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Joint engineering of SACE_Lrp and its target MarR enhances the biosynthesis and export of erythromycin in *Saccharopolyspora erythraea*

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Abstract

The Lrp and MarR families are two groups of transcriptional regulators widely distributed among prokaryotes. However, the hierarchical-regulatory relationship between the Lrp family and the MarR family remains unknown. Our previous study found that an Lrp (SACE_Lrp) from *Saccharopolyspora erythraea* indirectly repressed the biosynthesis of erythromycin. In this study, we characterized a novel MarR family protein (SACE_6745) from *S. erythraea*, which is controlled by SACE_Lrp and plays a direct regulatory role in erythromycin biosynthesis and export. SACE_Lrp directly regulated the expression of *marR* by specifically binding a precise site OM (5'-CTCCGGGAACCATT-3'). Gene disruption of *marR* increased the production of erythromycin by 45% in *S. erythraea* A226. We found that MarR has direct DNA-binding activity for the promoter regions of the erythromycin biosynthetic genes, as well as an ABC exporter *SACE_2701-2702* which was genetically proved to be responsible for erythromycin efflux. Disruption of *SACE_Lrp* in industrial *S. erythraea* WB was an efficient strategy to enhance erythromycin production. Herein, we jointly engineered SACE_Lrp and its target MarR by deleting *marR* in WBΔ*SACE_Lrp*, resulting in 20% increase in erythromycin yield in mutant WBΔ*Lrp*Δ*marR* compared to WBΔ*SACE_Lrp*, and 39% to WB. Overall, our findings provide new insights into the hierarchical-regulatory relationship of Lrp and MarR proteins and new avenues for coordinating antibiotic biosynthesis and export by joint engineering regulators in actinomycetes.

Key points

- *The hierarchical-regulatory relationship between SACE_Lrp and MarR was identified.*
- *MarR directly controlled the expression of erythromycin biosynthesis and export genes.*
- *Joint engineering of SACE_Lrp-MarR regulatory element enhanced erythromycin production.*

Keywords *Saccharopolyspora erythraea* · Lrp · MarR · Hierarchical regulation · Erythromycin biosynthesis and export · Joint engineering

Introduction

Transcription regulators can respond to alterations of environmental and physiological signals by tuning the expression of relevant genes (Liu et al. 2013). As typical representatives, the Lrp (leucine-responsive regulatory protein) and the MarR (multiple antibiotic resistance regulator) regulator families are both widely distributed and extremely well-characterized regulatory transcriptional factors among prokaryotes (Grove 2013; Peeters and Charlier 2010). Lrp family regulators (LFRs) are involved in diverse biological processes, especially in amino acid metabolism and transport (Brinkman et al. 2003; Peeters and Charlier 2010). LFRs are identified by an

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N-terminal helix-turn-helix (HTH) domain for DNA binding and a C-terminal αβ sandwich domain for ligand response (Deng et al. 2011; Peeters and Charlier 2010). MarR family regulators (MFRs) also modulate a variety of biological processes, mainly including antibiotic resistance, virulence, and stress responses (Grove 2013; Gupta et al. 2018). Similar to LFRs, MFRs are characterized by a winged HTH DNA-binding domain at the N-terminus, and the DNA-binding activity of MFRs is altered by conformational changes in response to structural ligands (Deochand and Grove 2017; Grove 2013).

Actinobacteria are well known for their ability to produce abundant antibiotics and related compounds (Bérdy 2005). Biosynthesis of these antibiotics involves sophisticated regulatory networks, which are finely adjusted by extracellular and intracellular signals (Liu et al. 2013; Martín and Liras 2010). Recently, studies on LFRs have made significant progress in providing insight into the molecular mechanism involved in the regulation of antibiotic biosynthesis by SACE_Lrp (Liu et al. 2017) and SACE_5717 (Liu et al. 2019) from *S. erythraea*, SCO3361 from *Streptomyces coelicolor* (Liu et al. 2017), SLCG_Lrp from *S. lincolnensis* (Xu et al. 2020) in our laboratory, and SSP_Lrp from *S. spiramyceticus* (Lu et al. 2019). MFRs are one of the most abundant groups of transcriptional regulators in antibiotic-producing actinobacteria; however, only a few members have been identified being involved in antibiotic biosynthesis, such as OhrR and PcaV from *S. coelicolor* (Davis et al. 2013; Oh et al. 2007), DptR3 from *S. roseosporus* (Zhang et al. 2015), SAV4189 and OhrR from *S. avermitilis* (Guo et al. 2018; Sun et al. 2018), and CtcS from *S. aureofaciens* (Kong et al. 2019).

In addition to antibiotic biosynthesis, antibiotic export is an important factor affecting antibiotic production. However, transcription regulators controlling antibiotic export in natural producers such as actinobacteria remain underexplored. TetR family regulator (TFR) GouR from *S. graminearus* modulates gougerotin export by directly controlling the major facilitator superfamily transporter gene *gouM* (Wei et al. 2014). TFR ActR from *S. coelicolor* regulates actinorhodin export and resistance by directly acting on the two putative export pumps *actAB* operon (Tahlan et al. 2008; Xu et al. 2012). Another TFR RifQ from *Amycolatopsis mediterranei* directly represses the expression of the rifamycin efflux pump RifP for regulation of rifamycin export (Lei et al. 2018).

S. erythraea is a model representative of actinomycetes that are used for industrial production of erythromycin A (Butler 2008). Erythromycin and its derivatives are widely used as clinical broad-spectrum polyketide antibiotics (Butler 2008; McDaniel et al. 2001). In *S. erythraea*, massive prediction of transcriptional regulators has offered the opportunity to elucidate specialized regulatory mechanisms for the biosynthesis and export of erythromycin, but for most of them, the specific biological functions have never been examined (Oliynyk et al. 2007). Bioinformatics analysis revealed 30 MFR genes in the *S. erythraea* genome (Marcellin et al. 2013; Oliynyk et al. 2007). However, the biological function of MFR links with erythromycin production in *S. erythraea* is unclear. Besides, it is also unknown whether the export of erythromycin is regulated. In this study, we characterized a novel MarR family protein (SACE_6745) from *S. erythraea*, which is regulated by SACE_Lrp and plays a significant role in the biosynthesis and export of erythromycin (Liu et al. 2017; Oliynyk et al. 2007). Furthermore, we successfully demonstrated a notable enhancement in erythromycin production through combinatorial engineering of SACE_Lrp and MarR regulatory elements in the *S. erythraea* industrial strain.

Materials and methods

Strains, plasmids, and primers

All bacterial strains and plasmids used in the present study are listed in Table 1, and all primers are listed in Supplementary Table S1.

Escherichia coli DH5α was used to construct plasmids, and *E. coli* BL21 (DE3) was used to overexpress proteins SACE_Lrp and MarR *S. erythraea*, and its derivatives were grown at 30°C on R5 solid medium for sporulation or in liquid TSBY medium for growth of mycelia with appropriate antibiotics as previously described (Wu et al. 2014). TSBY medium was also used for protoplast preparation and seed culture, and R5 agar was also used for protoplast regeneration and selection of transformants. R5 liquid medium was used for erythromycin fermentation (Wu et al. 2014).

Protein overexpression and purification

To express the MarR protein in *E. coli*, *marR* (*SACE_6745*) gene was amplified from *S. erythraea* A226 genome by PCR with the 6745-32a-F/R primers and inserted into the corresponding *Eco*RI/*Hin*dIII sites of pET32a obtaining the constructed pET32a-marR plasmid. pET32a-marR and pET28a-Lrp (Liu et al. 2017) was respectively introduced into *E. coli* BL21 (DE3) to express MarR and SACE_Lrp. Overexpression and purification of His_6-tagged proteins were performed as previously described (Liu et al. 2017).

Electrophoretic mobility shift assay (EMSA)

The EMSAs were performed as previously described (Hellman and Fried 2007). The putative promoter regions of *marR*, *SACE_2701-2702*, and the erythromycin biosynthetic gene cluster (*ery* cluster) genes were amplified from the A226 genome by PCR with appropriate primers (Table S1). The

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Sources
S. erythraea strains		
A226	An erythromycin low producer	China Pharmaceutical Culture Collection, CGMCC 8279
Δ*SACE_Lrp*	A226 with *SACE_Lrp* deleted	(Liu et al. 2017)
Δ*marR*	A226 with *marR* deleted	This study
Δ*marR/pIB139*	Δ*marR* carrying pIB139	This study
Δ*marR/pIB-marR*	Δ*marR* carrying *pIB-marR*	This study
Δ*2701-2702*	A226 with *SACE_2701-2702* deleted	This study
Δ*2701-2702/pIB139*	Δ*2701-2702* carrying pIB139	This study
Δ*2701-2702/pIB-2701-2702*	Δ*2701-2702* carrying pIB-*2701-2702*	This study
A226/*pIB139*	A226 carrying pIB139	(Liu et al. 2017)
A226/*pIB-2701-2702*	A226 carry pIB-2701-2702	This study
WB	An erythromycin industrial overproducer	Anhui Wanbei Pharmaceutical Co., Ltd. CGMCC 8280
WBΔ*SACE_Lrp*	WB with *SACE_Lrp* deleted	(Liu et al. 2017)
WBΔ*marR*	WB with *marR* deleted	This study
WBΔ*SACE_Lrp*Δ*marR*	WB/Δ*SACE_Lrp* with *marR* deleted	This study
E. coli strains		
DH5α	F *recA lacZ*M15	Invitrogen
BL21(DE3)	F-*ompThsd*SB (rB^-mB^-) *gal dcm* (DE3)	Novagen
Plasmids		
pBluescript II SK (+)	*lacZ* orif1	Stratagene
pUCTSR	pUC18 derivative containing a 1.36-kb fragment of a thiostrepton resistance cassette in the *Bam*HI/*Sma*I sites	(Han et al. 2011)
pUC-ΔmarR	pUCTSR derivative for *marR* deletion	This study
pUC-Δ2701-2702	pUCTSR derivative for *SACE_2701-2702* deletion	This study
pUC-apr-ΔmarR	pUC-ΔmarR derivative containing a 1.38-kb fragment of an apramycin resistance cassette in the *Xba*I/*Kpn*I sites	This study
pIB139	*aac(3)IV*, P*ermE**origin1	(Wilkinson et al. 2002)
pIB-marR	pIB139 derivative for expression of *marR*	This study
pIB-2701-2702	pIB139 derivative for expression of *SACE_2701-2702*	This study
pET28a-*Lrp*	pET28a derivative carrying *SACE_Lrp*	(Liu et al. 2017)
pET32a	*bla*, P_{T7}, His-tag	Novagen
pET32a-*marR*	pET32a derivative carrying *marR*	This study
pKC1139	*ori* (pSG5), *aac(3)IV*, *lacZ*	(Wilkinson et al. 2002)
pUPW-EGFP	*ori* (pJV1), *bla*, *tsr*, *egfp*	(Liu et al. 2017)
p*marR*-EGFP	pKC1139 derivative inserting *marR* promoter into upstream of *egfp*	This study
p*6744*-EGFP	pKC1139 derivative inserting *SACE_6744* promoter into upstream of *egfp*	This study
pLrp-*marR*-EGFP	p*marR*-GFP derivative inserting *SACE_Lrp* driven by *aac(3)IV* promoter	This study
pLrp-*6744*-EGFP	p*6744*-GFP derivative inserting *SACE_Lrp* driven by *aac(3)IV* promoter	This study

PCR products were purified for EMSA probes, and the concentration of probes was determined by the microplate reader (BioTek). The above probes and the $P_{Lrp\text{-}5387}$ probe were individually incubated with His_6-tagged protein as described previously (Liu et al. 2017).

DNase I footprinting assay

To determine the SACE_Lrp binding site within *SACE_Lrp-SACE_5387*-int, DNase I footprinting assays were performed as previously described (Liu et al. 2019). For each experiment,

100 ng of labeled probe (5'-FAM and 3'-HEX) was incubated with different concentrations of His_6-tagged SACE_Lrp in 40 μL of binding buffer. After incubation at room temperature for 20 min, DNase I digestion was performed at room temperature for 0.5 min. All reactions were stopped by adding 10 μL DNase I stop solution at 65°C for 10 min. All samples were extracted with phenol/chloroform and precipitated with ethanol for further sequencing. Electropherograms were then analyzed with GeneMarker v2.2 (Applied Biosystems).

GFP reporter assay in *E. coli*

To construct the GFP reporter plasmids, the enhanced green fluorescent protein gene (*egfp*) fragment was amplified from pUPW-EGFP (Liu et al. 2017) by PCR with DE-F/R primers (Xu et al. 2020) and digested with *Xba*I/*Bam*HI. In addition, a fragment containing the putative promoter region of *marR* was amplified from the A226 genome by PCR with P6745-F/R primers (Table S1) and digested with *Hin*dIII/*Xba*I. The two fragments were ligated into pKC1139 to create the reporter plasmid p*marR*-EGFP. To evaluate the regulatory effect of SACE_Lrp on the *marR* gene, the P*aac(3)IV* promoter with *Eco*RV/*Nde*I and *SACE_Lrp* with *Nde*I/*Eco*RI were amplified from pIB139 (Wilkinson et al. 2002) and A226 genome using primers Papr-F/R (Xu et al. 2020) and 5388P-F/R (Table S1), respectively. The two fragments were together joined with p*marR*-EGFP to create the reporter plasmid pLrp-*marR*-EGFP. The above method was also used to obtain the reporter plasmids p*6744*-EGFP and pLrp-*6744*-EGFP with the corresponding primers listed in Table S1. These plasmids were transformed into DH5α, detecting green fluorescence (excitation at 485 nm; emission at 510 nm, Molecular Devices). All fluorescence values were normalized to the growth rate (OD_{600}).

Gene disruption and complementation

To construct the *marR* disruption mutant in *S. erythraea* A226, two 1.5-kb DNA flanking fragments of *marR* were amplified by PCR with the 6745-up-F/R and 6745-down-F/R primers using A226 genomic DNA as a template (Table S1). The obtained fragments of PCR products were treated with *Hin*dIII/*Xba*I and *Kpn*I/*Eco*RI, and then inserted into the corresponding sites of plasmid pUCTSR (Han et al. 2011) to obtain pUC-Δ*marR*, as confirmed by DNA sequencing. Plasmid pUC-Δ*marR* was introduced into the parental strain A226 by PEG-mediated protoplast transformation. A 225-nt DNA fragment of the *marR* gene was replaced by the thiostrepton resistance gene (*tsr*) by homologous chromosomic recombination. The desired mutant Δ*marR* with thiostrepton-resistant was further determined by PCR with the 6745-C-F/R primers (Table S1). Using the same procedures, plasmid pUC-Δ*2701-2702* was constructed and introduced into A226 by PEG-mediated protoplast transformation. An 1800-nt DNA fragment of the SACE_*2701-2702* gene was replaced with the *tsr* gene, generating the deletion mutant Δ*2701-2702* in *S. erythraea*.

To construct the complementation strains Δ*marR*/pIB-*marR* and Δ*2701-2702*/pIB-*2701-2702*, the full-length *marR* gene of 384-bp and *SACE_2701-2702* gene of 2456-bp were amplified by PCR using the 6745-C-F/R and 2701-C-F/R primers (Table S1) with A226 genomic DNA as a template, respectively. The PCR products were cloned into the corresponding *Nde*I/*Xba*I sites of plasmid pIB139 to construct plasmids pIB-*marR* and pIB-*2701-2702*. These two plasmids were respectively introduced into Δ*marR* and Δ*2701-2702*, generating complemented Δ*marR*/pIB-*marR* and Δ*2701-2702*/pIB-*2701-2702* by screening with apramycin and PCR using the apr-test-F/R primers (Table S1). pIB-*2701-2702* was also introduced into A226 to generate the overexpressed strain A226/pIB-*2701-2702* using the same method.

In the industrial high-yield *S. erythraea* WB, the *marR* gene was also disrupted by the *tsr* gene through the above procedures, generating the WBΔ*marR* strain. To further knock out *marR* in WBΔ*Lrp* (Liu et al. 2017), a 1.38-kb DNA fragment of the apramycin resistance gene was prepared by PCR from pIB139 with the apr-F/R primers, treated with *Xba*I/*Kpn*I, and then replaced the *tsr* gene of pUC-Δ*marR* to obtain the pUC-apr-Δ*marR*. Similarly, the *marR* gene was displaced by the apramycin resistance gene using the pUC-apr-Δ*marR* by the above method. The desired mutant WBΔ*Lrp*Δ*marR* with apramycin-resistant was further determined by PCR with the 6745-C-F/R primers (Table S1).

Fermentation and erythromycin determination

Flask fermentation of *S. erythraea* A226, *S. erythraea* WB, and their derivatives was carried out as described previously (Wu et al. 2014). Isolation of erythromycin A and HPLC analysis of erythromycin A yield were performed as described previously (Wu et al. 2014).

RNA preparation and qRT-PCR assay

Cells of *S. erythraea* A226 and its derivatives were harvested by centrifugation, and total RNA was collected using the RNA extraction/purification kit (SBS) as described previously (Liu et al. 2017). All operation procedures were carried out following the manufacturer's instructions (MBI Fermentas). The specific primers used for the qRT-PCR assay are listed in Supplementary Table S1. The *hrdB* gene in *S. erythraea* served as an internal control to normalize samples.

Erythromycin resistance test

To determine the resistance of *S. erythraea* A226 and its derivatives against erythromycin, 10 μL spore suspension of A226, A/2701-2702, and Δ*marR* was inoculated into 5 mL of liquid TSBY with various concentrations of erythromycin A (0, 5, 10, 25, 50, and 100 mg/L) and cultured at 30°C for 24 h to compare their growth with OD_{600}.

Statistical analysis

The data of erythromycin production in the present study were stated as means ± standard error of mean (SEM) and analyzed by Student's *t*-test, with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ representing significant differences.

Results

Determination of the SACE_Lrp binding sequence in the *SACE_Lrp-SACE_5387* intergenic region

In our previous report, SACE_Lrp specifically interacted with the intergenic region between *SACE_Lrp* and *SACE_5387* (Liu et al. 2017). In order to elucidate the precise DNA-binding site of SACE_Lrp with its target, DNase I footprinting experiments were performed with the $P_{Lrp\text{-}5387}$ probe (Fig. 1a). The results showed that only one protected region (named site OL: 5'-CTCCGGGCAACATT-3') was identified from nucleotide position −39 to −26 relative to the putative *SACE_5387-5386* transcriptional start site (TSS) (Fig. 1b). The site OL-deletion analysis showed that the DNA-binding activity of SACE_Lrp to the mutated probe $P_{d(site\ OL)}$ completely disappeared as compared with the original $P_{Lrp\text{-}5387}$ probe by EMSAs (Fig. 1c). Taken together, these results demonstrated that the 14 bp asymmetric sequence (5'-CTCCGGGCAACATT-3') within the intergenic region between *SACE_Lrp* and *SACE_5387* is indispensable for SACE_Lrp binding activity.

SACE_Lrp directly interacts with the promoter region of a novel MarR homologue

The 14 bp consensus sequence (5'-CTCCGGGCAACATT-3') of site OL was used to search for SACE_Lrp putative target genes by scanning the genomic sequence of *S. erythraea* using PREDetector software (Hiard et al. 2007). A total of 31 upstream regions containing potential SACE_Lrp binding sites with high similarity to the site OL were identified (data not shown). Interestingly, PREDetector identified a 14

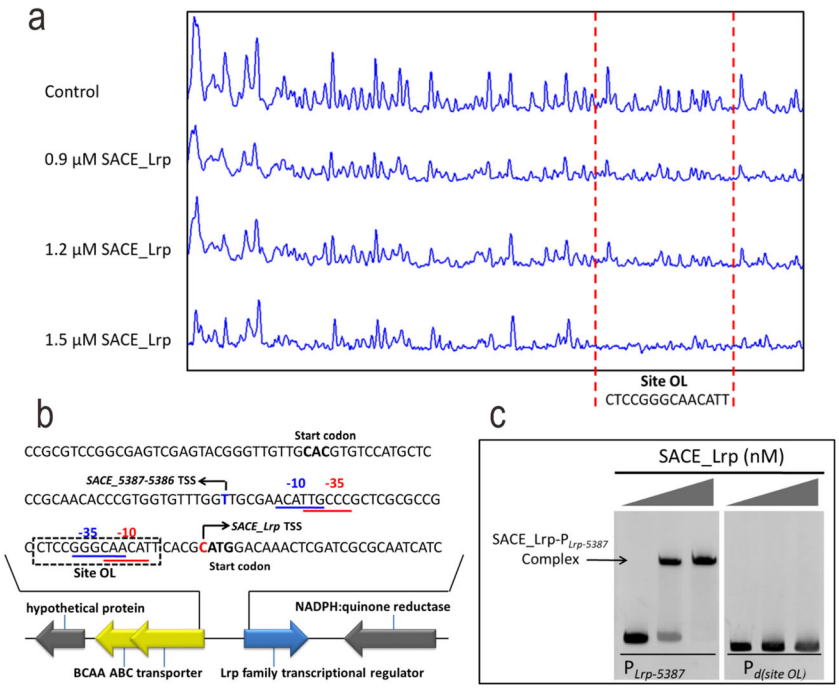

Fig. 1 DNase I footprinting for determination of the SACE_Lrp binding site. **a** The fluorograms correspond to the control DNA and to the protection reactions (with increasing concentrations of 0.9, 1.2, and 1.5 μM of SACE_Lrp respectively). **b** Nucleotide sequences of the intergenic segment between *SACE_Lrp* and *SACE_5387-5386*. The transcriptional start site (TSS) is indicated by a bent arrow. Sequence protected from DNase I digestion is indicated with box and labeled with Site OL. Presumptive −10 and −35 regions of the *SACE_Lrp* and *SACE_5387-5386* promoters are underlined. **c** EMSA of binding of SACE_Lrp to the probes $P_{Lrp\text{-}5387}$ and $P_{d(site\ OL)}$. The probe $P_{Lrp\text{-}5387}$ represents the intergenic segment between *SACE_Lrp* and *SACE_5387-5386*, and the $P_{d(site\ OL)}$ is derived from $P_{Lrp\text{-}5387}$ with deleting the site OL. The amounts of SACE_Lrp used were 0, 250, and 500 nM, respectively

bp sequence (5'-CTCCGGGAACCATT-3') that was highly similar to site OL, which lies in the putative promoter region of the *marR* gene (*SACE_6745*), encoding a MarR family regulator, so named as site OM (Fig. 2a). The site OM sequence is located at nucleotide positions −57 to −44 relative to the putative *marR* TSS (Fig. 2b).

In order to determine whether SACE_Lrp directly interacts with the site OM from the *marR* promoter, EMSAs were performed to evaluate the binding ability of SACE_Lrp to the original probe P_{marR} and the mutated probe $P_{d(site\ OM)}$. Results from EMSAs showed that a SACE_Lrp-P_{marR} complex specifically formed in the presence of probe P_{marR} but completely disappeared in the presence of probe $P_{d(site\ OM)}$ (Fig. 2c). We also utilized a biosensor system with green fluorescence in *E. coli* to verify the interaction of SACE_Lrp with the probe P_{marR} in vivo. As shown in Fig. 2d, plasmids p*marR*-EGFP and p*6744*-EGFP, in which the *egfp* gene was directly controlled by the promoters of *marR* and *SACE_6744*, were transformed into *E. coli* DH5α as controls. The *SACE_Lrp* gene driven by the promoter of the *aac(3)IV* gene (P*aac(3)IV*) was inserted into the control plasmids and transformed into *E. coli* DH5α. When the *SACE_Lrp* gene was inserted into the p*marR*-EGFP, the green fluorescence was enhanced by 64% compared to that without *SACE_Lrp* (Fig. 2e). However, when the *SACE_Lrp* gene was inserted into p*6744*-EGFP, the green fluorescence was almost unchanged compared to that without *SACE_Lrp* (Fig. 2e). Taken together, these findings indicate that SACE_Lrp specifically binds to the *marR* promoter by directly interacting with the site OM and indeed activates the expression of *marR* promoter in a heterologous *E. coli* host.

MarR negatively regulates biosynthesis of erythromycin

To further clarify the correlation between SACE_Lrp and *marR*, the transcription profile of *marR* in the deletion strain Δ*SACE_Lrp* (Liu et al. 2017) and the parent strain A226 was measured during the process of growth by qRT-PCR. The results showed that the transcription level of *marR* in Δ*SACE_Lrp* was reduced by 36% after 24 h and 50% after 48 h compared with A226 (Fig. 3a), suggesting that SACE_Lrp positively regulates the expression of *marR* in *S. erythraea*.

Given that the transcription of *marR* was decreased in Δ*SACE_Lrp* (Fig. 3a) and Δ*SACE_Lrp* resulted in a 25% increase in erythromycin production (Liu et al. 2017), it was reasonable to predict that *marR* deletion would increase erythromycin production. To test this concept, the *marR* gene was disrupted with *tsr* replacement (Han et al. 2011) in the parent strain A226 (Fig. 3b), and the resulting mutant Δ*marR* was confirmed by PCR (Fig. 3c) and tested for erythromycin production. In comparison with A226, the level of erythromycin production in Δ*marR* was improved by 45% ($p < 0.01$) (Fig. 3d). The complemented strain Δ*marR*/pIB-*marR* with a single copy of *marR* restored the production of erythromycin to the level of the parent strain

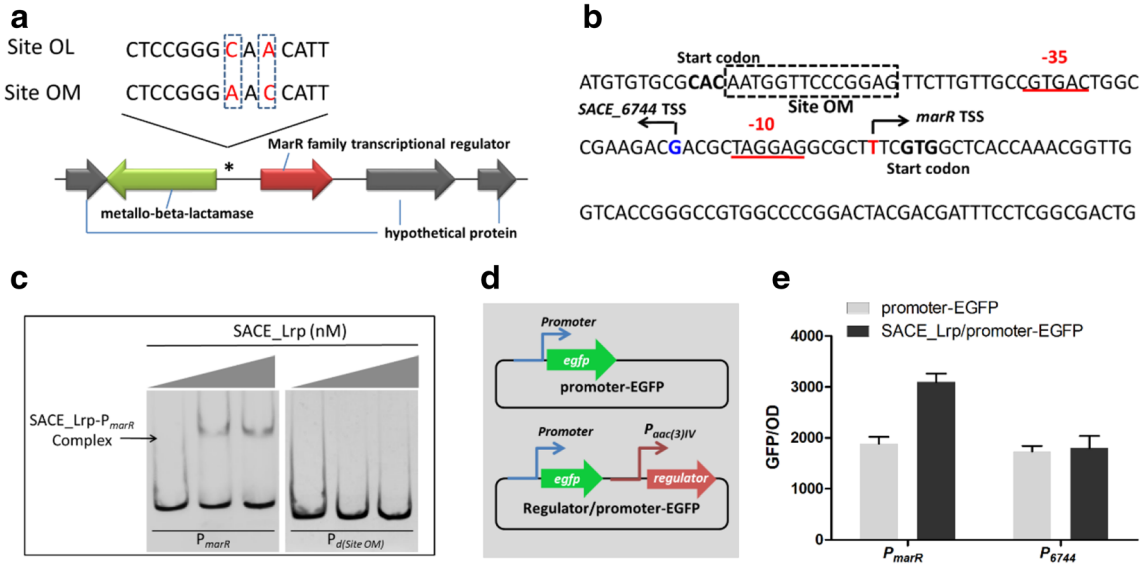

Fig. 2 SACE_Lrp directly controls the transcription of *marR* gene. **a** Blast analysis of site OL and site OM. **b** Nucleotide sequences of the promoter region of *marR* gene. The transcriptional start site (TSS) is indicated by a bent arrow. Presumptive sequence protected by SACE_Lrp is indicated with box and labeled with Site OM. Presumptive −10 and −35 regions of the *marR* promoter are underlined. **c** EMSA of binding of SACE_Lrp to the probes P_{marR} and $P_{d(site\ OM)}$. The probe P_{marR} represents the promoter region of *marR* gene, and the $P_{d(site\ OM)}$ is derived from P_{marR} with deleting the site OM. The amounts of SACE_Lrp used were 0, 500, and 1000 nM, respectively. **d** An illustration of the reporter plasmids in biosensor system. **e** Detection of the interaction of SACE_Lrp with the promoters of *marR* and its divergently transcribed *SACE_6744* using the relative fluorescence units (RFUs) in *E. coli*. The mean values of three replicates are shown, with the standard deviation indicated by error bars

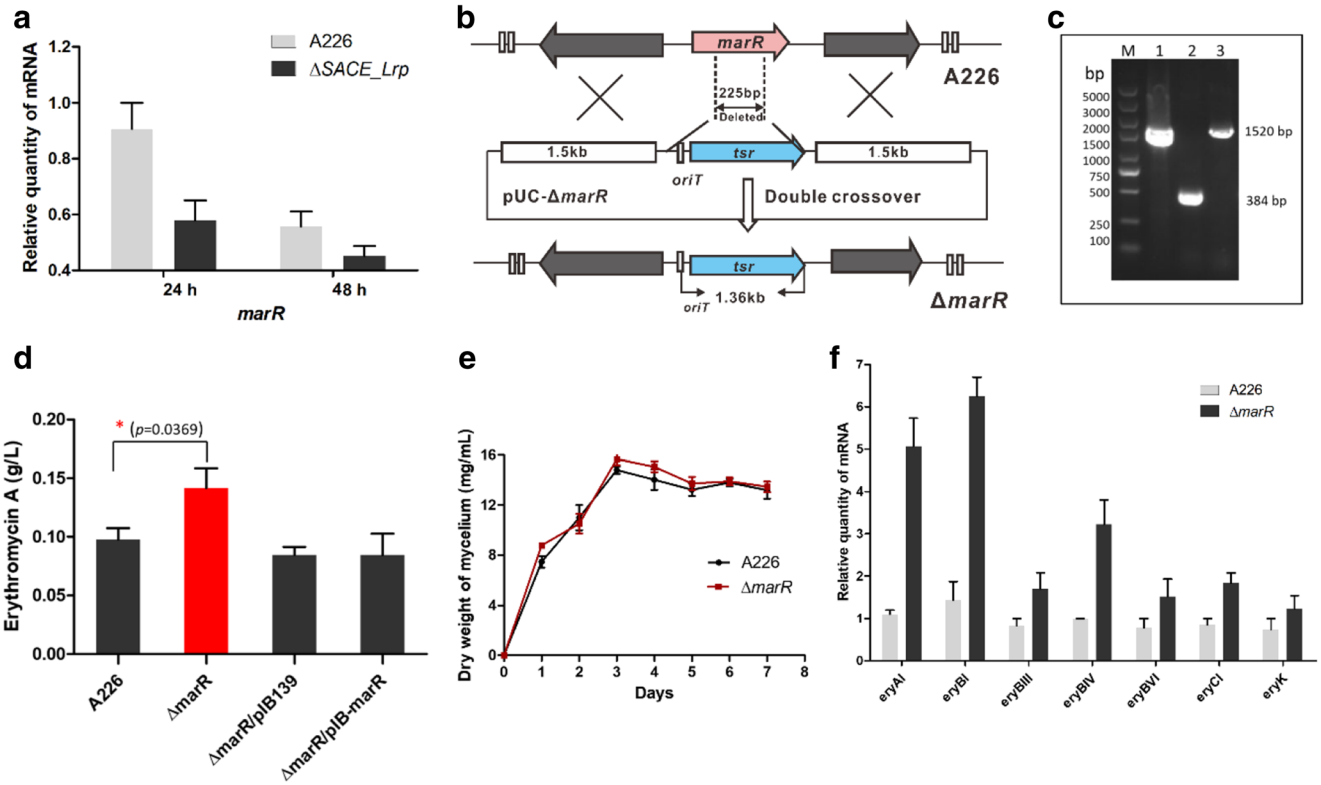

Fig. 3 Effects of *marR* deletion on erythromycin biosynthesis in *S. erythraea*. **a** Effects of *SACE_ Lrp* disruption on transcriptional levels of *marR*. qRT-PCR was used to quantify the amounts of transcripts in A226 and Δ*marR* cultured for 24 h and 48 h in liquid R5 medium. Mean values of three replicates are shown, with the standard deviation indicated by error bars. **b** Schematic deletion of *marR* by homologous recombination in *S. erythraea* A226. **c** PCR confirmation of the *marR* deletion mutant by the primers 6745-C-F/R. Lanes: M, 5000-bp DNA ladder, lane 1 of the size of 1520 bp was detected in pUC-Δ*marR* as a positive control, lane 2 of the size of 384 bp for the PCR amplified band was observed in A226 as a negative control, and lane 3 of the size 1520 bp was detected in mutant Δ*marR*. **d** Erythromycin A production in *S. erythraea* A226 and its derivatives by HPLC analysis. Mean values of three replicates are shown, with the standard deviation indicated by error bars. **e** Growth curves of A226 and Δ*marR*. The two strains were cultured in the R5 liquid medium, and their dry weights of mycelia were measured. **f** Effects of *marR* disruption on transcriptional levels of *ery* cluster. qRT-PCR was used to quantify the amounts of transcripts in A226 and Δ*marR* cultured for 24 h and 48 h in liquid R5 medium. Mean values of three replicates are shown, with the standard deviation indicated by error bars

A226 (Fig. 3d). Δ*marR* and A226 showed similar dry weight of mycelia cultured in R5 liquid medium (Fig. 3e), demonstrating that the increased erythromycin yield in Δ*marR* did not result from changes in cell growth. Taken together, these results indicate that the MarR from *S. erythraea* plays a significant repressor role in erythromycin production.

To investigate the relationship between MarR and erythromycin biosynthesis, we measured the transcription profile of Δ*marR* and A226 during the erythromycin production process. The qRT-PCR results showed that transcript levels of erythromycin biosynthetic genes in the *ery* cluster in Δ*marR* significantly increased by 1.7–4.6 fold compared with that in A226 (Fig. 3f), including *eryAI* (4.6 fold), *eryBI* (4.4 fold), *eryBIII* (2.0 fold), *eryBIV* (3.2 fold), *eryBVI* (1.9 fold), *eryCI* (2.1 fold), and *eryK* (1.7 fold). These results suggest that MarR negatively controls the expression of erythromycin biosynthetic genes in *S. erythraea*.

MarR negatively regulates export and resistance of erythromycin

Control of genes encoding antibiotic efflux pumps is a vital, well-documented role of MarR family regulators (Beggs et al. 2020). We scanned the genomic data of *S. erythraea* and found that only *SACE_2701-2702* encoded an ABC exporter of polyketide antibiotics, probably responsible for erythromycin export (Oliynyk et al. 2007). The qRT-PCR experiments showed that the transcripts of *SACE_2701-2702* in Δ*marR* increased by 3.0 fold at 24 h and 2.3 fold at 48 h compared with that in A226, respectively (Fig. 4a), suggesting that MarR indeed negatively controls the expression of the antibiotic efflux gene *SACE_2701-2702*. To confirm the physiological role of SACE_2701-2702 on erythromycin export, a *SACE_2701-2702* disruption mutant Δ*2701-2702* was constructed with *tsr* replacement in *S. erythraea* A226 (Fig. S1). We therefore assessed the extracellular amount of

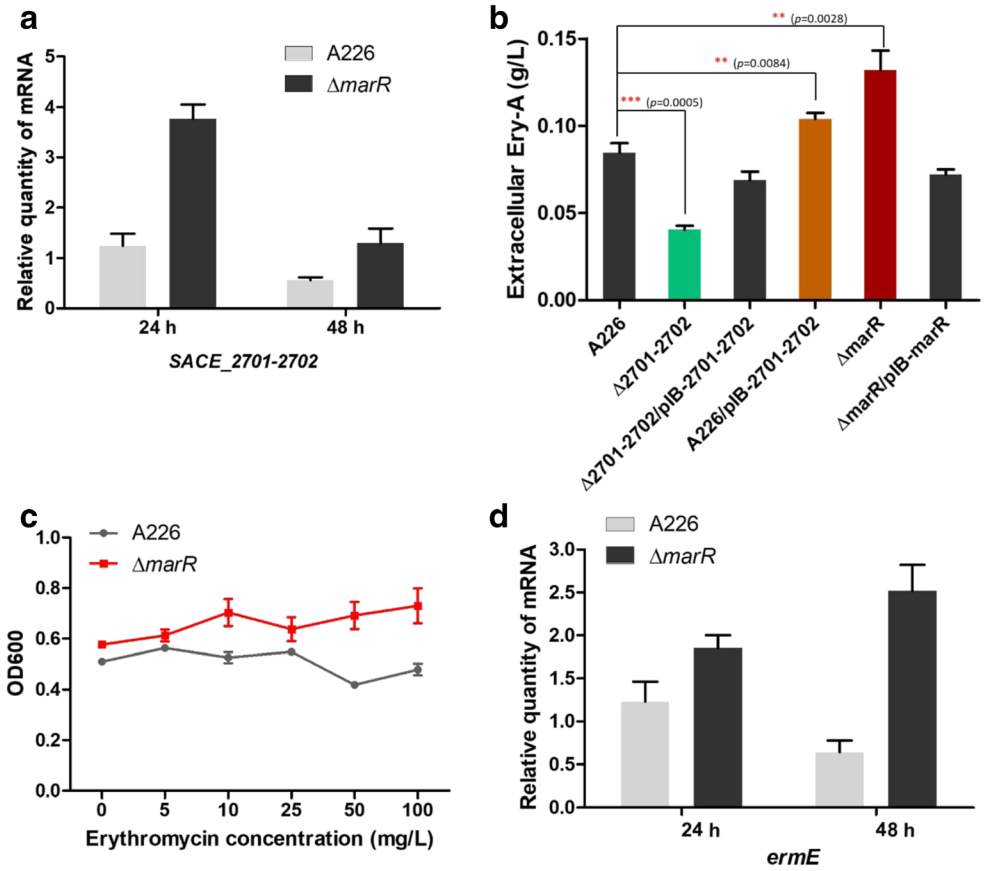

Fig. 4 Effects of *marR* deletion on erythromycin export in *S. erythraea*. **a** Effects of *marR* disruption on transcriptional levels of *SACE_2701-2702*. qRT-PCR was used to quantify the amounts of transcripts in A226 and Δ*marR* cultured for 24 h and 48 h in liquid R5 medium. Mean values of three replicates are shown, with the standard deviation indicated by error bars. **b** Concentrations of the extracellular erythromycin production in A226 and its derivatives by HPLC analysis. **c** Optical densities (OD600) of A226 and Δ*marR* with different concentration of erythromycin. The two strains were cultured in the R5 liquid medium for 24 h, and their optical densities of mycelia were measured. **d** Effects of *marR* disruption on transcriptional levels of *ermE*. qRT-PCR was used to quantify the amounts of transcripts in A226 and Δ*marR* cultured for 24 h and 48 h in liquid R5 medium. Mean values of three replicates are shown, with the standard deviation indicated by error bars

erythromycin in the mutant Δ*2701-2702* and the parent strain A226. The final extracellular accumulation of erythromycin was decreased by 52% (p <0.001) in Δ*2701-2702* compared with the parent strain A226 (Fig. 4b). The results were consisted with that the accumulation of extracellular erythromycin in Δ*marR*, and A226/pIB-*2701-2702* were enhanced by 55% (p <0.01) and 23% (p <0.01), respectively, due to the increased expression of the antibiotic efflux gene *SACE_2701-2702* (Fig. 4b). We also assessed its intracellular erythromycin content and found that there was little erythromycin in the cells and no obvious difference between the above strains (Fig. S2). These findings confirmed that the ABC exporter SACE_2701-2702 is responsible for the secretion of erythromycin outside the cell in *S. erythraea* and the MarR modulates erythromycin production by coordinating its biosynthesis in the cell and export from the cell to the medium.

Given the above fact that gene disruption of *marR* in *S. erythraea* A226 notably increased the extracellular production of erythromycin (Fig. 4b), it was reasonable to explore whether disruption of *marR* would affect the resistance against erythromycin of *S. erythraea*. Therefore, we tested the resistance of Δ*marR* and A226 against erythromycin. As shown in Fig. 4c, when the erythromycin concentration reached 10 mg/L, the growth state of Δ*marR* was obviously superior to that of the parent strain A226. These results suggested that deletion of *marR* effectively improved the resistance against erythromycin of *S. erythraea*, consistent with the qRT-PCR experiments, which showed that the transcripts of the resistance gene *ermE* in the *ery* cluster in Δ*marR* increased by 1.5 and 3.9 fold compared with that in A226 at 24 h and 48 h, respectively (Fig. 4d).

MarR directly controls genes of erythromycin biosynthesis, export, and resistance

There are five regulatory regions in the *ery* cluster, including the promoter regions of *eryBVI* and *eryK* and the intergenic regions of *eryAI-ermBIV*, *eryBI-ermBIII*, and *eryCI-ermE* (Fig. 5a). To determine whether MarR directly binds these

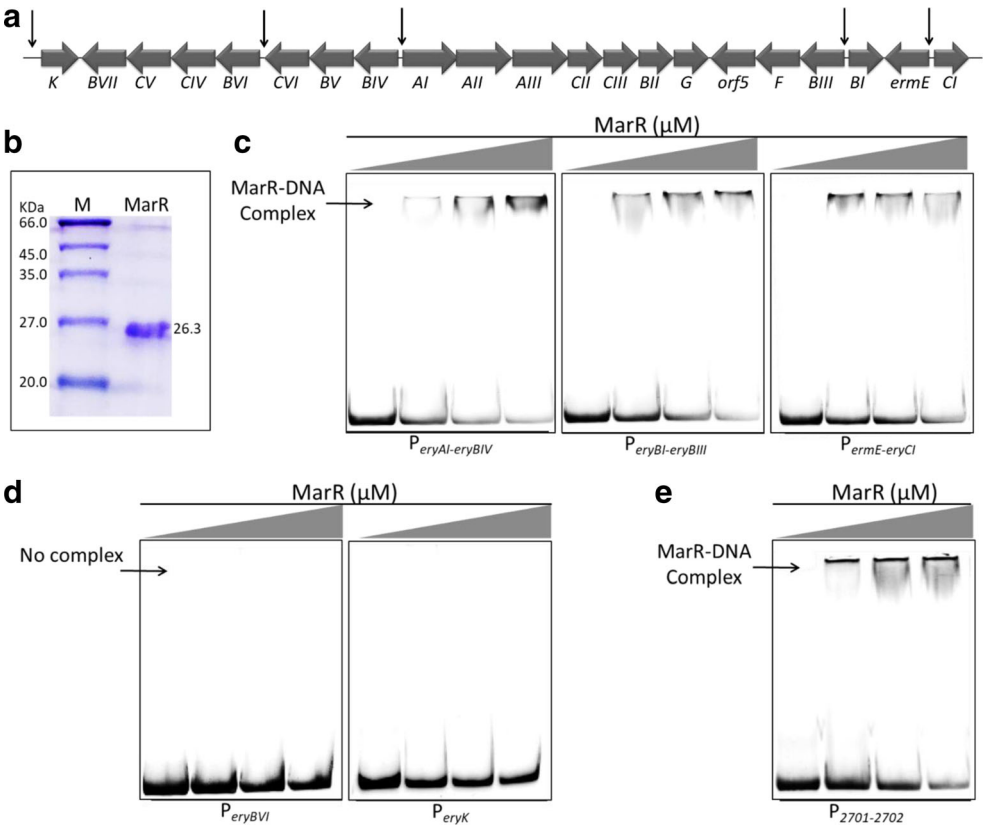

Fig. 5 Binding analyses of purified MarR to the putative targets. **a** Organization of the erythromycin biosynthetic gene cluster. **b** SDS-PAGE analysis of purified His_6-MarR. M, molecular mass marker. **c** EMSA assays of binding of MarR to $P_{eryAI\text{-}eryBIV}$, $P_{eryBI\text{-}eryBIII}$, and $P_{ermE\text{-}eryCI}$. The probe $P_{eryAI\text{-}eryBIV}$ represents the intergenic segment between *eryAI* and *eryBIV*, $P_{eryBI\text{-}eryBIII}$ represents the intergenic segment between *eryBI* and *eryBIII*, and $P_{ermE\text{-}eryCI}$ represents the intergenic segment between *ermE* and *eryCI*. **d** EMSA assays of binding of MarR to P_{eryBVI} and P_{eryK}. P_{eryBVI} and P_{eryK} represent the promoter regions of *eryBVI* and *eryK*, respectively. **e** EMSA assays of binding of MarR to $P_{2701\text{-}2702}$. $P_{2701\text{-}2702}$ represents the promoter region of *SACE_2701-2702*

regulatory regions of the *ery* cluster, we performed EMSA to evaluate the ability of MarR binding to the corresponding probes using purified His_6-tagged MarR protein (Fig. 5b). The results showed that MarR protein can bind to the intergenic regions of *eryAI-ermBIV*, *eryBI-ermBIII*, and *ermE-eryCI* (Fig. 5c), but did not bind to the promoter regions of *eryBVI* and *eryK* (Fig. 5d). With an increase in the protein concentration (0, 0.5, 1.0, and 2.0 μM), we observed a decrease in the abundance of the lower band and an increase in the abundance of the upper band (Fig. 5c). Similarly, MarR protein can bind to the promoter DNA of the erythromycin efflux gene *SACE_2701-2702* (Fig. 5e). These results demonstrate that MarR plays a direct regulatory role in erythromycin biosynthesis, export and resistance in *S. erythraea*.

Joint engineering of SACE_Lrp and MarR for industrial erythromycin overproduction

The above findings indicated that erythromycin production was distinctly improved by *marR* disruption in *S. erythraea* A226 (Fig. 3), so its practical application was explored in an industrial erythromycin high-yield strain. The *marR* gene was disrupted in industrial *S. erythraea* WB, and erythromycin production in shake-flask fermentation was increased by 18% ($p < 0.05$) in WBΔ*marR* in industrial fermentation medium (Fig. 6b).

In our previous study, gene deletion of *SACE_Lrp* in WB resulted in a 19% increase in erythromycin production (Liu et al. 2017). To evaluate the potential application of joint engineering of these two regulators for erythromycin overproduction, we further inactivated *marR* with *aac(3)IV* replacement in WBΔ*SACE_Lrp* (Fig. 6a). As expected, the engineered strain WBΔ*Lrp*Δ*marR* enhanced the yield of erythromycin A by 20% (p <0.01) and 39% ($p < 0.001$) relative to WBΔ*SACE_Lrp* and WB, respectively (Fig. 6b). Taken together, the above results indicate that this strategy of joint engineering the Lrp regulator and its target MarR regulator was effective in improving erythromycin production in industrial high-yield strains.

Discussion

It is vitally important to maintain the balance between the biosynthetic process and export process of antibiotics through fine-tuning (Severi and Thomas 2019). As shown in Fig. 7, we previously reported that SACE_Lrp is an efficient regulator for transporting branched-chain amino acids (BCAAs), playing an important role in regulating erythromycin production (Liu et al. 2017). In this study, we further identified the significant regulatory function of SACE_Lrp on a novel MarR protein, highlighting the key role of MarR as a repressor of erythromycin biosynthesis and export in *S. erythraea* as

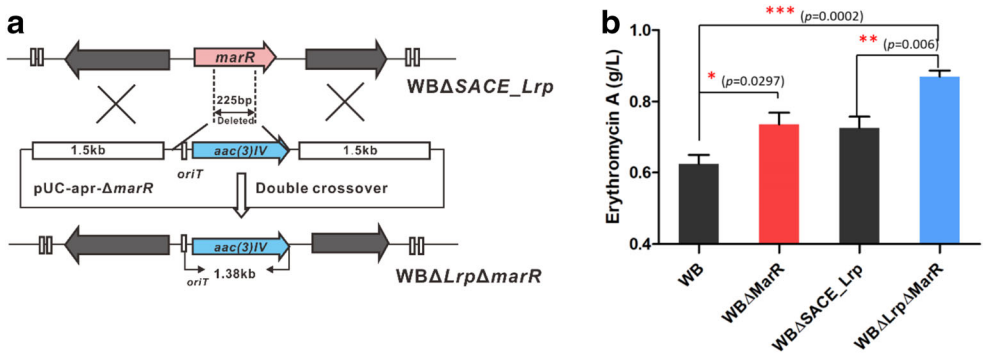

Fig. 6 Combinatorial deletion of *SACE_Lrp* and *marR* in the industrial *S. erythraea*. **a** Erythromycin A production of *S. erythraea* WB and its derivatives in flask fermentation by HPLC analysis. Mean values of three replicates are shown, with the standard deviation indicated by error bars. **b** Schematic deletion of *marR* by homologous recombination in the engineered industrial *S. erythraea* WBΔ*SACE_Lrp*

briefly epitomized in Fig. 7. Lrp proteins are widely distributed among prokaryotes and regulate various biological processes (Peeters and Charlier 2010). However, it is little known that the cascade regulation of Lrp family regulators controls other family regulators to form secondary- or hierarchical-regulatory networks. In *S. coelicolor*, our reported SCO3361 of the Lrp family protein directly regulates the cluster-situated regulator (CSR) gene *actII-ORF4*, controlling the biosynthesis of actinorhodin (Act) (Liu et al. 2017). In *S. spiramyceticus*, SSP_Lrp modulates the expression of three positive regulatory genes *bsm42*, *bsm23*, and *acyB2*, affecting the biosynthesis of spiramycin (SP) and bitespiramycin (BT) (Lu et al. 2019). For MarR family regulators, the cascade regulatory phenomenon is also limited. In *Neisseria gonorrhoeae*, FadR of MarR family protein was identified as being regulated by MtrR of TetR family members by directly binding to the promoter of FarR (Lee et al. 2003). As stated above, our study builds a previously unknown hierarchical-regulatory relationship between the LFR and MFR.

To our knowledge, MarR from *S. erythraea* is the first reported MFR showing explicit regulatory role on the export of antibiotic through a direct manner in antibiotic-producing actinobacteria. In previous researches, there were only three transcription regulators from the TetR family: *S. graminearus* GouR, *S. coelicolor* ActR, and *Amycolatopsis mediterranei* RifQ could modulate antibiotic export in actinobacteria (Lei et al. 2018; Wei et al. 2014; Xu et al. 2012). The TetR family is a large group of transcriptional regulators widely present in bacteria and controls a number of diverse processes (Cuthbertson and Nodwell 2013). The most common targets of TetR proteins are genes encoding the antibiotic efflux pumps, which are often situated right next to the *tetR* gene (Lei et al. 2018; Wei et al. 2014; Xu et al. 2012). Differently, *S. erythraea* MarR modulated the export of erythromycin by

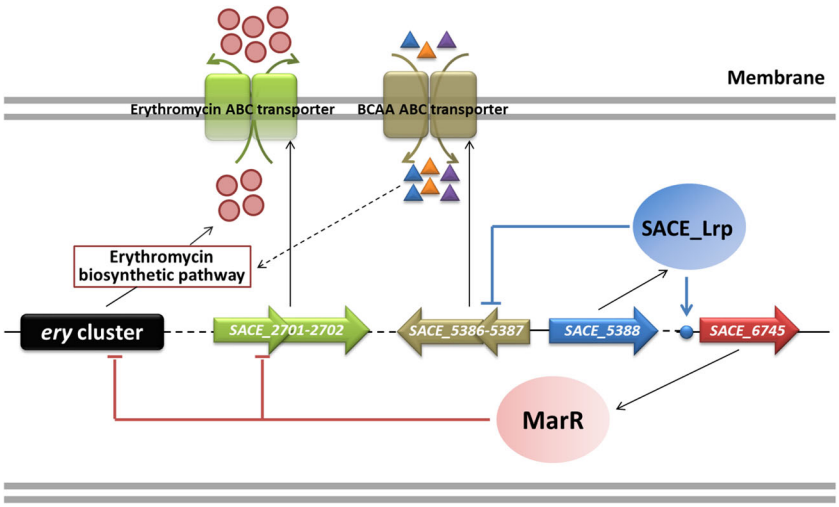

Fig. 7 Proposed model of the regulatory roles of SACE_Lrp and MarR in control of erythromycin biosynthesis and export in *S. erythraea*. *ery* cluster: the erythromycin biosynthetic gene cluster; *SACE_2701-2702*, encodes the ABC exporter of erythromycin (little circle); *SACE_5386-5387*, encodes the ABC exporter of branched-chain amino acid (BCAA) (triangle); *SACE_5388*, encodes the SACE_Lrp protein; *SACE_6745*, encodes the MarR protein; SACE_Lrp directly repressed (blue flat-headed arrow) the expression of *SACE_5386-5387* while directly activated (blue pointed arrow) the expression of *SACE_6745*. MarR directly repressed (red flat-headed arrow) the expressions of the erythromycin biosynthetic genes and *SACE_2701-2702*. Intracellular BCAA catabolism provides the precursors for erythromycin biosynthesis in *S. erythraea* (black dashed arrow)

controlling a faraway ABC exporter, SACE_2701-2702 (Fig. 4a). Our work indicates that there may be various complex regulatory mechanisms of the role of transcription regulators in antibiotic efflux in entire antibiotic-producing actinobacteria.

Most MarR regulators have been shown to control antibiotic biosynthesis through indirectly, such as AbsC of *S. coelicolor*, DptR3 of *S. roseosporus*, SAV4189 of *S. avermitilis*, and CtcS of *S. aureofaciens* (Deng et al. 2011; Guo et al. 2018; Hesketh et al. 2010; Kong et al. 2019). However, MarR of *S. erythraea* controlled erythromycin production by directly controlling the expression of erythromycin biosynthetic genes in the *ery* cluster (Fig. 4a and 5a). Similarly, OhrR of *S. avermitilis* controls avermectin production by directly repressing the expression of the pathway-specific activator gene *aveR* (Sun et al. 2018). In addition, in this study, we found that deletion of *marR* did not affect morphological differentiation in *S. erythraea* (Fig. S3). Similarly, MarR proteins CtcS from *S. aureofaciens*, SAV4189, and OhrR from *S. avermitilis* had no obvious effect on morphological differentiation (Guo et al. 2018; Kong et al. 2019; Sun et al. 2018). However, *S. roseosporus* MarR protein DptR3 deletion delayed aerial mycelium formation and sporulation (Zhang et al. 2015). These findings indicate that the regulatory roles and mechanisms of MFRs from different actinobacteria may not be entirely the same.

A common regulatory function of MarR proteins is directly regulation of its own gene's expression, such as PcaV and TamR of *S. coelicolor*, SAV4189 and OhrR of *S. avermitilis*, DptR3 of *S. roseosporus*, and CtcS of *S. aureofaciens* (Davis et al. 2013; Guo et al. 2018; Huang and Grove 2013; Kong et al. 2019; Sun et al. 2018; Zhang et al. 2015). In *S. erythraea*, the *marR* transcription level in Δ*marR* significantly decreased compared with A226 by qRT-PCR (Fig. S4a). Unexpectedly, MarR did not bind to P_{marR} including the entire *marR* promoter region by EMSA (Fig. S4b). This case is different from most MarR proteins, implying that the binding affinity of *S. erythraea* MarR be induced by an unknown cofactor or signal.

In this work, although we demonstrated the direct control of erythromycin biosynthesis, export, and resistance by MarR in *S. erythraea* (Figs. 4a and 5a), erythromycin is not the ligand of MarR protein (data not shown). In *S. aureofaciens*, the antibiotics CTC and TC could weaken the DNA-binding activity of CtcS (Kong et al. 2019). Pentalenolactone and two intermediates, pentalenolactones D and F, act as ligands of PenR of *S. exfoliatus* and its homologue PntR of *S. arenae* (Zhu et al. 2013). SAV4189 of *S. avermitilis* did not respond to antibiotics AveB1 and Oli but response to antibiotics HygB and Thi, which are produced by other *Streptomyces* species, to weaken its DNA-binding activity (Guo et al. 2018), implying that the MarR of *S. erythraea* can sense some other antibiotic, and it will be of interest to solve this question in the future.

Table 2 LFRs and MFRs distributed in typical antibiotic-producing actinomycetes

Family	Strain	ID/SM (%)	Protein accession number	Amino acids
Lrp	*Nocardia farcinica*	81/87	BAD59325.1	146
	Streptomyces griseus	80/85	WP_037682417.1	146
	Streptomyces mirabilis	78/86	WP_075027953.1	145
	Streptosporangium roseum	57/72	ACZ90523.1	150
	Streptomyces avermitilis	52/65	BAC71476.1	152
	Streptomyces venezuelae	51/66	CCA53511.1	154
	Streptomyces clavuligerus	51/63	EFG10736.1	147
	Streptomyces hygroscopicus	49/65	AEY89996.1	150
	Streptomyces bingchenggensis	49/61	ADI12526.1	147
	Streptomyces lincolnensis	46/61	AXG54283.1	150
	Streptomyces coelicolor	44/59	CAB40861.1	150
MarR	*Micromonospora eburnea*	67/74	WP_091118096.1	154
	Streptomyces azureus	58/72	GAP47817.1	141
	Streptomyces lincolnensis	56/69	WP_067443173.1	136
	Streptomyces virginiae	54/66	WP_033214083.1	135
	Streptomyces bingchenggensis	45/60	ADI11796.1	119
	Streptomyces avermitilis	44/58	BAC69718.1	218
	Streptomyces venezuelae	44/55	CCA55920.1	188
	Streptomyces hygroscopicus	43/57	AEY92550.1	208
	Streptomyces coelicolor	42/58	CAA20599.1	216
	Streptomyces griseus	42/61	BAG22378.1	236

ID stands for identity, and SM stands for similarity

Overall, these findings reveal the relatively high variability in the ligand-binding domain of MarR proteins and their complex mechanisms in response to different ligands.

Currently, genetic engineering of regulatory systems provides a potential approach for relevant antibiotic improvement (Li et al. 2015). Based on the regulatory properties of these transcription regulators, they have been employed for the overproduction of target metabolites by modulating transcription repression or activation (Niu and Tan 2013). For example, disruption of LFR SACE_Lrp combined with overexpression of its target *SACE_5387-5386* in industrial *S. erythraea* WB (Liu et al. 2017) and overexpression of MFR SAV4189 combined with disruption of its target gene *sav_4190* in industrial *S. avermitilis* A-144 (Guo et al. 2018) were efficient approaches to enhance the relevant antibiotic production. In this study, we found that deletion of *marR* in *S. erythraea* A226 reduced the expression of its own gene but promoted erythromycin production (Fig. 3d), implying that reduction of the *marR* expression level is an effective strategy for erythromycin overproduction. As expected, deletion of *SACE_Lrp* combined with deletion of its target *marR* improved erythromycin production of industrial *S. erythraea* strain by 39% in industrial strain WB (Fig. 6b). On the one hand, this strategy can improve the production erythromycin precursor by deleting *SACE_Lrp*; on the other hand, it can promote erythromycin biosynthesis and efflux by deleting *marR*. Based on bioinformatics analysis with SACE_Lrp and MarR, we found that LFRs and MFRs are widely present among typical antibiotic-producing actinomycetes (Table 2). To summarized, our present findings raise the knowledge of the molecular regulation of antibiotic biosynthesis and export by LFRs and MFRs and provide a potential use in large-scale industrial applications for target metabolite overproduction by joint engineering of these regulators.

To summarize, in our previous report, SACE_Lrp indirectly regulated the erythromycin production through directly controlling the BCAAs transport, and a question remains whether SACE_Lrp can control the erythromycin biosynthesis by secondary-or hierarchical-regulatory networks. Here in this study, our work revealed that the novel regulator MarR (SACE_6745) from *S. erythraea*, which is controlled by SACE_Lrp, plays a negative role in erythromycin biosynthesis and export. Dissection of the regulatory mechanism of MarR revealed that MarR directly controlled the genes that are responsible for erythromycin biosynthesis, export, and resistance. Moreover, joint engineering of the SACE_Lrp-MarR regulatory system for practical application on previously constructed high-yield WBΔ*SACE_Lrp* resulted in notable overproduction of erythromycin by further deletion of *marR*. Based on the above findings, we present new insights into the hierarchical-regulatory relationship of Lrp and MarR for the first time, and new avenues for coordinating antibiotic biosynthesis and export with combinatorial engineering regulators in actinobacteria.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-021-11228-8.

Author contribution JL, HW, and BZ conceived the study. JL designed the study. JL, LL, YW, BL, and XC performed the experiments. JL, LT, SD, EY, and HW analyzed the data. JL wrote the manuscript. HW and BZ checked the final version. All authors have read and approved the manuscript.

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Data availability All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

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

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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