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# Inactivation of SACE_3446, a TetR family transcriptional regulator, stimulates erythromycin production in *Saccharopolyspora erythraea*

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ABSTRACT

Erythromycin A is a widely used antibiotic produced by *Saccharopolyspora erythraea*; however, its biosynthetic cluster lacks a regulatory gene, limiting the yield enhancement via regulation engineering of *S. erythraea*. Herein, six TetR family transcriptional regulators (TFRs) belonging to three genomic context types were individually inactivated in *S. erythraea* A226, and one of them, SACE_3446, was proved to play a negative role in regulating erythromycin biosynthesis. EMSA and qRT-PCR analysis revealed that SACE_3446 covering intact N-terminal DNA binding domain specifically bound to the promoter regions of erythromycin biosynthetic gene *eryAI*, the resistant gene *ermE* and the adjacent gene *SACE_3447* (encoding a long-chain fatty-acid CoA ligase), and repressed their transcription. Furthermore, we explored the interaction relationships of SACE_3446 and previously identified TFRs (SACE_3986 and SACE_7301) associated with erythromycin production. Given demonstrated relatively independent regulation mode of SACE_3446 and SACE_3986 in erythromycin biosynthesis, we individually and concomitantly inactivated them in an industrial *S. erythraea* WB. Compared with WB, the WBΔ*3446* and WBΔ*3446*Δ*3986* mutants respectively displayed 36% and 65% yield enhancement of erythromycin A, following significantly elevated transcription of *eryAI* and *ermE*. When cultured in a 5 L fermentor, erythromycin A of WBΔ*3446* and WBΔ*3446*Δ*3986* successively reached 4095 mg/L and 4670 mg/L with 23% and 41% production improvement relative to WB. The strategy reported here will be useful to improve antibiotics production in other industrial actinomycete.



## 1. Introduction

*Saccharopolyspora erythraea* is a Gram-positive actinomycete utilized for the industrial-scale production of a macrolide antibiotic erythromycin A. Erythromycin and its semi-synthetic derivatives clarithromycin, azithromycin, dirithromycin, roxithromycin, and telithromycin are widely used in medicine to treat infections caused by pathogenic Gram-positive bacteria.[1] As worldwide sales of erythromycin and its derivatives reached billions of dollars per year,[2] systematic improvements in erythromycin production are of enormous importance. Over the past 60 years, erythromycin production has been frequently improved by utilizing traditional mutagenesis and metabolic engineering methodologies.[3,4] Genetic modulation of the overexpression of endogenous tailoring genes *eryK* (encoding a P450 hydroxylase for C-12 hydroxylation) and *eryG* (encoding an O-methyltransferase for C-3'' O-methylation) or exogenous genes *vhb* (encoding a hemoglobin from *Vitreoscilla*) and *metK* (encoding an S-adenosylmethionine synthetase) have enhanced erythromycin production and purity in industrial *S. erythraea* strains.[5–7] In recent years, extensive investigations provide insights into the genes involved in erythromycin biosynthesis.[4,8] However, unlike many *Streptomyces*, *S. erythraea* lacks a regulatory gene in the erythromycin

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biosynthetic gene cluster, compromising efforts to improve erythromycin production by regulation engineering.

In 2007, completion of the genome sequence of *S. erythraea* has offered the opportunity to identify regulators related to erythromycin production and understand the specialized regulatory mechanism of erythromycin biosynthesis.[9] A total of 1118 genes, corresponding to ~15% of the coding sequences of the *S. erythraea* genome, are predicted to be involved in regulation, including multiple sigma factors and other transcriptional regulators.[9] The developmental regulator BldD (SACE_2077) was found to directly control the synthesis of erythromycin by binding the promoter regions of erythromycin biosynthetic genes.[10] Recently, we have utilized the improved gSELEX (genomic systematic evolution of ligands by exponential enrichment) method to capture other target genes of BldD involved in the erythromycin production and morphogenesis in *S. erythraea*.[11] Additionally, a putative regulatory protein SACE_5599 was also identified to be positively involved in erythromycin production and morphological differentiation.[12] Nevertheless, the regulatory processes governing erythromycin biosynthesis in *S. erythraea* remains obscure.

The TetR family transcriptional regulators (TFRs) are widespread in bacteria, encoding proteins with a conserved helix-turn-helix (HTH) DNA-binding domain and a C-terminal ligand regulatory domain.[13,14] Members of the TetR family regulate a wide range of cellular activities, such as antibiotic production, the osmotic stress response, efflux pump production, multidrug resistance, and modulation of metabolism.[13,14] Several TFRs in *Streptomyces* control the biosynthesis or export of antibiotics, whether they are encoded inside the gene cluster for antibiotic biosynthesis or not.[15–19] TFRs are configured in the *Streptomyces* genome in three genome contexts, grouped according to their orientation relative to neighboring genes.[20] The first group is divergently oriented to a neighboring transcription unit. The second group is likely to be co-transcribed with an upstream or downstream neighboring gene when separated by 35 bp or less. The third group lacks a defined association of the TFR gene with adjacent genes. Also, a recent review of TRFs focusing on the nature and diversity of their ligands and related biochemical processes underscores their inducible and diverse regulatory systems.[21]

*In silico* analysis identified approximate 100 TFRs encoded by the *S. erythraea* genome,[9] suggesting an increased complexity to its regulatory networks. To date, except for two characterized TFRs (SACE_7040 and SACE_0012) that negatively controlled *S. erythraea* morphogenesis,[22,23] we recently identified two additional TFRs (SACE_3986 and SACE_7301) negatively or positively regulating the synthesis of erythromycin.[24,25] Nonetheless, little is still known about remaining TFRs encoded by the *S. erythraea* genome. Therefore, we herein selected six TFRs with three groups of genome contexts for gene inactivation to define potential regulators of erythromycin biosynthesis. Through *in vivo* and *in vitro* evidences, one of them, SACE_3446, was confirmed to repress erythromycin biosynthesis, and its interaction relationship to the other two TFRs (SACE_3986 and SACE_7301) were explored to guide the engineering of an industrial overproducer for enhancing erythromycin production.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and general techniques

The strains and plasmids used in this study are listed in Table 1. *S. erythraea* and its derivatives were grown either on solid R3M medium or in liquid TSB medium with appropriate antibiotics for recombinant strains at 30 °C.[28] *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 performed according to previously reported methods.[22,26]

### 2.2. Inactivation of the TetR family regulatory genes in S. erythraea *A226*

Six putative TetR family regulatory genes (*SACE_0820*, *SACE_1874*, *SACE_2947*, *SACE_3446*, *SACE_6589*, and *SACE_7325*) were individually inactivated in the parental strain A226 as follows: a region spanning ~1.5 kb of DNA on either side of each regulatory gene was amplified from the genome of A226. The upstream and downstream homologous fragments were amplified with their respective primer pairs P1/P2 and P3/P4 (Table S1), digested with different restriction enzymes (Table 1), and inserted into corresponding sites of pUCTSR (pUC18 derivative containing a 1.36 kb fragment of a thiostrepton resistance cassette in the *Bam*HI/*Sma*I sites),[22] respectively. Via the homologous recombination of linear DNA fragments,[22] those regulatory genes were then each replaced by the thiostrepton resistance gene (*tsr*), and the mutants were screened by thiostrepton and further confirmed by PCR analysis with their respective P5 and P6 primers. Correct disruption mutants were designated as Δ*SACE_0820*, Δ*SACE_1874*, Δ*SACE_2947*, Δ*SACE_3446*, Δ*SACE_6589*, and Δ*SACE_7325*.

### 2.3. Genetic complementation of the ΔSACE_3446 mutant

The *SACE_3446* gene (507 bp; GenBank Accession No. NC-009142; 3,802,179–3,802,685 nt) is a member of the TetR family, encoding a protein of 168 amino acids with a predicted molecular mass of 19 kDa. However, the re-annotation of *S. erythraea* genome in 2013 indicated that there was a predicted error of initiation codon position in SACE_3446.[29] We searched upstream sequences of the original *SACE_3446*, and found three additional initiation codons, resulting in 4 putative reading frames named *SACE_3446A* (168 aa, 507 bp), *SACE_3446B* (182 aa, 549 bp), *SACE_3446C* (220 aa, 663 bp), and *SACE_3446D* (239 aa, 720 bp).

To complement the Δ*SACE_3446* mutant, above four *SACE_3446* coding sequences were amplified with the primers 3446-P7 to 3446-P14 (Table S1) from the genomic DNA of A226. Then, the PCR products were cloned into the corresponding sites of pIB139,[27] successively generating pIB139-3446A, pIB139-3446B, pIB139-3446C, and pIB139-3446D. Then, these vectors were introduced into the Δ*SACE_3446* mutant by PEG-mediated protoplast transformation,[30] the correct transformants (Δ*SACE_3446*/pIB139-3446A, Δ*SACE_3446*/pIB139-3446B, Δ*SACE_3446*/pIB139-3446C, and Δ*SACE_3446*/pIB139-3446D) were selected with apramycin, and they were further confirmed by PCR analysis.

### 2.4. Construction of gene deletion/overexpression mutants in the industrial S. erythraea WB and its derivatives

In accord with above procedures, *SACE_3446* was also inactivated in WB strain, generating the WBΔ*3446* mutant. To further knock out *SACE_3986* in WBΔ*3446*, we first amplified the neomycin resistant gene (*neo*) from SupCos1[31] using the primers Neo-F/Neo-R (Table S1), digested with *Kpn*I/*Bgl*II, and replaced the *tsr* gene of pUCTSRΔ3986,[24] generating the pUCNEOΔ3986 plasmid. Likewise, through the homologous recombination of linear DNA fragments, *SACE_3986* was replaced by the *neo* gene, and the mutant WBΔ*3446*Δ*3986* with sensitivities to thiostrepton and kanamycin was obtained. Moreover, pSET152 carrying three extra copies of *SACE_7301* under the control of *PermE** was transferred into WBΔ*3446*Δ*3986* to yield WBΔ*3446*Δ*3986*/3 × *7301*.

**Table 1**
Bacterial strains and plasmids used in this study.

| Strains and plasmids | Description | Reference |
|---|---|---|
| *E. coli* | | |
| DH5α | F *recA lacZ*M15 | [26] |
| BL21 (DE3) | F⁻*ompThsdSB*(*rB*⁻ *mB*⁻) *dcmgal*λ(DE3) | Novagen |
| *S. erythraea* | | |
| A226 | CGMCC 8279, an erythromycin low producer | China Pharmaceutical Culture Collection |
| Δ*SACE_0820* | A226 derivative with *SACE_0820* deleted | This study |
| Δ*SACE_1874* | A226 derivative with *SACE_1874* deleted | This study |
| Δ*SACE_2947* | A226 derivative with *SACE_2947* deleted | This study |
| Δ*SACE_6589* | A226 derivative with *SACE_6589* deleted | This study |
| Δ*SACE_7325* | A226 derivative with *SACE_7325* deleted | This study |
| Δ*SACE_3446* | A226 derivative with *SACE_3446* deleted | This study |
| Δ*SACE_3446*/pIB1393446A | Δ*SACE_3446* carrying pIB1393446A | This study |
| Δ*SACE_3446*/pIB1393446B | Δ*SACE_3446* carrying pIB1393446B | This study |
| Δ*SACE_3446*/pIB1393446C | Δ*SACE_3446* carrying pIB1393446C | This study |
| Δ*SACE_3446*/pIB1393446D | Δ*SACE_3446* carrying pIB1393446D | This study |
| WB | CGMCC 8280, an erythromycin industrial overproducer | Anhui Wanbei Pharmaceutical Co., Ltd. |
| WBΔ*3446* | WB derivative with *SACE_3446* deleted | This study |
| WBΔ*3446*Δ*3986* | WB derivative with *SACE_3446*/*SACE_3986* deleted | This study |
| WBΔ*3446*Δ*3986*/3 × *7301* | WBΔ*3446*Δ*3986* derivative carrying pSET152-3 × 7301 | This study |
| Plasmids | | |
| pUCTSR | pUC18 derivative containing a 1.36-kb fragment of a thiostrepton resistance gene in *Bam*HI/*Sma*I sites | [22] |
| pUCTSRΔ3446 | pUCTSR derivative containing two 1.5-kb fragments, the upstream and downstream regions of *SACE_3446* | This study |
| pUCNEO | pUC18 derivative containing a 0.79-kb fragment of a neomycin resistance gene in *Bam*HI/*Sma*I sites | This study |
| pUCTSRΔ3986 | pUCTSR derivative containing two 1.5-kb fragments, the upstream and downstream regions of *SACE_3986* | [24] |
| pUCNEOΔ3986 | pUCTSRΔ3986 derivative with *tsr* replaced by *neo* | This study |
| pSET152-3 × *7301* | pSET152 carrying three extra copies of *SACE_7301* under the control of P*ermE** | [25] |
| pIB139 | *E. coli* – *S. erythraea* integrative shuttle vector containing a strong constitutive *ermE** promoter | [27] |
| pIB1393446A | pIB139 carrying an extra *SACE_3446A* for the gene complementation | This study |
| pIB1393446B | pIB139 carrying an extra *SACE_3446B* for the gene complementation | This study |
| pIB1393446C | pIB139 carrying an extra *SACE_3446C* for the gene complementation | This study |
| pIB1393446D | pIB139 carrying an extra *SACE_3446D* for the gene complementation | This study |
| pET28a | T7 promoter, His-tag, *kan* | Novagen |
| pET28a-3446A | pET28a-derived plasmid carrying *SACE_3446A* | This study |
| pET28a-3446B | pET28a-derived plasmid carrying *SACE_3446B* | This study |
| pET28a-3446C | pET28a-derived plasmid carrying *SACE_3446C* | This study |
| pET28a-3446D | pET28a-derived plasmid carrying *SACE_3446D* | This study |

The symbol * represents changed version of original *ermE* promoter.

### 2.5. Fermentation and erythromycin assay

Flask Fermentation of A226, WB and their derivatives were done as previously described.[25] For bioassay-based erythromycin analysis, 5 μL above fermentation supernatants were added to LB agar plates, which were sprayed with an overnight culture of *B. subtilis* PUB110. The plates were incubated at 37 °C for 12 h, and the erythromycin production was estimated by detecting and measuring the growth-inhibition zones. Fed-batch cultures of WB, WBΔ*3446* and WBΔ*3446*Δ*3986* were performed in a 5-L fermentor (Baoxing, Shanghai, China). Samples (50 mL) were taken every 24 h and erythromycin A was extracted from above cultures in terms of previous reported method.[25] HPLC analysis was carried out 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 mol/L, 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.

### 2.6. Quantitative real-time PCR (qRT-PCR)

The relative transcriptional levels of *eryAI*, *ermE*, *SACE_3447*, and three TetR family regulatory genes (*SACE_3446*, *SACE3986* and *SACE_7301*) were determined by qRT-PCR. Specific primers were designed as listed in Table S1. Total RNA was extracted from A226 and its derivatives after 2 and 4 days of growth on R5 agar medium.[32] As for WB and its derivatives, total RNA was isolated when they were cultured for the 4 days. 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 Step-One 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 the comparative cycle threshold ($C_T$) method.[33]

### 2.7. Expression and purification of recombinant SACE_3446 in E. coli

Based on re-annotation of *S. erythraea* genome,[29] three additional initiation codons within upstream sequences of *SACE_3446* were detected, thereby generating 4 putative SACE_3446 coding sequences (SACE_3446A, SACE_3446B, SACE_3446C, and SACE_3446D). Using the genomic DNA of *S. erythraea* A226 as a template, we amplified these DNA fragments by PCR with the primers 3446-P17 to 3446-P24 (Table S1). Then, the PCR products were cloned into the *Nde*I and *Xba*I sites of pET28a to acquire pET28a-3446A,

pET28a-3446B, pET28a-3446C, and pET28a-3446D, respectively. Those plasmids were transformed into *E. coli* BL21 (DE3) for protein production. Gene expression was induced with 0.5 mM IPTG for 5 h at 30 °C. Cells were harvested and lysed by sonication treatment. Purification of $His_6$-SACE_3446 fusion protein was performed by Ni-NTA Sepharose chromatography.[34] The concentration of purified fusion protein was analyzed by BCA assays, and its purity was judged by SDS-PAGE analysis.

### 2.8. Electrophoretic mobility shift assays (EMSAs)

DNA fragments harboring *eryAI*, *ermE*, or *SACE_3447* promoter regions were independently obtained by PCR amplification with their respective primers (Table S1), and 5′-end labeled with 6-carboxyfluorescein (6-FAM). Those labeled DNA fragments were successively mixed with above purified $His_6$-tagged SACE_3446 proteins. The binding reaction system contained 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 50 mM EDTA, 60 mM KCl, 10 mM DTT, 10% glycerol, 150 ng labeled probe, and 0.2–1.4 μM purified protein. 7.5 μg unlabeled DNA fragments or 7.5 μg poly dIdC were respectively added into the reaction system for competitive assays. After incubation on ice for 10 min, the reactants were run on an 8% TBE polyacrylamide gel (Bio-Rad) buffered in 0.5 X TBE at 30 mA for 1 h.

## 3. Results

### 3.1. Genomic and bioinformatics analysis of TFRs in S. erythraea

Actinomycete chromosomes are mostly linear, with a conserved 'core' region that contains most of the essential genes, and variable 'non-core' regions at both ends, primarily including non-essential species-specific genes.[35] The 8.2-Mb circular chromosome of *S. erythraea* has a 'core' region containing two parts (6.3–8.2 Mb and 0–2.5 Mb) around the origin of replication *oriC*. The remaining region from 2.5 to 6.3 Mb is defined as the 'non-core' region. By combining protein BLAST analysis with the consensus sequence for Pfam PF00440 (TetR_N) and the genome annotation of *S. erythraea*,[9,20] we identified 97 genes encoding putative TFRs, which were distributed evenly over its chromosomes with a slight enrichment in the non-core (3.8 Mb, 12.6 TFRs/Mb), relative to the core

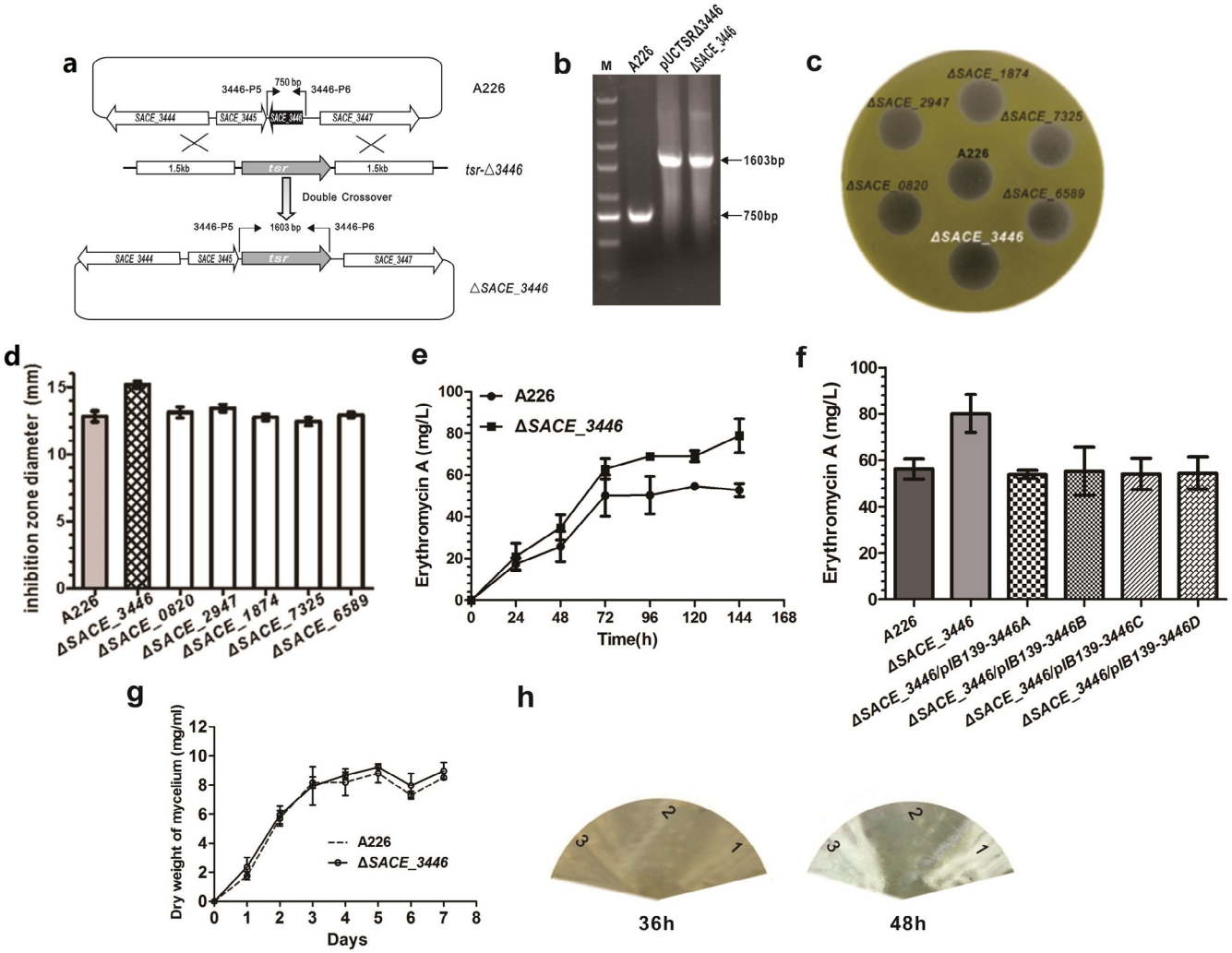


**Fig. 1.** Inactivation of *SACE_3446* in *S. erythraea* A226. (a) Schematic deletion of *SACE_3446* by linearized fragment homologous recombination in A226. (b) PCR confirmation of the *SACE_3446* deletion mutant by the primers 3446-P5/3446-P6. A 750-bp PCR-amplified band was observed in A226, while a 1603-bp band was detected in the Δ*SACE_3446* mutant. (c) Inhibition tests of deletion mutants of six TetR family regulators against *B. subtilis*. (d) Comparison of the erythromycin titer estimated by scoring the growth-inhibition zones. (e) Time course of erythromycin A yield in A226 and Δ*SACE_3446* by HPLC analysis. The mean values of at least three replicates are shown, with the standard deviation indicated by error bars. (f) Erythromycin A production in Δ*SACE_3446* expressing *SACE_3446* from four different initiation codons by HPLC analysis. (g) Growth curves of A226 and Δ*SACE_3446*. The two strains were cultured in R5 liquid medium, and their dry weights of mycelia (DWM) were measured. (h) Morphological differentiation of *S. erythraea* A226 and its derivatives. All strains were grown on R3M agar medium at 30 °C for 36 and 48 h. 1, A226; 2, Δ*bldD*; and 3, Δ*SACE_3446*.

regions (4.4 Mb, 11.1 TFRs/Mb). Based on the genome context analysis of individual TFR,[20] 69% (67/97), 14% (14/97), and 17% (16/97) of TFRs are in the first, second, and third groups, respectively. Thus, the orientation of most TFRs in *S. erythraea* is divergent from their neighboring genes. Herein, we randomly chose two members of each group (SACE_2947 and SACE_3446, group one; SACE_6589 and SACE_7325, group two; and SACE_0820 and SACE_1874, group three) to search for candidate TFRs related to erythromycin biosynthesis in *S. erythraea*.

### 3.2. SACE_3446 negatively regulates erythromycin biosynthesis

Through the homologous recombination of linearized fragments,[22] aforementioned six genes of TetR family were individually inactivated in *S. erythraea* A226, and the desired mutants were obtained by thiostrepton resistant screening and PCR confirmation (Fig. 1a–b, with the currently studied *SACE_3446* as an example). By fermentation and bioassay analysis, we demonstrated that the Δ*SACE_2947*, Δ*SACE_0820*, and Δ*SACE_3446* mutants created a larger inhibition zone against *B. subtilis* PUB110 than their parental strain A226. Among these mutants, Δ*SACE_3446* presented the highest inhibition activity (Fig. 1c–d) and was selected for further investigation.

To confirm its negative effect on erythromycin production, the Δ*SACE_3446* mutant and its parental strain A226 were cultivated in the liquid R5 medium for 6 days, and extracts of those cultures were quantified by HPLC analysis. Compared with A226 (52.8 mg/L), Δ*SACE_3446* displayed an increased erythromycin A yield by 49.4% (78.9 mg/L) (Fig. 1e). Intriguingly, when Δ*SACE_3446* was complemented with 4 predicted *SACE_3446* genes under the control of *PermE** promoter, erythromycin A yield of these desired mutants all recovered to nearly original levels (Fig. 1f). We further compared the mycelium dry weight of A226 and Δ*SACE_3446* cultured in the liquid R5 medium, and found that the two strains had comparable growth rates and cell densities (Fig. 1g), demonstrating that elevated erythromycin yield in Δ*SACE_3446* was not caused by enhancement of cell growth. Also, when cultured on R3M agar medium, Δ*SACE_3446* was identical to A226 in the rate of aerial mycelium formation, whereas Δ*bldD* introduced the expected defect of aerial hyphae (Fig. 1h). Taken together, these findings indicate that SACE_3446 exclusively acts as a repressor to regulate the synthesis of erythromycin in *S. erythraea*.

### 3.3. SACE_3446 specifically binds to the promoter regions of eryAI, ermE, and SACE_3447

To determine whether SACE_3446 play a direct role in regulating erythromycin biosynthesis, EMSAs were performed with the promoter regions of erythromycin biosynthetic gene *eryAI* and the resistant gene *ermE*. During above 4 putative SACE_3446, only 2 longer protein-coding sequences (SACE_3446C and SACE_3446D) completely covered the DNA-binding domain (Fig. 2a). Based on the pET28a vector, we successfully expressed SACE_3446B, SACE _3446C, and SACE _3446D in *E. coli* BL21 (DE3); however, it failed to express SACE _3446A, although we performed the experiments with various conditions. In light of the similar phenomenon reported previously,[12] the shortest SACE_3446A might be rapidly degraded during its expression process.

Then, the 3 recombinant $His_6$-SACE_3446 proteins were purified from *E. coli* BL21 (DE3) harboring pET28a-3446B, pET28a-3446C and pET28a-3446D (Fig. 2b). The results from EMSAs showed that $His_6$-SACE_3446C and $His_6$-SACE_3446D, but not $His_6$-SACE_3446B, could bind to the promoter regions of *eryAI* and *ermE* (Fig. 2c). To examine the binding specificity, EMSAs with an excess of unlabeled specific or nonspecific competitor DNA (poly dIdC) were performed (Fig. 2c), demonstrating that purified $His_6$-SACE_3446C and $His_6$-SACE_3446D bound specifically to *eryAI* and *ermE* promoter regions. In addition, given SACE_3446 is classified as the group I TFR because it shows a divergent orientation to its adjacent gene

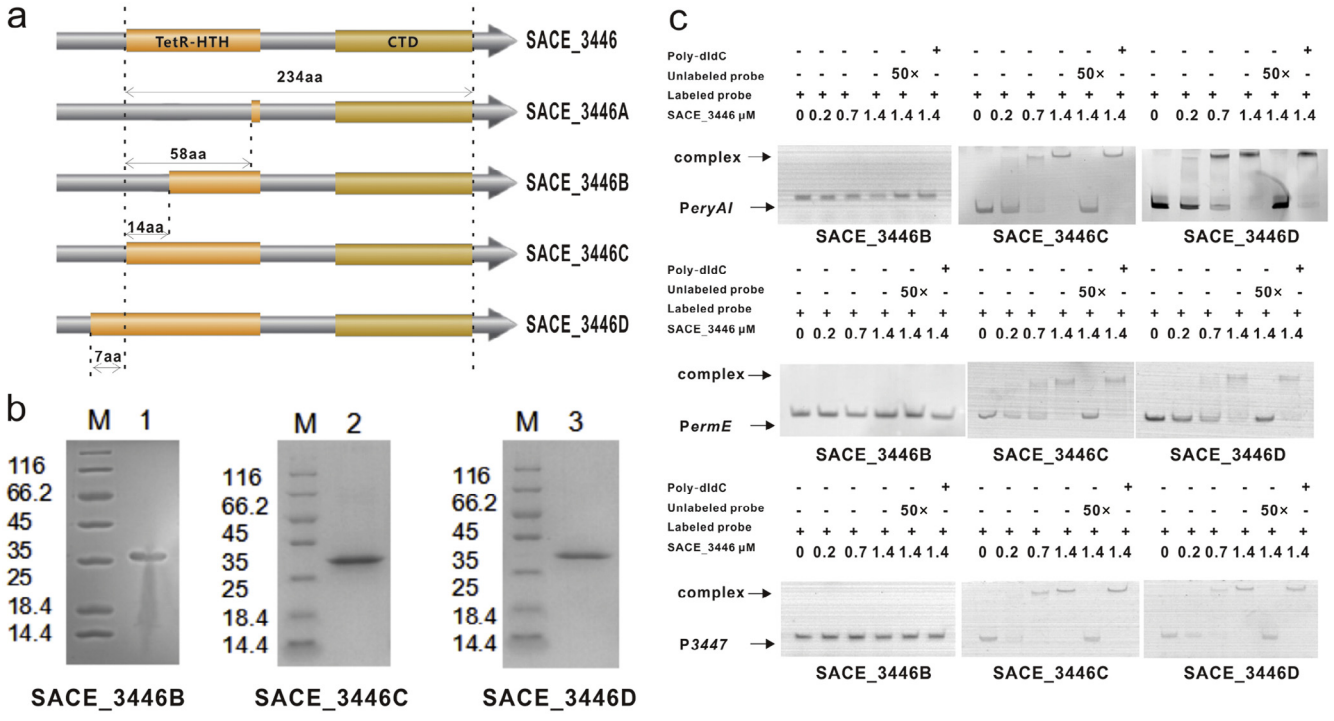


**Fig. 2.** Binding of purified SACE_3446 proteins to the promoter regions of *eryAI*, *ermE* and *SACE_3447*. (a) Bioinformatics analysis indicating that 3 alternative initiation codons for *SACE_3446*, resulting in 4 predicted coding proteins. (b) SDS-PAGE analysis of purified $His_6$-SACE_3446 proteins. Left lane, 116 KDa protein ladder; right lane, recombinant protein (1, purified SACE_3446B; 2, purified SACE_3446C; and 3, purified SACE_3446D). (c) EMSAs with the promoter regions of *eryAI*, *ermE*, and *SACE_3447* and 3 $His_6$-SACE_3446 proteins.

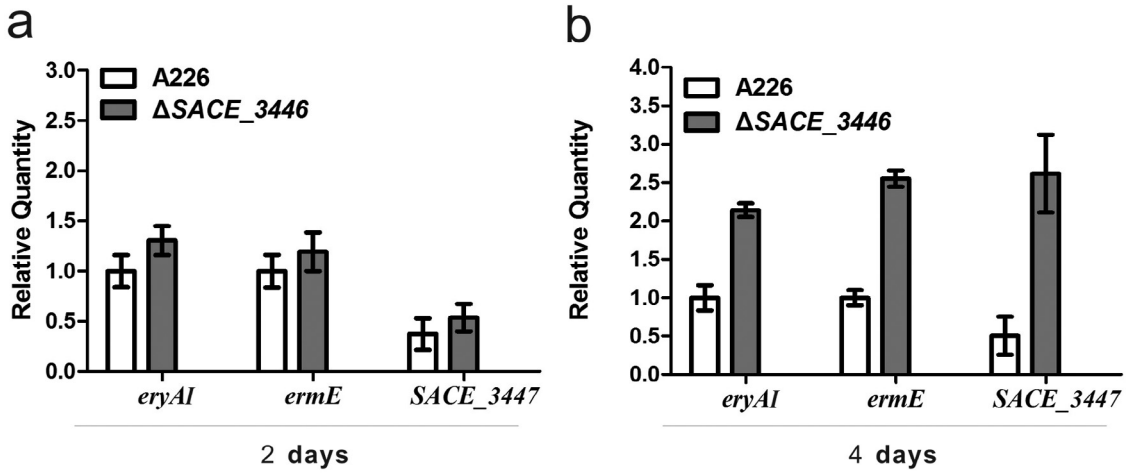


**Fig. 3.** Effects of *SACE_3446* disruption on the transcription of *eryAI*, *ermE*, and *SACE_3447*. qRT-PCR was used to quantify the amounts of transcripts produced by A226 and Δ*SACE_3446* cultured for 2 days (a) and 4 days (b). The mean values of three independent experiments are shown, with the standard deviation indicated by error bars.

*SACE_3447* (encoding a long-chain fatty-acid CoA ligase); we also performed EMSAs with the promoter region of *SACE_3447*. The gel shift bands were obviously detected when successively adding purified $His_6$-SACE_3446C and $His_6$-SACE_3446D (Fig. 2c). Consequently, it was proved that the SACE_3446 protein with entire N-terminal DNA binding domain could specifically bind to the promoter regions of *eryAI*, ermE, and *SACE_3447*.

### 3.4. *SACE_3446 represses the transcription of eryAI, ermE, and SACE_3447*

To evaluate the regulatory significance of SACE_3446 for its targets as mentioned above, we chose *eryAI*, *ermE* and *SACE_3447* for transcriptional comparison between A226 and Δ*SACE_3446* grown for 2–4 days by qRT-PCR. In the 2-day growth period, the transcriptions of *eryAI*, *ermE,* and *SACE_3447* were not remarkably different among the two strains (Fig. 3a). When grown for 4 days, compared with the wild-type A226, Δ*SACE_3446* displayed increased transcriptional levels of *eryAI*, *ermE* and *SACE_3447* by 2.1-, 2.6-, and 5.2-fold, respectively, (Fig. 3b). Consequently, those results, together with EMSA evidences, indicated that SACE_3446 repressed the expression of *eryAI* and *ermE*, as well as the neighboring gene *SACE_3447* by directly interacting with their promoter regions.

### 3.5. *Interaction relationships of three TFRs associated with erythromycin biosynthesis*

We previously identified and dissected the two TFRs, SACE_3986 and SACE_7301, involved in the erythromycin production, and *SACE_3986* inactivation or *SACE_7301* overexpression in the industrial *S. erythraea* WB resulted in the yield improvement.[24,25] Herein, we examined their interaction relationship to SACE_3446 via qRT-PCR and EMSA analysis. Compared with wild-type A226, Δ*SACE_3446* did not exhibit differential expression of *SACE_7301* and *SACE_3986* (Fig. 4a), the same pattern as *SACE_3446 and SACE_7301* in ΔSACE_*3986* (Fig. 4b). Intriguingly, the transcriptions of *SACE_3446* and *SACE_3986* in Δ*SACE_7301* were both significantly increased relative to that in A226 (Fig. 4c). SACE_7301 did not bind to the upstream regions of *SACE_3446* and *SACE_3986* (data not show). These data suggested that the regulation by SACE_3446 and SACE_3986 is relatively independent; however, SACE_7301 played an indirect role in negatively regulating the expression of *SACE_3446* and *SACE_3986*.

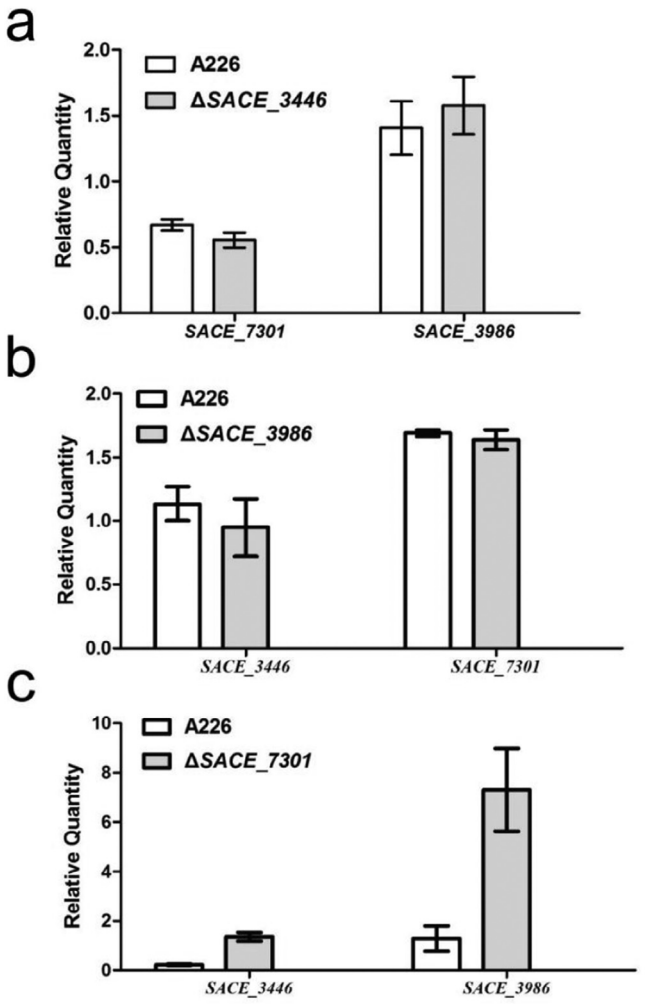


**Fig. 4.** SACE_7301 indirectly regulated the transcriptions of *SACE_3446* and *SACE_3986*. Gene transcription was compared between A226 and deletion mutants of three TetR family genes (*SACE_3446*, *SACE_3986*, and *SACE_7301*) cultured for 4 days via qRT-PCR assay. (a) Effects of *SACE_3446* disruption on the transcriptions of *SACE_3986* and *SACE_7301*. (b) Effects of *SACE_3986* disruption on the transcriptions of *SACE_3446* and *SACE_7301*. (c) Effects of *SACE_7301* disruption on the transcriptions of *SACE_3446* and *SACE_3986*. The mean values of three independent experiments are shown, with the standard deviation indicated by error bars.

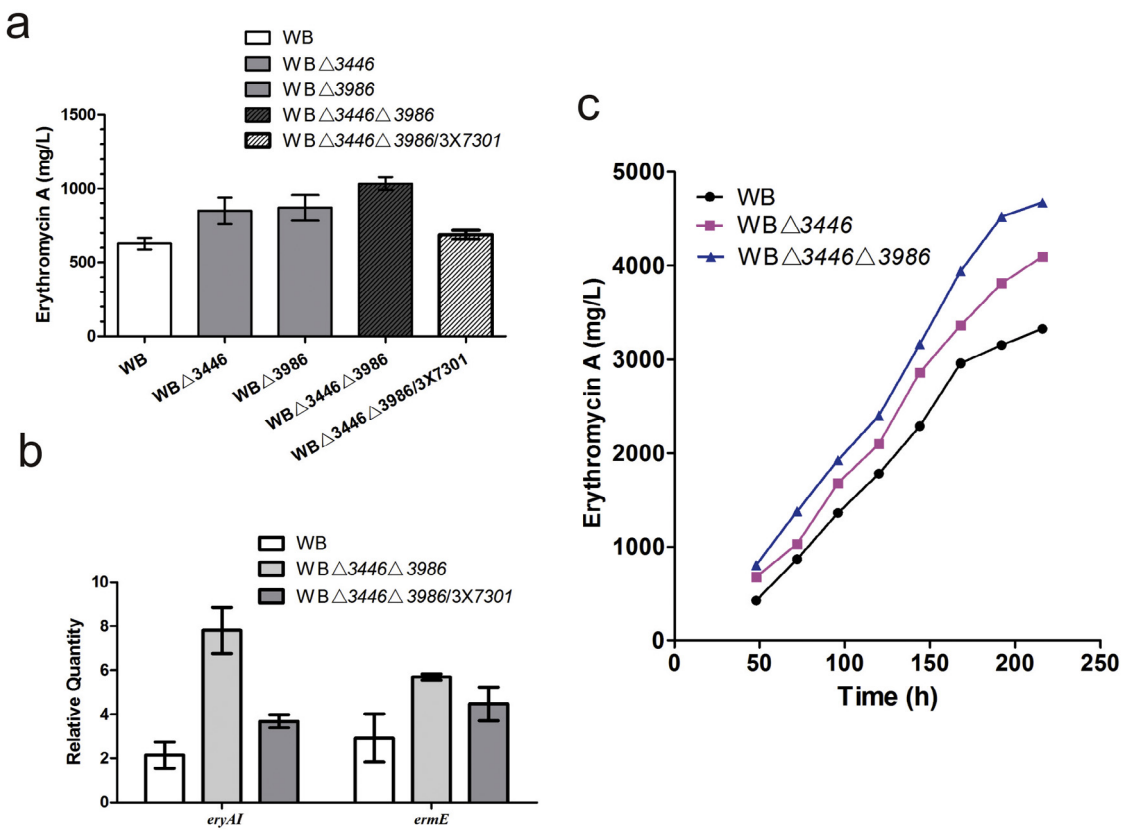


**Fig. 5.** Effects of *SACE_3446/SACE_3986* deletion and *SACE_7301* overexpression on erythromycin production and gene transcription in the industrial strain WB. (a) Erythromycin A yield of WB and its derivatives cultured in flasks for 6 days. The mean values of at least three replicates are shown, with the standard deviation indicated by error bars. (b) qRT-PCR analysis of *eryAI* and *ermE* in WB, WBΔ*3446Δ3986*, and WBΔ*3446Δ3986*/3 × *7301* cultured for 4 days. (c) Time course of erythromycin A production of WB, WBΔ*3446* and WBΔ*3446Δ3986* in a 5-L fermentor. One of the representative datasets is shown.

### *3.6. Double deletion of SACE_3446/SACE_3986 in the industrial strain WB enhances erythromycin yield*

To examine its practical application, *SACE_3446* was likewise inactivated in the high-yield *S. erythraea* WB strain, generating the WBΔ*3446* mutant. When cultured in a flask with the liquid industrial medium for 6 days, WBΔ*3446* displayed enhanced erythromycin A production by 36% over WB (Fig. 5a). As SACE_3446 and the other identified TFR (SACE_3986) played the independent role in the negative regulation of erythromycin biosynthesis, we further inactivated *SACE_3986* in WBΔ*3446*. As expectedly, erythromycin A production of the WBΔ*3446Δ3986* double mutant was 1033 mg/L, separately approaching 21% and 19% enhancement over WBΔ*3446* (851 mg/L) and WBΔ*3986* (871 mg/L), especially the highest 65 % in comparison to WB (628 mg/L) (Fig. 5a). A previous study demonstrated that overexpression of *SACE_7301* in WB dramatically elevated its erythromycin A yield,[25] thereby we expressed *SACE_7301* with 3 extra copies under the control of P*ermE** in the WBΔ*3446Δ3986*, to yield WBΔ*3446–3986*/3 × *7301*. Interestingly, erythromycin A yield in WBΔ*3446–3986*/3 × *7301* was dramatically decreased relative to that in WBΔ*3446–3986*, but a little higher than that in WB (Fig. 5a). Furthermore, we compared the transcriptional levels of *eryAI* and *ermE* among WB, WBΔ*3446Δ3986* and WBΔ*3446–3986*/3 × *7301* by qRT-PCR assay. Compared to WB, the transcription of *eryAI* and *ermE* in WBΔ*3446Δ3986* was up-regulated by 3.7- and 1.9-fold; however, that in Δ*3446Δ3986*/3 × *7301* slightly increased by nearly 1.5-fold (Fig. 5b), in which the transcriptional pattern was well correlated with the trend of yield alteration.

In addition, we tested the engineered strains WBΔ*SACE_3446* and WBΔ*3446Δ3986* in a larger scale fermentation. The time course of erythromycin fermentation in those strains at a 5-L fermentor is shown in Fig. 5c. At the end of fermentation, the yield of erythromycin A in WBΔ*3446* reached 4095 mg/L, an approximately 23% production increment over its original productivity (3322 mg/L) (Fig. 5c). WBΔ*3446Δ3986* (4670 mg/L) exhibited the highest yield, which was 14% and 41% higher than WBΔ*3446* and WB, respectively (Fig. 5c).

## 4. Discussion

In previous studies, we have identified two TFRs, SACE_3986 and SACE_7301, to successively repress and induce erythromycin production;[24,25] however, knowledge of the members of this family associated with erythromycin biosynthesis in *S. erythraea* remained limited. Herein, through genome context analysis, we systematically analyzed the TFRs in *S. erythraea*, and found that most of them are classified as the group I TFR, consistent with TFRs analysis in three *Streptomyces*.[20] During the type I TFRs, SACE_3446 was proved to repress erythromycin biosynthesis, the same as identified TFR SACE_3986.[24]

In terms of the re-annotation of *S. erythraea* genome in 2013,[29] three alternative initiation codons for SACE_3446 were detected, but the shorter SACE_3446A and SACE_3446B did not cover intact N-terminal DNA binding domain. To our surprise, the genetic complementation of *SACE_3446* with any of above four coding proteins in Δ*SACE_3446* all restored original levels of erythromycin A yield. Actually, in the construction process of *SACE_3446*-disrupted mutant, the DNA-binding core domain was not deleted. Thus, it might fuse with SACE_3446A or SACE_3446B for full activities.

Similar to SACE_3986,[24] we proved that SACE_3446 bound specifically to promoter region of the adjacent gene *SACE_3447*, and reduced its transcription. Differently, it repressed the transcription of *eryAI* and *ermE* by interacting with their promoters, while SACE_7301 acted as a positive regulator directly modulating the synthesis of erythromycin.[10,25] Furthermore, we assessed the interaction relationships of three aforementioned TFRs and demonstrated that there was no cross-talk regulation between SACE_3446 and SACE_3986, while SACE_7301 indirectly repressed the expression of the other two TFRs.

To evaluate the potential application of above interaction relationships, an industrial *S. erythraea* WB strain was engineered via deletion or overexpression of the three TFRs. Our data confirmed that individual and combined deletion of *SACE_3446* and *SACE_3986* had stepwise enhancement in erythromycin A yield, probably owing to their independent regulatory roles in erythromycin biosynthesis. However, when SACE_7301 was expressed with 3 extra copies under P*ermE** in the WBΔ*3446*Δ*3986*, a reduced production and corresponding decreased transcriptions of *eryAI* and *ermE* were detected in WBΔ*3446–3986*/3 × *7301*. To be specific, in WB, SACE_7301 directly activated the expression of *eryAI* and *ermE*, whereas it repressed their transcriptions when *SACE_3446* and *SACE_3986* were absent. As SACE_7301 also exerted indirect transcriptional repression of above two TFRs, we proposed its regulatory pattern was dependent on SACE_3986 and SACE_3446. The regulation mechanism seems to be more complicated than previously expected, and remains to be elucidated. Capturing of more targets of these TFRs with the improved gSELEX method[11] are being performed to construct the global regulatory network for uncovering aforementioned mysteries, and will be contribute to systematically improve the productivity of erythromycin in *S. erythraea*.

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## Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.synbio.2016.01.004.

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