J Ind Microbiol Biotechnol (2014) 41:1159–1167
DOI 10.1007/s10295-014-1449-9

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SACE_3986, a TetR family transcriptional regulator, negatively controls erythromycin biosynthesis in *Saccharopolyspora erythraea*

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Received: 7 March 2014 / Accepted: 17 April 2014 / Published online: 3 May 2014

Abstract Erythromycin, a medically important antibiotic, is produced by *Saccharopolyspora erythraea*. Unusually, the erythromycin biosynthetic gene cluster lacks a regulatory gene, and the regulation of its biosynthesis remains largely unknown. In this study, through gene deletion, complementation and overexpression experiments, we identified a novel TetR family transcriptional regulator SACE_3986 negatively regulating erythromycin biosynthesis in *S. erythraea* A226. When *SACE_3986* was further inactivated in an industrial strain WB, erythromycin A yield of the mutant was increased by 54.2 % in average compared with that of its parent strain, displaying the universality of SACE_3986 as a repressor for erythromycin production in *S. erythraea*. qRT-PCR analysis indicated that SACE_3986 repressed the transcription of its adjacent gene *SACE_3985* (which encodes a short-chain dehydrogenase/reductase), erythromycin biosynthetic gene *eryAI* and the resistance gene *ermE*. As determined by EMSA analysis, purified SACE_3986 protein specifically bound to the intergenic region between *SACE_3985* and *SACE_3986*, whereas it did not bind to the promoter regions of *eryAI* and *ermE*. Furthermore, overexpression of *SACE_3985* in A226 led to enhanced erythromycin A yield by at least 32.6 %. These findings indicate that SACE_3986 is a negative regulator of erythromycin biosynthesis, and the adjacent gene *SACE_3985* is one of its target genes. The present study provides a basis to increase erythromycin production by engineering of SACE_3986 and SACE_3985 in *S. erythraea*.

Keywords *Saccharopolyspora erythraea* · Erythromycin · TetR family transcriptional regulator · SACE_3986 · SACE_3985

Introduction

Saccharopolyspora erythraea is an actinomycete used industrially for production of erythromycin A, a potent macrolide antibiotic. Semi-synthetic derivatives of erythromycin, such as clarithromycin and azithromycin, are widely used in medicine to treat infections caused by pathogenic Gram-positive bacteria. Due to its high industrial and medical importance, efforts toward erythromycin overproduction have been undertaken by genetic/metabolic engineering as well as classical mutagenesis methods [1, 3, 24, 29].

Extensive genetic and biochemical investigations have provided insights into the structural genes involved in erythromycin biosynthesis [2, 30]. The erythromycin biosynthetic (*ery*) cluster contains 20 genes arranged in four major polycistronic units, spanning over 60 kb of DNA [25]. In contrast to the commonly occurring cluster-situated regulators (CSRs) in actinomycetes [17], *S. erythraea* lacks a regulatory gene in the *ery* cluster, compromising efforts to

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detect regulators affecting erythromycin production and to understand its specialized regulation mechanisms. Guided by in vitro and in vivo experiments, BldD (SACE_2077), a key developmental regulator in actinomycetes [8], was proved to directly control erythromycin biosynthesis by binding to five promoter regions of the *ery* cluster [5]. Lately, a putative regulatory protein, SACE_5599, was shown to positively control erythromycin production and morphological differentiation [16]. Yet, our understanding of the molecular mechanisms regulating erythromycin biosynthesis in *S. erythraea* remains limited.

The TetR family transcriptional regulators (TFRs) are widespread in bacteria, with a conserved helix-turn-helix DNA-binding domain and a C-terminal ligand regulatory domain, and they control a variety of processes including antibiotic production, osmotic stress response, efflux pump expression, and multidrug resistance [7, 23]. Recently, several TFRs have been demonstrated to control the biosynthesis of multiple antibiotics or morphogenesis in *Streptomyces* [4, 10, 12, 14, 19, 31]. Previously, based on homologous recombination with linearized DNA fragments, we developed a rapid gene inactivation technology in *S. erythraea* [18] and identified two TFRs, SACE_7040 and SACE_0012, shown to negatively affect morphogenesis of *S. erythraea* [11, 33]. However, it is not known whether any of the TFRs from *S. erythraea* is related to erythromycin production. In this study, we identified and characterized a novel TFR (SACE_3986) that negatively regulates erythromycin biosynthesis in *S. erythraea*.

Materials and methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. *S. erythraea* was grown either on solid R3M medium (gram per liter: 103 g sucrose, 4 g yeast extract, 4 g tryptone, 4 g casamino acids, 0.25 g K_2SO_4, 2.2 g agar and 40 μl trace element solution) or in liquid TSB medium (3 g/L tryptone soya broth powder) with appropriate antibiotics at 30 °C [27]. *Escherichia coli* and *Bacillus subtilis* strains were cultured in Luria–Bertani (LB) liquid medium or on LB plates at 37 °C [26]. DNA isolation and manipulation in *E. coli* and *S. erythraea* were carried out according to the standard methods [11, 15].

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Description	References
E. coli		
DH5α	F *recA lac*ZM15	[26]
BL21 (DE3)	F^- *ompT hsdSB*(rB^- mB^-) *dcm gal* λ(*DE3*)	Novagen
S. erythraea		
A226	CGMCC 8279, an erythromycin low producer	China Pharmaceutical Culture Collection
Δ*SACE_3986*	A226 derivative with *SACE_3986* deleted	This study
Δ*SACE_3986*/pIB139	Δ*SACE_3986* carrying pIB139	This study
Δ*SACE_3986*/ pIB139-3986	Δ*SACE_3986* carrying pIB139-3986	This study
A226/pIB139	A226 carrying pIB139	This study
A226/pIB139-3986	A226 carrying pIB139-3986	This study
A226/pIB139-3985	A226 carrying pIB139-3985	This study
WB	CGMCC 8280, an erythromycin industrial overproducer	Anhui Wanbei Pharmaceutical Co., Ltd.
WBΔ*SACE_3986*	WB derivative with *SACE_3986* deleted	This study
Plasmids		
pUCTSR	pUC18 derivative containing a 1.36-kb fragment of a thiostrepton resistance gene in *Bam*HI/*Sma*I sites	[11]
pUCTSRΔ3986	pUCTSR derivative containing two 1.5-kb fragments, the upstream and downstream regions of *SACE_3986*	This study
pIB139	*E. coli–S. erythraea* integrative shuttle vector containing a strong constitutive *ermE** promoter	[32]
pIB139-3986	pIB139 carrying an extra *SACE_3986* for the gene complementation and overexpression	This study
pIB139-3985	pIB139 carrying an extra *SACE_3985* for the gene overexpression	This study
pET28a	T7 promoter, His-tag, *kan*	Novagen
pET28a-3986	pET28a derivative carrying *SACE_3986*	This study

Table 2 Primers used in this study

Name	Sequence (5′–3′) (restriction site underlined)	Use
3986-P1	GCGGAATTCGCGTGCGGGAACACCGC	Inactivation of *SACE_3986*
3986-P2	TCTGGTACCGGGCCATCACGAGGCTACGATAACGGC	
3986-P3	CGCTCTAGATTAGTGGTACGCCGCTGCTACGGTGC	
3986-P4	CCGAAGCTTGTGCGCGCCACCAAGCG	
3986-P5	CGGTGACCACCGCGACCCTGCCCGC	Confirmation of Δ*SACE_3986* by PCR
3986-P6	AGTAGCCGGTCATGCGTTGCCACCTGCCTCGG	
3986-P7	AGCCATATGATGGCCCGAGTCAAGAGCA	Complementation and overexpression of *SACE_3986*
3986-P8	GGCTCTAGACTAACTTCCGGCGATCACGAC	
3986-P9	AGCCATATGATGGCCCGAGTCAAGAGCA	Expression of SACE_3986 In *E. coli*
3986-P10	GGCAAGCTTACTTCCGGCGATCACGAC	
3985-P1	GCGTTGTAGGCGCTCTGC	qRT-PCR analysis of *SACE_3985*
3985-P2	GCGTGTCATGGACGTCAACTT	
3985-P3	CTTCATATGTTGGCGAGGCGGGAATCCTG	Overexpression of *SACE_3985*
3985-P4	CACTCTAGACTAACGCACGAGCCTGCCGG	
3985-3986-F	GCCTCGCCAACGCCGCAAGC	EMSA of intergenic region of *SACE_3985* and *SACE_3986*
3985-3986-R	CGACCTGCCGTCCGCCGGTG	
eryAI-P1	CCGCTGATGCCGAACGAC	qRT-PCR analysis of *eryAI*
eryAI-P2	CACCCTTCCCCGCACTCTG	
eryAI-P3	CGGAGCATTTGCTCGCTTTCCAGG	EMSA of *eryAI* promoter
eryAI-P4	GCGTCCCCCTACTCGACGACCAC	
ermE-P1	CCTCCAGGCACCAGTCCAC	qRT-PCR analysis of *ermE*
ermE-P2	AGTCGTTGCGGGAGAAGCT	
ermE-P3	GCGAGTGTCCGTTCGAGTGGCGG	EMSA of *ermE* promoter
ermE-P4	CGCTGGATCCTACCAACCGGCAC	
hrdB-F	GGTCACGCCGTAGACCTGGC	qRT-PCR analysis of *hrdB* as an internal control
hrdB-R	CGGTGTCGTTCACGCTGCTG	

Gene deletion, complementation and overexpression

Two 1.5 kb DNA fragments flanking the *SACE_3986* gene were respectively obtained from the genome of *S. erythraea* A226 by PCR with the primer pairs 3986-P1/3986-P2 and 3986-P3/3986-P4 (Table 2). Then, the amplified upstream and downstream homologous fragments were digested with *Eco*RI/*Kpn*I and *Xba*I/*Hin*dIII restriction enzymes, and inserted into corresponding sites of pUCTSR to generate pUCTSRΔ3986. By the homologous recombination of linearized fragments [11], *SACE_3986* was replaced by the thiostrepton resistance gene *tsr*. The desired mutant Δ*SACE_3986* was obtained by thiostrepton-resistant screening, and further confirmed by PCR using the primers 3986-P5 and 3986-P6 (Table 2). Similarly, *SACE_3986* was disrupted in the high-yield *S. erythraea* WB, generating the mutant WBΔ*SACE_3986*.

For complementation of Δ*SACE_3986* mutant, a 672-bp *SACE_3986* was amplified by PCR with the primers 3986-P7 and 3986-P8 (Table 2). The PCR product was cloned into the *Nde*I/*Xba*I sites of pIB139 [32], a φC31-derived integrative vector containing the strong constitutive promoter *ermEp**, to generate pIB139-3986. Then, pIB139-3986 was introduced into Δ*SACE_3986* mutant, and the desired complementation strain Δ*SACE_3986*/pIB139-3986 was obtained by apramycin-resistant screening and PCR confirmation. Furthermore, pIB139 and pIB139-3986 were introduced into A226, generating control strain A226/pIB139 and overexpressed strain A226/pIB139-3986, respectively.

For overexpression of SACE_3985 in *S. erythraea*, an 828-bp *SACE_3985* was amplified with the primers 3985-P3 and 3985-P4 (Table 2). Then, the *Nde*I/*Xba*I digested fragment was ligated into the corresponding sites of pIB139, and the constructed vector pIB139-3985 was introduced into A226 to obtain the overexpressed strain A226/pIB139-3985.

Fermentation and HPLC analysis of erythromycin A production

For fermentation of A226 and its derivatives, spores from the R3M plates cultured for 4 days were inoculated into the 250-ml baffled flasks containing 30-ml TSB medium, and incubated for 2 days at 30 °C (220 rpm). Then, three

milliliters of those cultures were transferred into 250-ml baffled flasks containing 30-ml R5 liquid fermentation medium (gram per liter: 103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2{\cdot}6H_2O$, 0.1 g casein hydrolysate, 10 g glucose, 5 g yeast extract, 5.73 g TES, 3 g L-proline, 0.05 g KH_2PO_4, 2.94 g $CaCl_2{\cdot}2H_2O$, 0.28 g NaOH and 2 ml trace element solution) [15], and incubated for 6 days at 30 °C (220 rpm). For fermentation of the erythromycin high-yield industrial strain WB and its derivatives, a liquid industrial medium (gram per liter: 40 g cornstarch, 30 g soybean flour, 30 g dextrin, 2 g $(NH_4)_2SO_4$, 10 g soybean oil, 6 g $CaCO_3$, pH 6.8–7.0) was used. After 1-day fermentation, 0.3 ml of n-propanol was added into the cultures, and further incubated for 5 days at 30 °C (220 rpm).

According to the method previously described [28], erythromycin A extracted from those cultures was quantified by high-performance liquid chromatograph (HPLC). HPLC analysis was performed on Agilent 1260 HPLC system equipped with an Agilent Extend-C18 column (5 μm; 250 × 4.6 mm), which was equilibrated with 40 % solution A (acetonitrile, chromatographic grade) and 60 % solution B (potassium dihydrogen phosphate, 0.032 M, pH 6.8). An isocratic program was carried out at a flow rate of 1.0 ml/min at 29 °C using UV detector at 210 nm.

Quantitative real-time PCR (qRT-PCR) assay

The relative transcriptional levels of *eryAI*, *ermE*, and *SACE_3985* were determined by qRT-PCR assay. Specific primers were designed as listed in Table 2. Following the manufacturer's instructions of a RNA extraction/purification kit (SBS), the total RNA was extracted from *S. erythraea* A226 or Δ*SACE_3986* mutant after 4 days of growth on R5 agar medium. Isolated RNA was treated with DNase I (MBI Fermentas), and reverse transcription was achieved using a cDNA synthesis kit (MBI Fermentas). qRT-PCR reactions were performed on the Applied Biosystems StepOne Plus system with Maxima™ SYBR Green/ROX qPCR Master Mix (MBI Fermentas). The *hrdB* gene encoding the major sigma factor in *S. erythraea* was used as an internal control, and relative quantification was evaluated using a comparative cycle threshold (C_T) method [20].

Heterologous expression and purification of SACE_3986

A DNA fragment encoding 224 amino acids of SACE_3986 was obtained by PCR using the primers 3986-P9 and 3986-P10 (Table 2). The PCR product was digested with *Nde*I/*Hin*dIII restriction enzymes and inserted into the corresponding sites of the expression vector pET-28a (Novagen) to generate pET28a-3986, which was introduced into *E. coli* BL21 (DE3) for protein expression. After induction by 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the resulting recombinant His_6-tagged SACE_3986 protein was extracted and purified on a Ni^{2+}-NTA spin column (BIO-RAD) according to the manufacturer's instructions. The concentration of purified protein was quantified by BCA protein assay kit (Thermo) by following the manufacturer's instructions, and the purity was judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Electrophoretic mobility shift assays (EMSAs)

EMSA was performed according to the method as previously described [13]. Using the genomic DNA from *S. erythraea* A226 as a template, the promoter regions of *eryAI* and *ermE*, and the intergenic region between *SACE_3985* and *SACE_3986* were amplified by PCR with their respective primers (Table 2) labeled at 5′ end by the 6-isomer of carboxyfluorescein (6-FAM). These labeled DNA fragments were individually mixed with purified His_6-tagged SACE_3986 protein. The binding reaction system consisted of 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 50 mM EDTA, 60 mM KCl, 10 mM DTT, 10 % glycerol, 150 ng labeled probes and 100–400 nM purified SACE_3986 protein. For competitive inhibition of the binding reaction, 7.5 μg unlabeled fragments or 7.5 μg poly dIdC was added into this reaction system, respectively. After incubation on ice for 10 min, the reactants were run on an 8 % TBE polyacrylamide gel (Bio-Rad) with 0.5 × TBE as a running buffer at 30 mA for 1 h.

Results

SACE_3986 acts as a negative regulator for erythromycin production

Given the key roles played by the TFRs in antibiotic biosynthesis and morphological differentiation in actinomycetes [6], we carried out gene inactivation studies, biological activity analyses and phenotype observations, and have identified several TFRs involved in erythromycin production or morphological differentiation in *S. erythraea*. These include SACE_7040 and SACE_0012, related to the formation of aerial mycelium [11, 33], and the currently studied SACE_3986, which regulates erythromycin biosynthesis.

According to the genome annotation of *S. erythraea*, the *SACE_3986* gene (672 bp, GenBank Accession No. NC-009142. 4,373,306–4,373,977 nt) encodes a classical TFR of 223 amino acids with a molecular mass of approximately 26 kDa, containing N-terminal helix-turn-helix DNA-binding domain [23]. To investigate its function, we replaced *SACE_3986* in *S. erythraea* A226 with

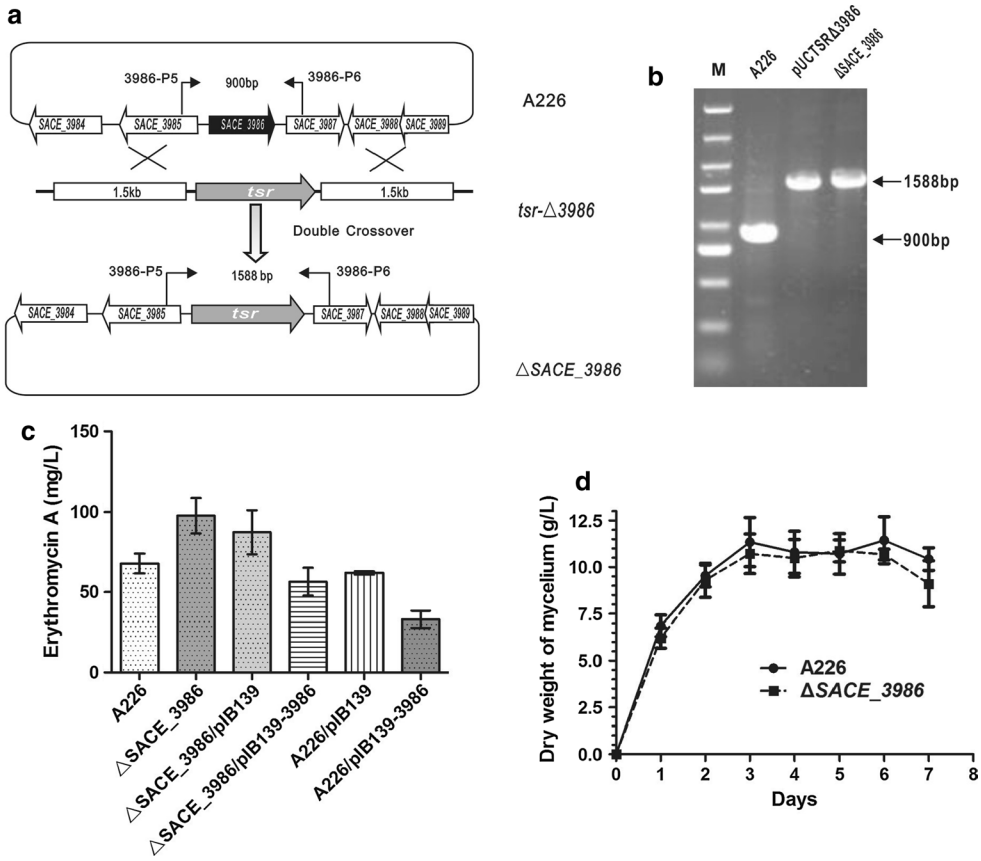

Fig. 1 Inactivation of *SACE_3986* in *S. erythraea* A226. **a** Schematic deletion of *SACE_3986* by linearized fragment homologous recombination in A226. **b** PCR confirmation of the *SACE_3986* deletion mutant by the primers 3986-P5 and 3986-P6. The size of 900 bp for the PCR-amplified bands was observed in A226, while a band of the size 1,588 bp was detected in mutant Δ*SACE_3986*. **c** Erythromycin A production in A226, Δ*SACE_3986*, Δ*SACE_3986*/pIB139, Δ*SACE_3986*/pIB139-3986, A226/pIB139, and A226/pIB139-3986 by HPLC analysis. Mean values of three replicated experiments are shown, with the standard deviation indicated by *error bars*. **d** Growth curves of A226 and Δ*SACE_3986* in the liquid R5 medium

a thiostrepton resistance gene *tsr* via linearized fragment homologous recombination (Fig. 1a). A thiostrepton-resistant mutant Δ*SACE_3986* was screened and confirmed by PCR analysis (Fig. 1b).

The parent strain A226 and Δ*SACE_3986* mutant were cultivated in the liquid fermentation medium R5 for 6 days, and the extracts of those cultures were quantified by HPLC analysis. Erythromycin A yield in the Δ*SACE_3986* mutant (97.8 mg/L) was increased by 44.2 % in comparison to the parental productivity (67.8 mg/L). (Fig. 1c). When Δ*SACE_3986* was complemented with a cloned copy of *SACE_3986*, the amount of erythromycin A was recovered almost to the original level (Fig. 1c), demonstrating that SACE_3986 played a negative regulatory role in erythromycin production. We also compared the mycelium dry weight of A226 and Δ*SACE_3986* cultured in R5 liquid medium, and found that the two strains had comparable growth rates and cell densities (Fig. 1d), implying that enhanced erythromycin production in Δ*SACE_3986* was not caused by the improvement of cell growth.

To further confirm the negative regulatory role of SACE_3986 in erythromycin production, *SACE_3986* was overexpressed in A226. The plasmids pIB139 and pIB139-3986 were, respectively, introduced into A226 to generate control strain A226/pIB139 and the overexpressed derivative A226/pIB139-3986. HPLC analysis showed that A226/pIB139-3986 (33.1 mg/L) had 51.2 and 46.6 % reduction in erythromycin A yield, compared with A226 (67.8 mg/L) and A226/pIB139 (62.0 mg/L), respectively (Fig. 1c). In addition, when grown on the R3M agar medium, A226 and its derivatives had no significant phenotypic differences (data not shown). These findings, together with *SACE_3986* inactivation and complementation experiments, indicated that SACE_3986 acted as a negative regulator for erythromycin production, and had no effect on cell growth and morphological differentiation in *S. erythraea*.

Enhanced erythromycin A yield by *SACE_3986* disruption in an industrial strain WB

To examine the universality of SACE_3986 as a negative regulator for erythromycin biosynthesis in *S. erythraea*, we further inactivated *SACE_3986* in an erythromycin high-yield industrial *S. erythraea* strain WB, generating two separate isolates of the desired mutant, WBΔ*SACE_3986*A and WBΔ*SACE_3986*B. When cultured in a liquid industrial fermentation medium for 6 days, erythromycin A yields of the two mutants, respectively, reached 930.3 and 975.7 mg/L with 50.5 and 57.9 % increase in comparison

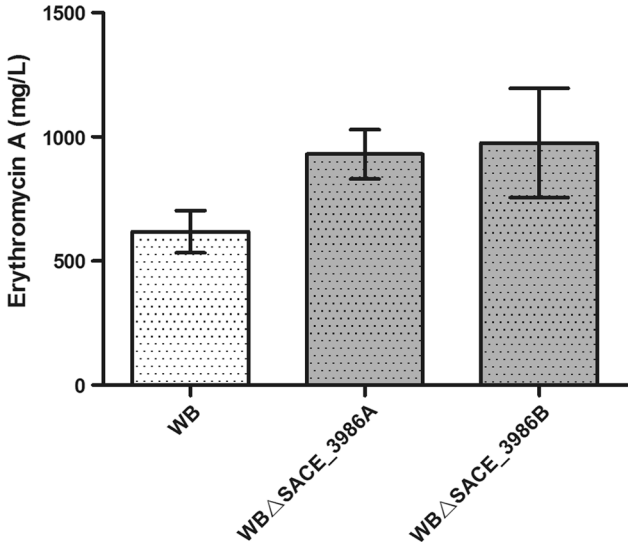

Fig. 2 Inactivation of *SACE_3986* in the industrial strain *S. erythraea* WB leading to a higher production of erythromycin A. Mean values of three replicated experiments are shown, with the standard deviation indicated by *error bars*. WBΔ*SACE_3986*A and WBΔ*SACE_3986*B are two independent mutants derived from the industrial strain WB

to that of WB (618.1 mg/L) (Fig. 2). As WB is already an industrial strain, it is likely that the deletion of *SACE_3986* in other erythromycin high-yield *S. erythraea* strains will have commercial value.

The deletion of *SACE_3986* increases the transcription of *eryA*, *ermE* and *SACE_3985*

To search for potential target genes regulated by *SACE_3986*, we performed qRT-PCR using RNAs isolated from A226 and Δ*SACE_3986* mutant on the fourth day of growth. Initially, the *ery* gene *eryAI* (encoding polyketide synthase I) and the resistance gene *ermE* (encoding rRNA methyltransferase) were selected for transcriptional comparison between A226 and Δ*SACE_3986*. The results showed that the transcription of *eryAI* and *ermE* in Δ*SACE_3986* was increased by 3.7- and 1.7-folds relative to that of A226, respectively (Fig. 3), implying that SACE_3986 had a negative effect on erythromycin biosynthesis by potentially repressing the transcription of its biosynthetic gene and the resistance gene. In addition, the transcriptional level of *SACE_3985*, adjacent to *SACE_3986*, was examined using the same RNA preparations, revealing that *SACE_3985* was transcriptionally up-regulated by 5.7-fold in Δ*SACE_3986* in comparison to A226 (Fig. 3). To sum up, these results demonstrated that SACE_3986 could repress the transcription of *eryAI* and *ermE* as well as its adjacent gene *SACE_3985*.

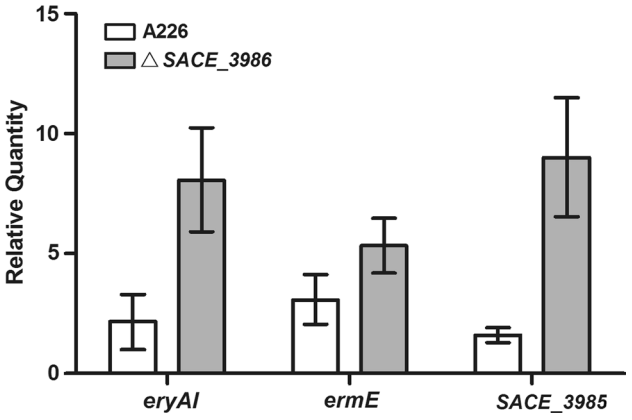

Fig. 3 Effects of *SACE_3986* disruption on transcriptional levels of *eryAI*, *ermE* and *SACE_3985*. qRT-PCR was used to quantify the amounts of transcripts produced by A226 and Δ*SACE_3986* cultured for 4 days. Mean values of three replicated experiments are shown, with the standard deviation indicated by *error bars*

SACE_3986 binds to the intergenic region between *SACE_3985* and *SACE_3986*

Most TetR family members are transcriptional repressors and regulate transcription of target genes by specifically binding to their promoters [6]. To determine whether SACE_3986 directly binds to above-mentioned gene promoters, a full-length SACE_3986 gene was expressed in *E. coli* BL21 (DE3), and the purified His_6-SACE_3986 protein was used for in vitro EMSAs (Fig. 4a).

By incubating 6-FAM labeled *eryAI* or *ermE* promoter DNA with purified SACE_3986 protein, no obvious gel shift band was detected (Fig. 4b), suggesting that SACE_3986 might indirectly regulate the expression of erythromycin biosynthetic and resistant genes of *S. erythraea*.

The entire intergenic region between *SACE_3985* and *SACE_3986* was 85 bp in length, and a probe (140 bp) labeled with 6-FAM was designed to cover this region. As detected by EMSA, two gel shift bands were obviously detected, implying that there were two SACE_3986 binding sites within this region (Fig. 4b). When a 50-fold excess of unlabeled probe was added into the reaction system to evaluate binding specificity, it dramatically competed with labeled probe for binding to SACE_3986 (Fig. 4b). As a negative control, a non-specific DNA, poly dIdC, was used to compete with the 6-FAM labeled probe, and the two shifted bands did not disappear, thereby confirming that SACE_3986 specifically bound to the *SACE_3985* -*SACE_3986* bidirectional promoter regions. Taken together, it was determined that SACE_3986 repressed the transcription of *SACE_3985* by interacting with its promoter region.

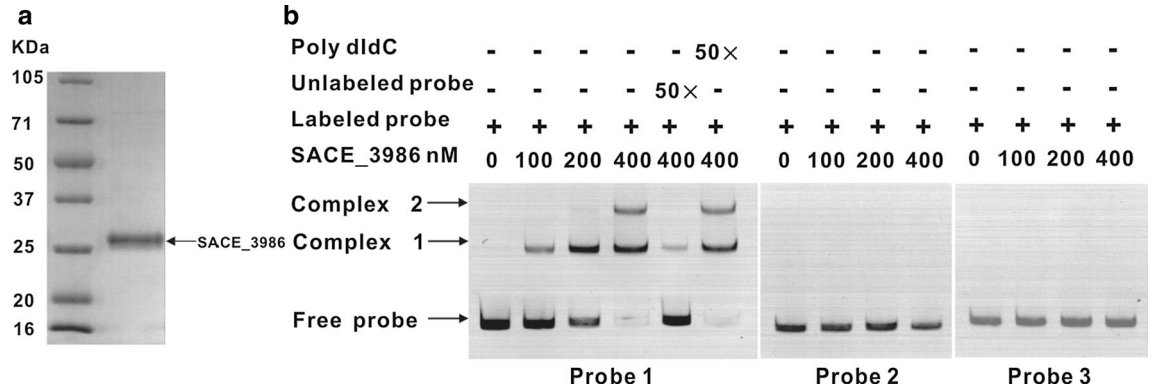

Fig. 4 EMSAs of purified SACE_3986 protein with the *SACE_3985-SACE_3986* intergenic region and the promoter regions of *eryA* and *ermE*. **a** Purification of His_6-tagged SACE_3986. *Left lane* 105 KDa protein marker. *Right lane* purified SACE_3986. **b** Assay of DNA binding of SACE_3986. 6-FAM Labeled Probes 1, 2, 3 represent the intergenic region between *SACE_3985* and *SACE_3986*, *eryA* promoter region and *ermE* promoter region. The varying concentrations of SACE_3986 used in the assays are as indicated. Competing assays were performed using 50-fold unlabeled probe 1 and 50-fold non-specific DNA poly dIdC, respectively. Free probe and protein–probe complexes are shown by *arrows*

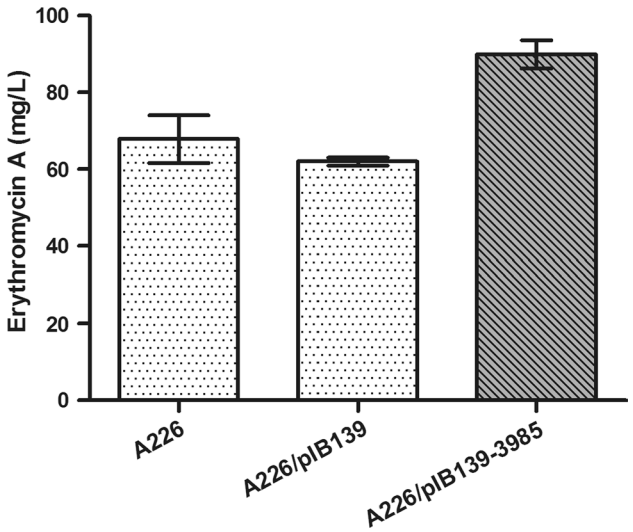

Fig. 5 Effect of *SACE_3985* overexpression on erythromycin A production in *S. erythraea* A226. Mean values of three replicated experiments are shown, with the standard deviation indicated by *error bars*

Overexpression of *SACE_3985* increases erythromycin A production

Given that *SACE_3985* was identified as a target gene of SACE_3986, we investigated its relationship to erythromycin production by overexpressing *SACE_3985* in A226. The plasmid pIB139-3985 was transformed into A226, and the overexpressed strain A226/pIB139-3985 was obtained by screening for apramycin-resistant transformants and then confirmed by PCR. As expected, the production of erythromycin A by A226/pIB139-3985 (89.9 mg/L) showed 32.6 and 45.0 % improvements relative to A226 (67.8 mg/L) and A226/pIB139 (62.0 mg/L), respectively (Fig. 5), indicating that SACE_3985 displayed a positive effect on erythromycin production.

Discussion

The present study identified and characterized a novel TFR, SACE_3986, which was negatively involved in the erythromycin biosynthesis in *S. erythraea*. Inactivation of *SACE_3986* and overexpression of *SACE_3985*, the SACE_3986 target gene, resulted in improved erythromycin A productivity, which provided a valuable way for the rational engineering of *S. erythraea* to improve erythromycin production. Meanwhile, a similar result was recently reported in *Streptomyces avermitilis*, where avermectin production was increased by engineering of the TFR, SAV151, and its target genes [12]. Therefore, the employed strategy may be generally applicable for the improvement of production of other antibiotics in industrial actinomycetes.

Transcription and EMSA experiments confirmed that SACE_3986 inhibited the transcription of its upstream gene *SACE_3985* and specifically bound to the intergenic region between *SACE_3985* and *SACE_3986*, indicating that SACE_3986 might affect the biosynthesis of erythromycin by directly repressing the transcription of *SACE_3985*. Bioinformatic analysis shows that SACE_3985 belongs to the family of short-chain dehydrogenase/reductase SDR [EC:1.1.1.303] likely involved in the butanoate metabolism [9, 22]. The reaction catalyzed by SACE_3985 may indirectly provide energy or precursors for erythromycin biosynthesis. Moreover, it was determined that inactivation of *SACE_3986* in *S. erythraea* increased the transcription of *eryA* and *ermE*, but SACE_3986 did not bind to their promoter regions, suggesting that SACE_3986 might

also regulate the production of erythromycin by inhibition of yet-unknown downstream factors that directly affected the expression of *ery* gene in *S. erythraea*. Therefore, SACE_3986 might negatively regulate erythromycin biosynthesis through at least two separate mechanisms. On the one hand, by affecting precursor availability or energy metabolism, SACE_3986 may redirect cellular metabolic flux to control erythromycin production. On the other hand, SACE_3986 may interact with other transcriptional factors to regulate the expression of erythromycin biosynthetic and resistance genes.

Previous studies revealed that the key developmental regulator, BldD (SACE_2077), directly controlled the production of erythromycin by binding to the promoter regions of the *ery* cluster [5]. However, the action of BldD on the promoters of *ery* genes was much weaker than on its own promoter, and *bldD* exhibited an opposite transcription pattern with respect to most of the *ery* genes in a high-yield *S. erythraea* relative to its wild-type strain. Moreover, the recently identified regulator, SACE_5599, acted as a pleiotropic regulator affecting both erythromycin biosynthesis and morphological differentiation in *S. erythraea* [16], but its regulatory mechanism remains to be elucidated. Thus, the molecular basis of regulation of erythromycin biosynthesis is complicated [21]. Together with BldD and SACE_5599, the identification of SACE_3986 provides an additional step in understanding the specialized regulatory mechanisms controlling erythromycin production in *S. erythraea*. In this study, except for *SACE_3985*, the other target genes of SACE_3986 in *S. erythraea* remain unknown. In future studies, it may be possible to identify more target genes of *SACE_3986* using system-level methods, such as genome searching for binding sites of SACE_3986, and transcriptome analyses. Improved knowledge of SACE_3986 target genes and their functions will be valuable to further dissect the molecular mechanisms regulating erythromycin biosynthesis and develop more useful strategies to enhance erythromycin yield in *S. erythraea*.

Acknowledgments This work was supported by the National Program on Key Basic Research Project (973 program, 2013CB734000), Open Funding Project of the State Key Laboratory of Bioreactor Engineering (2013), The National Natural Science Foundation of China (31300081, 30870069), The Natural Science Foundation of Anhui Province (1208085MC46), The Initial Foundation of Doctoral Scientific Research in Anhui University (01001904), and The Youth Foundation of Anhui University (02303305).

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