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

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A B S T R A C T
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 erylA, 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 WBA3446Δ and WBA3446Δ3986 mutants respectively displayed 36% and 65% yield enhancement of erythromycin A, following significantly elevated transcription of erylA and ermE. When cultured in a 5 L fermentor, erythromycin A of WBA3446 and WBA3446Δ3986 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 actinomycetes.

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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. As worldwide sales of erythromycin and its derivatives reached billions of dollars per year, 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. Genetic modulation of the overexpression of endogenous tailoring genes eryl (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. In recent years, extensive investigations provide insights into the genes involved in erythromycin biosynthesis. However, unlike many Streptomyces, S. erythraea lacks a regulatory gene in the erythromycin
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. 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. The developmental regulator BldD (SACE_2077) was found to directly control the synthesis of erythromycin by binding the promoter regions of erythromycin biosynthetic genes. 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*. Additionally, a putative regulatory protein SACE_5599 was also identified to be positively involved in erythromycin production and morphological differentiation. 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. 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. Several TFRs in *Streptomyces* control the biosynthesis or export of antibiotics, whether they are encoded inside the genome or as expression plasmids. Recently, we have utilized the improved gSELEX method to capture other target genes of BldD involved in the erythromycin production and morphogenesis in *S. erythraea*. Additionally, a putative regulatory protein SACE_5599 was also identified to be positively involved in erythromycin production and morphological differentiation. Nevertheless, the regulatory processes governing erythromycin biosynthesis in *S. erythraea* remains obscure.

In *S. erythraea* genome, suggesting an increased complexity to its regulation systems.

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. *Escherichia coli* and *Bacillus subtilis* strains were cultured in Luria–Bertani (LB) liquid medium or on LB plates at 37 °C. DNA isolation and manipulation in *E. coli* and *S. erythraea* were performed according to previously reported methods.

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 pUC18 derivative containing a 1.36 kb fragment of a thiostrepton resistance cassette in the BamHI/SmaI sites, respectively. Via the homologous recombination of linear DNA fragments, those regulatory genes were then 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 ΔSACE_0820, ΔSACE_1874, ΔSACE_2947, ΔSACE_3446, ΔSACE_6589, and ΔSACE_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. 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 ΔSACE_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, successively generating pIB139-3446A, pIB139-3446B, pIB139-3446C, and pIB139-3446D. Then, these vectors were introduced into the ΔSACE_3446 mutant by PEG-mediated protoplast transformation, the correct transformants (ΔSACE_3446/pIB139-3446A, ΔSACE_3446/pIB139-3446B, ΔSACE_3446/pIB139-3446C, and ΔSACE_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 WBAΔ3446 mutant. To further knock out SACE_3986 in WBAΔ3446, we first amplified the neomycin resistant gene (*neo*) from SupCos1 using the primers Neo-F/Neo-R (Table S1), digested with KpnI/BglII, and replaced the *tsr* gene of pUCTSRA3986, generating the pUCNEO3986 plasmid. Likewise, through the homologous recombination of linear DNA fragments, SACE_3986 was replaced by the neo gene, and the mutant WBAΔ3446Δ3986 with sensitivities to thiostrepton and kanamycin was obtained. Moreover, pSET152 carrying three extra copies of SACE_7301 under the control of Pmsre in WBAΔ3446Δ3986 was transferred into WBAΔ3446Δ3986 to yield WBAΔ3446Δ3986Δ7301.

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Table 1

| Strains and plasmids | Description | Reference |
|----------------------|-------------|-----------|
| E. coli | DH5α | F recA lacZ/M15 | 26 |
| | BL21 (DE3) | F ompT hsdS (B- mB ) dcm gal (DE3) | Novagen |
| S. erythraea | A226 | CGMCC 8279, an erythromycin low producer | China Pharmaceutical Culture Collection |
| | ΔSACE_0820 | A226 derivative with SACE_0820 deleted | This study |
| | ΔSACE_1874 | A226 derivative with SACE_1874 deleted | This study |
| | ΔSACE_2947 | A226 derivative with SACE_2947 deleted | This study |
| | ΔSACE_6589 | A226 derivative with SACE_6589 deleted. This study deleted | This study |
| | ΔSACE_7325 | A226 derivative with SACE_7325 deleted | This study |
| | ΔSACE_3446 | A226 derivative with SACE_3446 deleted | This study |
| | ΔSACE_3446/ΔpIB1393446A | ΔSACE_3446 carrying pIB1393446A | This study |
| | ΔSACE_3446/ΔpIB1393446B | ΔSACE_3446 carrying pIB1393446B | This study |
| | ΔSACE_3446/ΔpIB1393446C | ΔSACE_3446 carrying pIB1393446C | This study |
| | ΔSACE_3446/ΔpIB1393446D | ΔSACE_3446 carrying pIB1393446D | This study |
| | WB | CGMCC 8280, an erythromycin industrial overproducer | Anhui Wanbei Pharmaceutical Co., Ltd. |
| | WBA3446 | WB derivative with SACE_3446 deleted | This study |
| | WBA3446/Δ3986 | WB derivative with SACE_3446/SACE_3986 deleted | This study |
| | WBA3446/Δ3986/3 × 7301 | WBA3446/Δ3986 derivative carrying pSET152-3 × 7301 | This study |
| Plasmids | pUCTSR | pUC18 derivative containing a 1.36-kb fragment of a thioestrepton resistance gene in BamHI/Smal sites | This study |
| | pUCTSR/Δ3446 | 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 BamHI/Smal sites | This study |
| | pUCTSR/Δ3446 | pUCTSR derivative containing two 1.5-kb fragments, the upstream and downstream regions of SACE_3446 | This study |
| | pUCTSR/Δ3446 | pUCTSR derivative containing two 1.5-kb fragments, the upstream and downstream regions of SACE_3446 | This study |
| | pSET152-3 × 7301 | pSET152 carrying three extra copies of SACE_7301 under the control of Permeα™ | This study |
| | pBI139 | E. coli – S. erythraea integrative shuttle vector containing a strong constitutive ermE™ promoter | 27 |
| | pBI1393446A | pBI139 carrying an extra SACE_3446A for the gene complementation | This study |
| | pBI1393446B | pBI139 carrying an extra SACE_3446B for the gene complementation | This study |
| | pBI1393446C | pBI139 carrying an extra SACE_3446C for the gene complementation | This study |
| | pBI1393446D | pBI139 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 |

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, WBA3446 and WBA3446/Δ3986 were performed in a 5-L fermentor (Bauoxing, Shanghai, China). Samples (50 mL) were taken every 24 h and erythromycin A was extracted from above cultures in terms of previous reported method. 26 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 qRTPCR. 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), 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 (Ct) 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 NdeI and XhoI 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<sub>6</sub>-SACE_3446 fusion protein was performed by Ni-NTA Sepharose chromatography.<sup>34</sup> 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 (<sup>Table S1</sup>), and 5’-end labeled with 6-carboxyfluorescein (6-FAM). Those labeled DNA fragments were successively mixed with above purified His<sub>6</sub>-tagged SACE_3446 proteins. The binding reaction system contained 10 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 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.<sup>35</sup> 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,<sup>9,20</sup> 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 region.
regions (4.4 Mb, 11.1 TFRs/Mb). Based on the genome context analysis of individual TFR, 20% (69/362), 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 ΔSACE_2947, ΔSACE_0820, and ΔSACE_3446 mutants created a larger inhibition zone against *B. subtilis* PUB110 than their parental strain A226. Among these mutants, ΔSACE_3446 presented the highest inhibition activity (Fig. 1c–d) and was selected for further investigation.

To confirm its negative effect on erythromycin production, the ΔSACE_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), ΔSACE_3446 displayed an increased erythromycin A yield by 49.4% (78.9 mg/L) (Fig. 1e). Intriguingly, when ΔSACE_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 ΔSACE_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 ΔSACE_3446 was not caused by enhancement of cell growth. Also, when cultured on R3M agar medium, ΔSACE_3446 was identical to A226 in the rate of aerial mycelium formation, whereas ΔbldD 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 His6-SACE_3446 proteins were purified from *E. coli* BL21 (DE3) harboring pET28a-SACE_3446B, pET28a-SACE_3446C and pET28a-SACE_3446D (Fig. 2b). The results from EMSAs showed that His6-SACE_3446C and His6-SACE_3446D, but not His6-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 His6-SACE_3446C and His6-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.
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<sub>6</sub>-SACE_3446C and His<sub>6</sub>-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 ΔSACE_3446 grown for 2–4 days by qRT-PCR. In the 2-day growth period, the transcript levels 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, ΔSACE_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. Herein, we examined their interaction relationship to SACE_3446 via qRT-PCR and EMSA analysis. Compared with wild-type A226, ΔSACE_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 transcripts of SACE_3446 and SACE_3986 in ΔSACE_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.
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 WBΔ3446 mutant. When cultured in a flask with the liquid industrial medium for 6 days, WBΔ3446 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 WBΔ3446. As expectedly, erythromycin A production of the WBΔ3446Δ3986 double mutant was 1033 mg/L, separately approaching 21% and 19% enhancement over WBΔ3446 (851 mg/L) and WBΔ3986 (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; 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. During the type I TFRs, SACE_3446 was proved to repress erythromycin biosynthesis, the same as identified TFR SACE_3986.

In terms of the re-annotation of S. erythraea genome in 2013, 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 ΔSACE_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.

4. Discussion

In previous studies, we have identified two TFRs, SACE_3986 and SACE_7301, to successively repress and induce erythromycin production; 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. During the type I TFRs, SACE_3446 was proved to repress erythromycin biosynthesis, the same as identified TFR SACE_3986.

In terms of the re-annotation of S. erythraea genome in 2013, 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 ΔSACE_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. 16,26 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 PerM 15 in the WBA3446Δ3986, a reduced production and corresponding decreased transcriptions of eryAI and ermE were detected in WBA3446Δ3986(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 13 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

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