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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*.