the amplified disruption cassette into *E. coli* BW25113/pKD46 (with the genes encoding the λ Red system) containing fosmid 7G12. Gene deletion was verified by PCR analysis (primers

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Description	Source/Reference
<i>Streptomyces</i>		
<i>S. albus</i>	Wild-type producer for salinomycin	Zhejiang ShenghuaBiok Biological Company (China)
<i>S. albus</i> slnRDM	<i>slnR</i> mutant, construct with <i>slnR</i> replaced by <i>aac(3)IV-oriT</i> , Apr ^R	This study
<i>S. albus</i> slnRCOM	slnRDM complemented with a <i>slnR</i> under the control of its own promoter, Apr ^R , Thio ^R	This study
<i>S. albus</i> slnROE	<i>slnR</i> gene overexpression strain under the control of the <i>ermE</i> [*] promoter, Apr ^R	This study
<i>E. coli</i>		
TG1	Used for usual transformation in plasmid Construction	Stratagene, USA
BL21(DE3)	Strain for recombinant protein expression	Novagen, USA
BW25113/pKD46	Strain used for PCR-targeted mutagenesis	Gust et al. (2003)
ET12567/pUZ8002	Non-methylating ET12567 containing non-transmissible RP4 derivative plasmid pUZ8002	Gust et al. (2003)
<i>Bacillus subtilis</i>		
<i>Subsp. subtilis</i>	Indicator strain for salinomycin bioassays	CGMCC 1.3358
Plasmids		
pTA2	General cloning T vector	Toyobo, Japan
pTA2-N	T vector with probe1 DNA fragment	This study
pTA2-O	T vector with probe2 DNA fragment	This study
pTA2-A1	T vector with probe3 DNA fragment	This study
pTA2-F	T vector with probe4 DNA fragment	This study
pTA2-B3	T vector with probe5 DNA fragment	This study
pTA2-R1	T vector with probe6 DNA fragment	This study
pTA2-R2	T vector with probe7 DNA fragment	This study
pTA2-D2	T vector with probe8 DNA fragment	This study
pGEX2T	Amp, expression vector (GST tag)	Amersham
pGEX2T-DBD	pGEX2T with the <i>slnR</i> -DBD (803–906) inserted between the <i>Bam</i> HI and <i>Eco</i> RI sites	This study
pGEX2T- <i>slnR</i>	pGEX2T with the <i>slnR</i> ORF inserted between the <i>Bam</i> HI and <i>Eco</i> RI sites	This study
PIJ8600	Shuttle vector	Liu et al. (2015)
pIJ8660	Site-specific integration vector carrying <i>ermEp</i> [*] , <i>φC31 int</i> , and <i>attP</i>	Sun et al. (1999)
pIJ8661	pIJ8660 with extra MCS including <i>Nde</i> I and <i>Kpn</i> I restriction sites.	This study
7G12	Fosmid carrying the <i>slnR</i> gene	This study
pIJ8600- <i>slnR</i>	pIJ8600 with a 3.2-kb <i>Xba</i> I/ <i>Kpn</i> I DNA fragment	This study
pIJ8661- <i>slnR</i>	pIJ8661 with a 2.7-kb <i>Nde</i> I/ <i>Kpn</i> I DNA fragment	This study

test-F/test-R). The resulting mutated fosmid was first transformed into the non-methylating *E. coli* ET12567 using plasmid pUZ8002 and then transferred into *S. albus* by intergeneric conjugation. Apramycin-resistant exconjugants were selected, and then *slnR*-knockout mutants were confirmed by PCR using primer pair test-F/test-R and sequence analysis.

Complementation of the *slnR* mutant

Using the genomic DNA of *S. albus* as the template, a 3260-bp fragment carrying the complete *slnR* gene and its possible promoter was amplified by PCR using the primers 8600-F/8600-R. The PCR product was then digested by *Xba*I/*Kpn*I. The 3.2-kb *Xba*I/*Kpn*I digested fragment was cloned into the

integrative vector pIJ8600 to give the complementation plasmid pIJ8600-*slnR*. By intergeneric conjugation, the plasmid pIJ8600-*slnR* was introduced into the *slnR* mutant in an *S. albus* background. This resulted in a thiostrepton-resistant exconjugant. The correct integration in the exconjugants was confirmed by Southern hybridization and PCR analysis.

Overexpression of the *slnR* gene in *S. albus*

A 2718-bp fragment carrying the full-length *slnR* gene was amplified by PCR using the primers 8660-F/8660-R. The PCR fragment was digested with *Nde*I/*Kpn*I and then inserted into the corresponding sites in an integrative vector pIJ8661 to produce the recombinant vector pIJ8661-*slnR*. The resultant

plasmid was introduced by intergenic conjugation into *S. albus*. This generated the apramycin-resistant exconjugants. The genotype of the exconjugants was confirmed by PCR analysis.

Expression and purification of protein SlnR-DBD

The DNA-binding domain (DBD) region (residues 803–906) of SlnR was amplified using the primers 2T-F/2T-R. It was then cloned into expression plasmid pGEX2T to generate the plasmid pGEX2T::DBD (pGEX2T-DBD). The overnight culture of *E. coli* BL21 (DE3), containing either expression vector pGEX2T or pGEX2T-DBD, was inoculated at 1:100 concentration in 200-ml LB with 100 µg/ml ampicillin. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM when the culture was grown to OD₆₀₀ 0.4–0.6 at 37 °C. After a further 18 h of incubation at 16 °C, the cells were harvested by centrifugation and disrupted by sonication on ice. The supernatant was then recovered by centrifugation (4 °C, 13,000×g for 30 min). Soluble GST and GST-SlnR^{DBD} were purified with GST-binding resin, respectively, as described by the manufacturer's protocol (Novagen, USA).

Electrophoretic mobility gel shift assays

Promoter regions were amplified by PCR (the primers are listed in Table S1), then cloned into a pTA2 vector and verified by sequence analysis. Biotin-labeled probes used for electrophoretic mobility gel shift assays (EMSAs) were obtained by PCR using the biotin-labeled T3/T7 universal primer pair and were then gel-purified. A standard binding reaction contained 1 ng of labeled DNA probe and incubated with 0, 0.2, 0.4, 0.8, and 1 µM of purified GST-SlnR^{DBD}, respectively, at 30 °C for 30 min in a binding buffer (10 mM Tris-Cl (pH 8.0), 100 mM Na₂HPO₄, 50 µg/ml sheared sperm DNA). EMSA analysis then followed as previously described (Liu et al. 2015).

DNase I footprinting assays

DNase I footprinting assays were performed using a fluorescence labeling procedure (Zianni et al. 2006). DNA fragments were prepared by PCR using the fluorescence-labeled primer 28 forward and primer 29 reverse (Table S1) and were purified from the agarose gel. Labeled DNA fragments (70 ng) and purified GST-SlnR^{DBD} protein (8 µM) were added to a final reaction mixture volume of 50 µl and incubated at 25 °C for 30 min. DNase I digestions (0.01 U of DNaseI with 10 mM MgCl₂ and 1 mM CaCl₂) were carried out for 1 min at 25 °C and stopped with 50 µl of 0.1 M EDTA

(pH 8.0). After phenol-chloroform extraction and ethanol precipitation, the samples were loaded into an ABI 3130 sequencer and electropherograms were analyzed using the Genemapper v4.0 software (Applied Biosystems, USA) to align and determine the protected region. A DNA sequencing ladder was prepared as previously described (He et al. 2010; Liu et al. 2015). The alignment and consensus of the binding sequences was obtained by clustalX software.

RNA extraction and quantitative RT-PCR analysis

The total RNA of *S. albus* strains was prepared from mycelium grown in YMG liquid cultures using an RNA extraction kit (Ailaide, China) following the manufacturer's instructions. To avoid chromosomal DNA contamination, the RNA sample was treated with DNaseI (Takara, Japan) and subsequently confirmed by PCR using three different primer pairs. The RNA concentration was subsequently determined by measuring the A₂₆₀ using a spectrophotometer (Thermo ND LITE, USA). The relative expression level was calculated using the comparative threshold cycle (*C_t*) method. Results were normalized to the expression of *hrdB*. Each experiment was performed in triplicate.

The DNase I-treated RNA sample (2 µg) was used as a template for complementary DNA (cDNA) preparation using M-MLV reverse transcriptase (Takara, Japan). Quantitative real-time PCR (qRT-PCR) was carried out using a SYBR Premix Ex Taq kit (Takara, Japan) according to the manufacturer's instructions. The synthesized cDNA was then used for qRT-PCR using sequence-specific primers (listed in Table S1). The *hrdB* gene, which encodes the major σ factor in *Streptomyces*, was used as the internal control. The fold changes of target gene expression were quantified with the 2^{-ΔΔC_t} method, and the mRNA level of the genes in the WT strain was assigned a value of 1.

Salinomycin bioassay and HPLC analysis

Salinomycins, produced by either *S. albus* or the mutant strains, were measured using a disk agar diffusion method with *B. subtilis* as the indicator strain. 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 was directly applied to a high-performance liquid chromatography (HPLC) system (Agilent 1260 infinity) with a C18 column (Eclipse XDB-C18, 4.6 × 150 mm) developed with methanol (91 %) and 0.2 M NaAC (pH 5.8) (9 %) at a flow rate of 1.0 ml/min. Salinomycin was detected by UV absorption at 210 nm where authentic samples of salinomycin were used as internal standards.

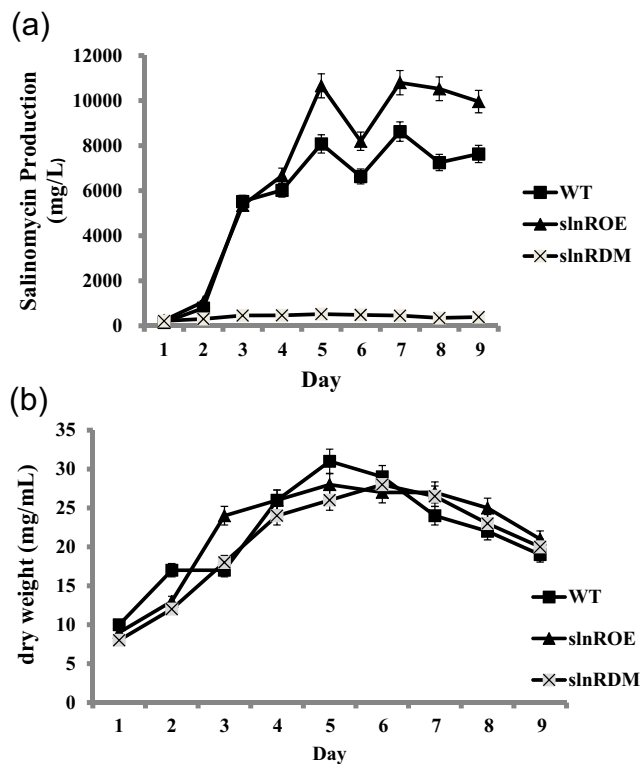


Fig. 2 Overexpressing *slnR* improves salinomycin production **(a)** time course of salinomycin production in WT, *slnR*-deletion strain (*slnRDM*), and *slnR* overexpression strain (*slnROE*). **(b)** Growth curves in liquid culture

Results

SlnR, a cluster-situated regulator, positively regulates salinomycin production

The entire *sal* biosynthetic gene cluster contains 25 ORFs spanning a distance of 104 kb (Yurkovich et al., 2012; Jiang et al. 2012) (Fig. 1a; Fig. S1). Computer-assisted analysis of the *slnR* gene for the putative regulator SlnR has revealed that *slnR* contains 2721 nucleotides and encodes a protein of 906 amino acids (with a predicted molecular mass of 97 kDa). Initial analysis using the NCBI BLASTP program showed that this protein is homologous to many other putative transcriptional regulators of the LAL family. Further analysis of SlnR was performed using the SwissProt protein motif identification software. Query returns revealed a hypothetical N-terminal NTP-binding domain (a.a. 3–127) and Walker A motif (GXXGXGKT) (a.a.32–39) (Fig. 1b), as well as a possible C-terminal HTH DNA-binding domain (a.a.843–893) belonging to the LuxR family (Evelyne Richet and Olivier Raibaud 1989; Wilson et al. 2001) (Fig. 1c). Members of this family almost always act as positive pathway-specific regulators to their corresponding biosynthetic clusters (Wilson et al. 2001; Widdick et al. 2003).

In order to begin probing SlnR's function, the *slnR* gene was deleted from the chromosome using an apramycin resistant gene (*aac(3)IV*) replacement cassette (Fig. 2a). The *slnR* deletion mutant strain (named *S. albus slnRDM*) was identified by PCR (Fig. S2). To confirm that the deletion of *slnR*

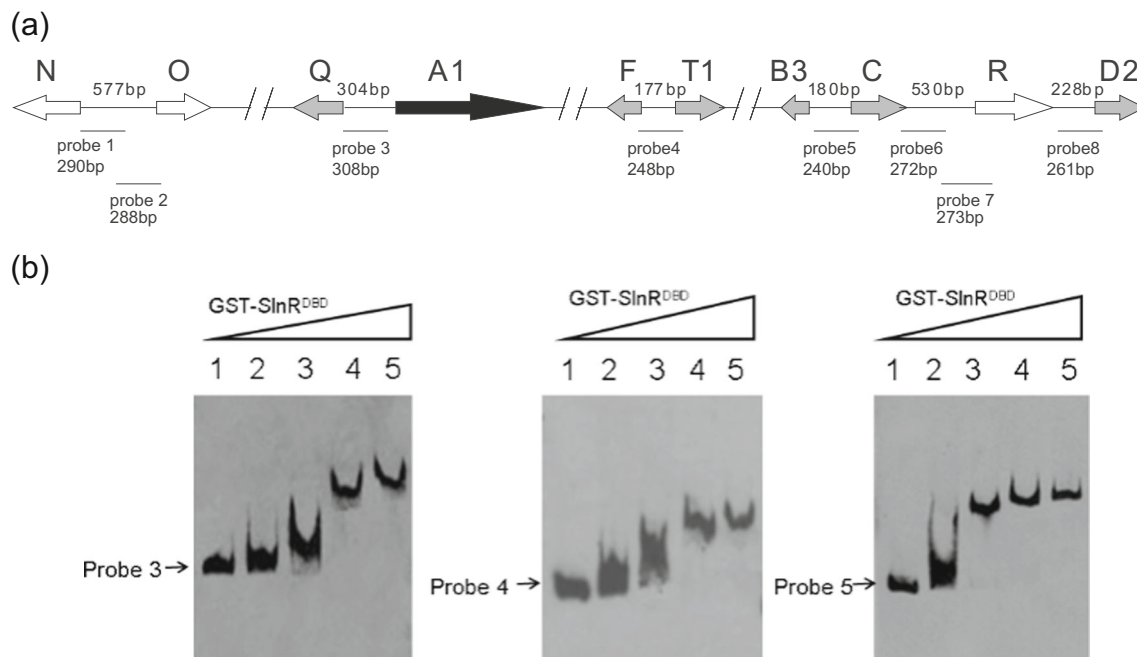


Fig. 3 Gel electrophoretic mobility shift assay (EMSA) for determination of SlnR binding sites. **(a)** Schematic representation of the probes used for EMSAs. The lengths and positions of the eight probes are shown. **(b)**

EMSAs with probes 3, 4, and 5. Each lane contained 1 ng probe. Lanes 1 to 5 contain 0, 0.2, 0.4, 0.8, and 1 μM of purified GST-SlnR^{DBD}, respectively

was the sole reason for the loss of salinomycin production, a 3.2-kb fragment containing *slnR* and its own promoter region was reintroduced into *slnRDM* by the pIJ8600 plasmid. The complementation strain (named *S. albus slnRCOM*) restored salinomycin production (Fig. S4), suggesting that the *slnR* gene is required for salinomycin biosynthesis. Meanwhile, morphological observation revealed that the morphological characteristics of *slnRDM* strain are identical to wild type of *S. albus* when grown on solid media (Fig. S3). It means that *SlnR*, as a pathway-specific regulator, cannot affect the morphological differentiation of *S. albus*.

Salinomycin bioassays using *Bacillus subtilis* as an indicator strain lacked any inhibition zone in the *slnRDM* strain in contrast to the wild type where an obvious inhibition zone was observed (Fig. S4). Furthermore, HPLC detection of salinomycin production during fermentation revealed a lack of salinomycin peaks in the fermentation culture of the *slnRDM* strain. In contrast, the wild-type strain, *slnRCOM*, and *slnR* overexpression strain (named *S. albus slnROE*) produced obvious salinomycin peaks (Fig. S5). It indicated that salinomycin production level was restored in *slnRCOM* strain.

Many LAL family pathway-specific positive regulators play key roles in the production of secondary metabolites. For instance, *PikD* of the pikromycin cluster of *S. venezuelae* (Wilson et al. 2001) is the pathway-specific activator in that particular biosynthetic gene cluster. *AveR* of the avermectin cluster in *S. avrimtilis* (Guo et al. 2010) is also described as a positive pathway-specific regulator. *SlnR*, which has been assigned to the LAL family, has also now been discovered to positively regulate salinomycin production in this work.

SlnR protein binds specifically to the intergenic region of *slnQ-slnA1*, *slnF-slnT1*, and *slnC-slnB3*

The recombinant soluble GST-SlnR^{DBD} was expressed in *E. coli* BL21 (DE3) and has been purified by GST binding resin (Fig. S6a). In addition, soluble GST protein has also been purified and used as control protein (Fig. S6b). In electrophoretic mobility shift assays (EMSAs), we designed eight biotin-labeled probes (probes 1–8) containing all possible promoter regions for the EMSAs (Fig. 3a). Of the eight probes tested, probe 3 (308 bp of the *slnQ-slnA1* intergenic region), probe 4 (248 bp of the *slnF-slnT1* intergenic region), and probe 5 (240 bp of the *slnC-slnB3* intergenic region) all gave retarded signals (Fig. 3b). However, no retarded signals occurred with probes 1, 2, 6, 7, 8, or 9 (pTA2 vector sequence, as a negative control) (Fig. S7). Moreover, the GST protein also did not bind to any of the promoters tested (Fig. S8). It confirms the binding specificity of *SlnR* to the three promoter regions.

To determine the *SlnR*^{DBD} binding sequence, the intergenic regions shown above to be retarded in EMSAs were studied by DNase I protection analysis. GST-SlnR^{DBD} protein (8 μM) was tested using 5'-end FAM-labeled DNA fragment. The DNase I footprinting assay revealed that there were one binding sites for probe 3, probe 4, and probe 5 (Fig. 4a, c, e) Nucleotide sequences of the *slnQ-slnA1*, *slnF-slnT1*, and *slnC-slnB3* intergenic regions and the *SlnR*-binding sites are showed in Fig. 4b, d, f. Nucleotide sequence consensus of three binding sites is shown in Fig. 4g. These sites were analyzed and identified a consensus-directed repeat sequence 5'-ACCCCT-3'. In order to validate the consensus binding site, three mutant DNA probes were constructed with the repeat

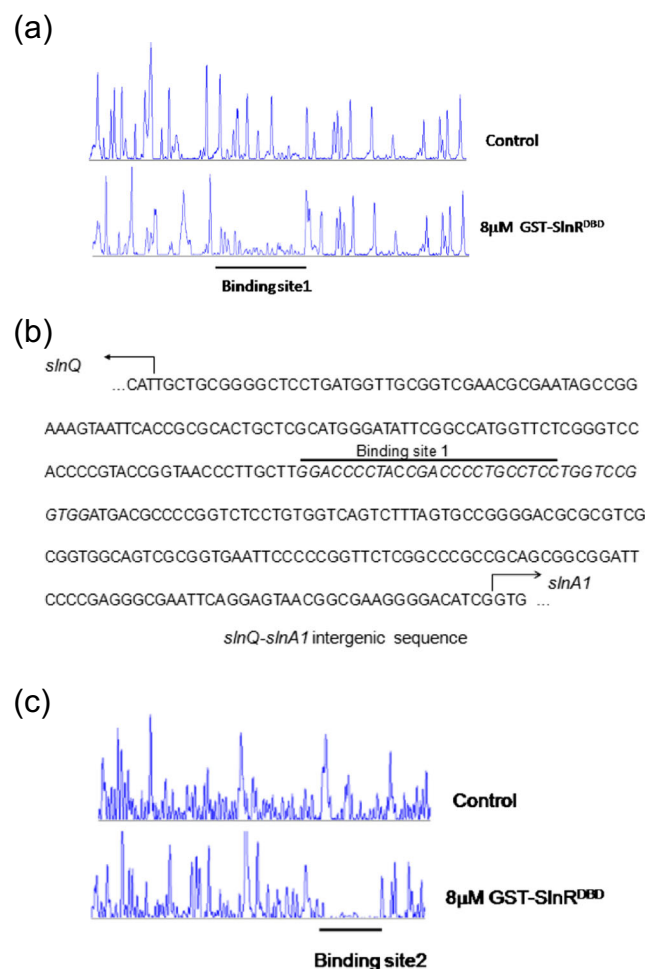


Fig. 4 DNase I footprinting assay for *SlnR* binding site determination 5'-FAM-labeled probe3 amplified from pTA2-A1 (a), 5'-FAM-labeled probe 4 amplified from pTA2-F (c), and 5'-FAM-labeled probe 5 amplified from pTA2-B3 (e) were used in the DNase I footprinting assay with or without purified GST-SlnR^{DBD} (8 μM), respectively. The protected region is *underlined*. Nucleotide sequences of the *slnQ-slnA1* intergenic region (b), *slnF-slnT1* intergenic region (d), and *slnC-slnB3* intergenic region (f); the *SlnR*-binding sites are *overlined* and the initiation codons are marked with *arrows*. **g** Nucleotide sequence consensus of three binding sites. A consensus-directed repeat sequence 5'-ACCCCT-3' was displayed with *underline*

sequence (5'-ACCCCT-3') deleted. The binding assay was then studied by EMSAs. Interestingly, the mutant probes cannot form a complex with the SlnR protein (Fig. S9). This result validates the proposed binding sites.

Therefore, the SlnR binding sites were seen to be located at the *slnQ-slnA1*, *slnF-slnT1*, and *slnC-slnB3* intergenic regions, and the hypothetical regulatory pathway of SlnR related to salinomycin production in *S. albus* was shown in Fig. 6. In addition, SlnR cannot bind to probe 6 or probe 7 which infers that SlnR cannot regulate its own expression.

There have been few previous reports of soluble LAL regulators which have been expressed in the *E. coli* system for its large molecular weight and complex structures. Understanding the regulation mechanism of this novel regulatory protein family by EMSA is therefore quite a challenge. Only one example of a truncated form of AveR, containing the C-terminal HTH domain (AveRc) fused to a his₆-tag at its N-terminus, has been previously noted to have been expressed as an inclusion body (Guo et al. 2010; Kitani et al. 2009). As the inclusion body has no native protein structure, it is very difficult to verify the function of its DNA-binding domain by EMSA. In this study, a soluble GST-SlnR^{DBD} (residues 803-

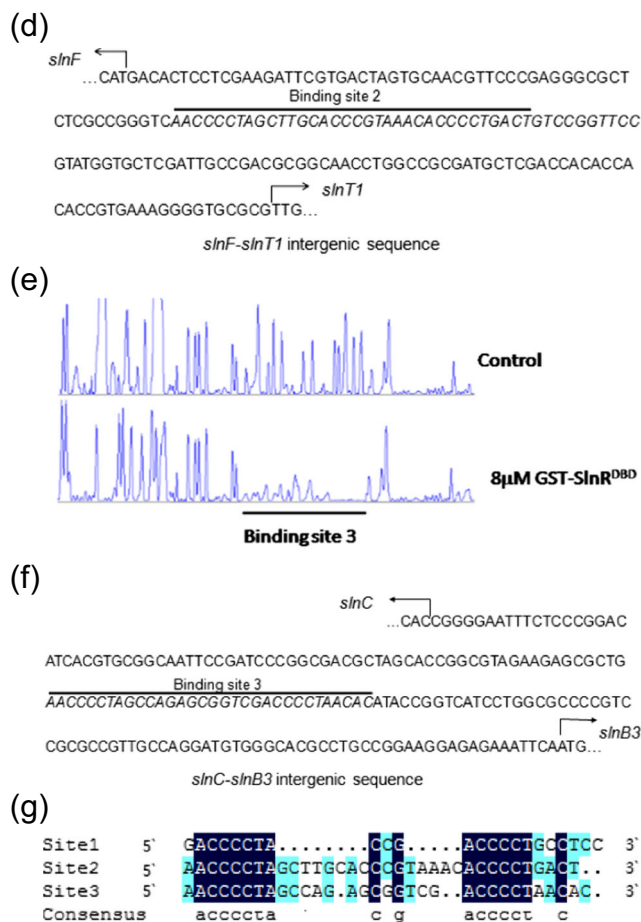


Fig. 4 (continued)

906) was successfully expressed and EMSAs have been used here to prove the direct binding of the SlnR^{DBD} to the multiple binding sites in its target promoters.

Regulation of SlnR on expression of salinomycin biosynthetic genes

The abolished production of salinomycin in the *slnR*-deletion mutant (*slnRDM*) and the overproduction of salinomycin in the *slnR*-overexpression strain (*slnROE*) both indicate the possible positive regulatory roles of SlnR in the pathway. To test whether SlnR regulates salinomycin production through affecting the transcriptional levels of *sal* biosynthetic genes, we performed quantitative RT-PCR (qRT-PCR) analysis. As compared to those of the wild-type strain, the transcript levels of most of the genes (*slnQ*, *slnA1*, *slnF*, *slnT1*, *slnB3* and *slnC*), with the exception of *slnN*, *slnO*, and *slnD2*, were greatly decreased in the *slnRDM* strain (Fig. 5a). In contrast, most of the genes' expression levels (including *slnO*, *slnQ*, *slnA1*, *slnF*, *slnT1*, *slnB3*, *slnC*, *slnR*, and *slnD2*) were obviously increased in the *slnROE* strain (Fig. 5b). However, *slnN* transcript level has little change whether in the *slnRDM* or *slnROE* strains.

The above results demonstrate the correlation between qRT-PCR and three binding sites. From the hypothetical

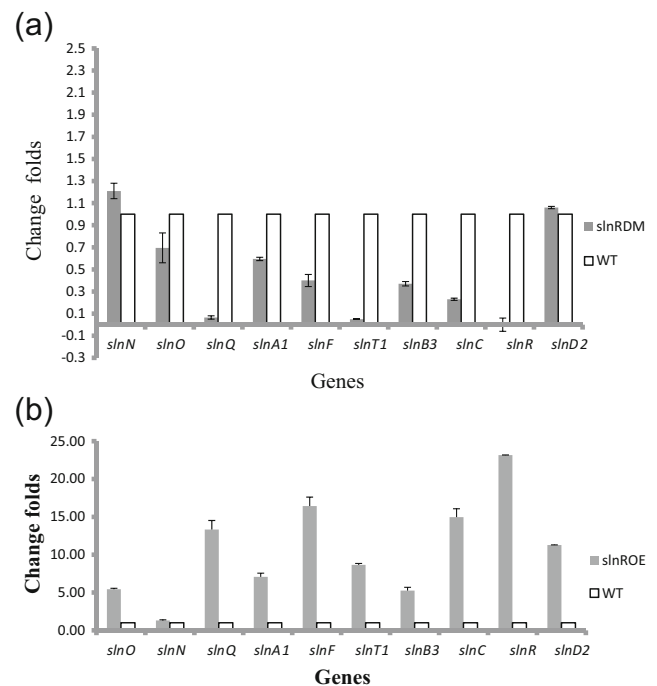


Fig. 5 Compared to wild-type strain (*S. albus*), fold changes of relative mRNA expression of salinomycin biosynthetic genes by qRT-PCR. The mRNA transcription level of genes in WT was assigned a value of 1 (a) *slnR*-deletion strain (*slnRDM*) and WT; (b) *slnR*-overexpressed strain (*slnROE*) and WT

scheme of the regulatory pathway of SlnR (Fig. 6), we can see the transcription direction of all genes in *sal* cluster. The three binding sites, which were located in the three intergenic regions, just situated in three important DNA regions. The promoters in three intergenic regions can control most of gene transcription in *sal* cluster according to the transcriptional direction.

Overexpression of *slnR* leads to overproduction of salinomycin

In general, overexpression of a pathway-specific transcriptional activator leads to increased production of the corresponding antibiotic (Pan et al. 2011). To test this possibility, the *slnR* gene was cloned into the integration vector (pIJ8661) containing the *aac3* (*IV*) gene and *ermE***p* while empty pIJ8661 also as control. The *slnR*-over expression plasmid pIJ8661-*slnR* and empty pIJ8661 were then introduced into the wild-type strain. The *slnR* overexpressed strain (slnROE) was confirmed by quantitative RT-PCR analysis and analyzed for its effects on cell growth, bioassay, and salinomycin productivity in the fermentation medium. The production levels of salinomycin in slnROE were performed by comparing antibacterial activity using an agar diffusion assay with *B. subtilis* as the indicator strain (Chen et al. 2008). The results showed that samples from the pIJ8661-*slnR* transformant had a much larger growth inhibition zone than did that of the wild-type strain (Fig. S4).

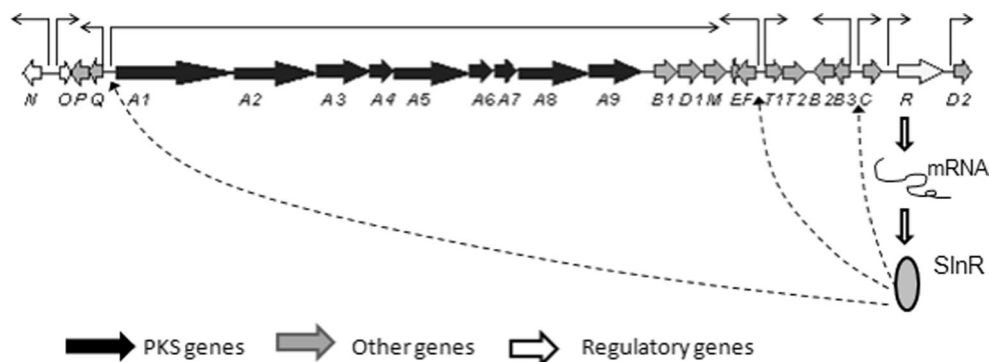
Shake-flask fermentation followed by HPLC analysis showed that the overexpression strain had raised salinomycin production by 10 % (at 4 days) to 25 % (at 7 days) as compared to the wild type (Fig. 2a). The cell concentration of the overexpression strain, and the wild type was measured every 24 h during the fermentation process. No apparent difference in the dry cell weight between the overexpression strain and wild type was observed (Fig. 2b). However, the salinomycin productions of two control strains which contain empty pIJ8600 or pIJ8661 have no significant change compared with the WT strain (Fig. S10).

Discussion

This study provides a valuable initial understanding of the first pathway-specific regulatory gene, *slnR*, in *S. albus*. We demonstrated that a LAL family regulator, SlnR, has a positive effect on the salinomycin production. EMSA results indicated that the SlnR protein could bind three intergenic regions and control up to 21 of the 25 genes within the *sal* cluster. The binding sites also have been identified by DNaseI footprinting assay. Analysis of the SlnR-binding region revealed consensus-directed repeat sequence (5'-ACCCCT-3'); it may be a feature of the SlnR-binding target. In addition, the significantly enhanced production of salinomycin (up to 25 %) in the genetically engineered strain slnROE in which the positive regulatory gene was overexpressed in the background of wild-type strain demonstrates the potential of this approach for strain development (Pan et al. 2011). It may be possible to upregulate the entire salinomycin antibiotic pathway to further increase the production of known metabolites.

Up to now, numerous regulatory proteins involved in antibiotic production have been identified in *Streptomyces*. Pathway-specific regulators specifically regulate the transcription of certain biosynthetic genes (Wietzorrek and Bibb 1997). Many pathway-specific regulators belong to SARP family proteins (e.g., ActII-orf4, RedD, and TylS), and the mechanism of action is very clear (Arias et al. 1999; Bate et al. 2002). On the other hand, the LAL family proteins of pathway-specific regulators have been reported limited in recent years (Wilson et al. 2001; Kitani et al. 2009), and little is known about their precise mechanisms of regulation. The large size of LAL family regulator is a “bottleneck” in high-level expression and purification of intact LAL protein (Guo et al. 2010; Santos-Aberturas et al. 2011). This makes it more difficult to understand the regulation mechanism of this novel regulatory family. In this study, GST-SlnR^{DBD} fusion proteins were successfully expressed and used for EMSAs. The DNA-binding domain (SlnR^{DBD}) was found to bind with high affinity to the three intergenic regions, thus suggesting that binding specificity is dependent of the DBD domain. However, the effect of N-terminal NTP-binding motif of SlnR in regulation

Fig. 6 Hypothetical scheme of the regulatory pathway of SlnR related to salinomycin production in *S. albus*



of salinomycin production is known little. The ATPase/GTPase activity of SlnR N-terminus may affect the DNA-binding of SlnR and its target promoters; it also needs further research and exploration. This report provides important clues toward understanding the pathway-specific regulation of salinomycin biosynthesis and provides a useful means for controlling the expression of genes involved in the salinomycin biosynthesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

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