

SACE_0012, a TetR-Family Transcriptional Regulator, Affects the Morphogenesis of *Saccharopolyspora erythraea*

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Received: 26 February 2013 / Accepted: 10 May 2013 / Published online: 23 June 2013
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Abstract *Saccharopolyspora erythraea*, a mycelium-forming actinomycete, produces a clinically important antibiotic erythromycin. Extensive investigations have provided insights into erythromycin biosynthesis in *S. erythraea*, but knowledge of its morphogenesis remains limited. By gene inactivation and complementation strategies, the TetR-family transcriptional regulator SACE_0012 was identified to be a negative regulator of mycelium formation of *S. erythraea* A226. Detected by quantitative real-time PCR, the relative transcription of *SACE_7115*, the *amfC* homolog for an aerial mycelium formation protein, was dramatically increased in *SACE_0012* mutant, whereas erythromycin biosynthetic gene *eryA*, a pleiotropic regulatory gene *bldD*, and the genes *SACE_2141*, *SACE_6464*, *SACE_6040*, that are the homologs to the sporulation regulators WhiA, WhiB, WhiG, were not differentially expressed. *SACE_0012* disruption could not restore its defect of aerial development in *bldD* mutant, and also did not further accelerate the mycelium formation in the mutant of *SACE_7040* gene, that was previously identified to be a morphogenesis repressor. Furthermore, the transcriptional level of *SACE_0012* had not markedly

changed in *bldD* and *SACE_7040* mutant over A226. Taken together, these results suggest that SACE_0012 is a negative regulator of *S. erythraea* morphogenesis by mainly increasing the transcription of *amfC* gene, independently of the BldD regulatory system.

Introduction

During its life cycle, the soil-inhabiting *Actinomycetes* undergoes a complex morphological differentiation to adapt to adverse environments [1]. Growth of *Actinomycetes* begins with spore germination and hyphal outgrowth, leading to the formation of a vegetative, or substrate mycelium. Sensing of nutrient deprivation stimulates reproductive growth resulting in the development of aerial hyphae and spore chains [2]. *Saccharopolyspora erythraea* could form the aerial hyphae, and produce erythromycin, which is a macrolide antibiotic with broad-spectrum antimicrobial activity. Extensive genetic and biochemical studies have provided detailed insights into the genes involved in erythromycin biosynthesis in *S. erythraea* [3, 4], yet its morphological differentiation remains poorly understood.

In recent years, the availability of the complete genome sequence of *S. erythraea* allowed a deeper exploration of the molecular processes controlling its morphogenesis [5]. Guided by in vitro and in vivo investigations, BldD (SACE_2077) was discovered to be a key developmental regulator in actinomycetes [1], controlling erythromycin biosynthesis and morphological differentiation in *S. erythraea* [6]. Furthermore, we identified a TetR-family transcriptional regulator (SACE_7040) involving in *S. erythraea* mycelium formation, and established genetic evidence for the crosstalk between SACE_7040 and BldD

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Electronic supplementary material The online version of this article (doi:10.1007/s00284-013-0410-x) contains supplementary material, which is available to authorized users.

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[7]. In this study, we have used gene inactivation, complementation, and transcriptional analysis to delineate the role of a new TetR-family regulator (*SACE_0012*) in the development differentiation of *S. erythraea*. Deletion of *SACE_0012* principally influences the transcription of a putative aerial mycelium formation gene *SACE_7115*, that is homologous to *amfC* of *Streptomyces*.

Materials and Methods

Strains and Growth Conditions

Saccharopolyspora erythraea A226 and its derivatives were incubated in TSB medium at 30 °C for DNA extraction, protoplast preparation, and in liquid fermentation medium R5 for analysis of erythromycin production. R3M agar medium was used for protoplast regeneration, phenotypic observations, and RNA extraction [7]. *Escherichia coli* DH5 α was the host for plasmid construction [8]. *Bacillus subtilis* PUB110 was used for an inhibition test of erythromycin production in *S. erythraea*.

Plasmid, DNA Isolation, and Manipulation

Plasmid pUCTSR [9] was a pUC18 derivative containing a 1.36 kb fragment of a thiostrepton resistance cassette (*tsr*) cloned into the *Bam*HI/*Sma*I sites. The *E. coli*-*S. erythraea* integrative shuttle expression vector pZMW [4, 10] was used for the gene complementation. DNA isolation and manipulation in *E. coli* and *S. erythraea* were carried out according to the standard methods [8, 11].

Disruption of *SACE_0012* in *S. erythraea* A226, *bldD*, and *SACE_7040* Mutant

Two 1.5 kb fragments flanking the *SACE_0012* gene were amplified from genomic DNA of *S. erythraea* A226 by PCR using the primer pairs P1/P2 (5'-TGC GAA TTC CTC CTC

GGC CGG TGA GCA GC-3'; 5'-GAT GGT ACC ATA CGA GCG GCC CCA ACC CGA AAG CCC-3') and P3/P4 (5'-ATT TCT AGA ACA CGC CCG CCA CCG GCT TCG C-3'; 5'-ACC AAG CTT AAG GGC TCG ATC GAC TCC TGG CGG-3'). Then, the two DNA PCR products were inserted into the *Eco*RI/*Kpn*I and *Xba*I/*Hind*III sites of pUCTSR, respectively, yielding pUCTSR Δ 0012. By linearized fragment homologous recombination [7], the *SACE_0012* gene was replaced with the thiostrepton resistance gene in the *S. erythraea* A226 chromosome, and the selected mutants were verified by PCR using the primers P1/P4 (Fig. 1a, b). Similarly, *SACE_0012* disruption was formed in the *bldD* and *SACE_7040* mutant strains.

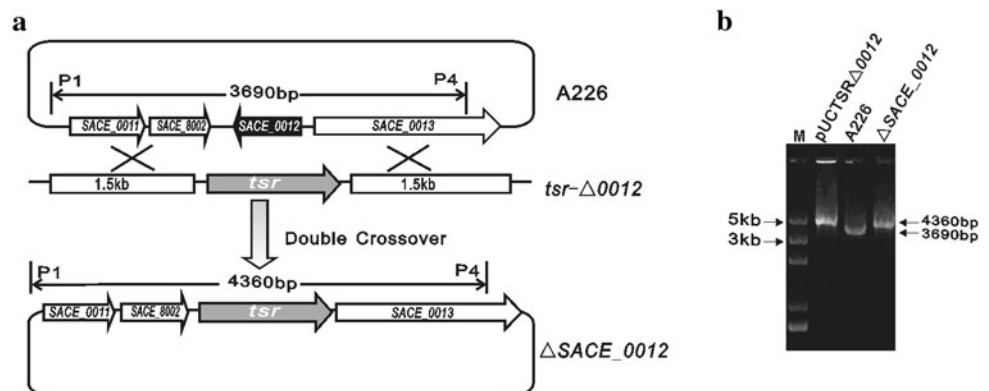
Genetic Complementation of the *SACE_0012* Mutant

For complementation, the *SACE_0012* gene was amplified by the primers P5 (5'-TAA CAT ATG TTG AAA ACG GCG TCA ATC CTC ATC CCG-3') and P6 (5'-CGC GAT ATC TCA GCG ATC GGC GGT AGT CG-3') from genomic DNA of *S. erythraea* A226, and was ligated into the *Nde*I/*Eco*RV sites of pZMW [9] to generate pZMW-0012. Then, pZMW-0012 was introduced into *SACE_0012* mutant by PEG-mediated protoplast transformation, generating the complemented strain Δ *SACE_0012*/pZMW-0012.

Quantitative Real-Time PCR (qRT-PCR)

The transcriptional levels of *eryA*, *bldD*, *SACE_0012*, and homologous genes of *whiA*, *whiB*, *whiG*, and *amfC* associated with morphogenesis in *Streptomyces* (Table S1) [12], were determined by qRT-PCR. Specific primers were designed as listed in Table S2. Total RNA was isolated from *S. erythraea* A226 and the mutants of *SACE_0012*, *bldD*, and *SACE_7040* after 2 or 4 days growth on R3M agar medium. Then, extracted RNA was treated with DNase I (Fermentas), and reverse transcription was accomplished using a cDNA synthesis kit (Fermentas).

Fig. 1 Inactivation of the TetR-family regulatory gene *SACE_0012*. **a** Schematic deletion of *SACE_0012* in *S. erythraea* A226. **b** Confirmation of *SACE_0012* deletion mutant by PCR analysis with the primer pair P1/P4. The size of 3.69 kb for the PCR-amplified bands was observed in wild-type A226, while a band of the size 4.36 kb was observed in mutant Δ *SACE_0012*, suggesting that the *SACE_0012* gene was completely deleted



qRT-PCR reactions were performed on the Applied Biosystems StepOnePlus system with Maxima™ SYBR Green/ROX qPCR Master Mix (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 method as described by Livak and Schmittgen [13].

Fermentation and Analysis

Wild-type strain A226, $\Delta SACE_0012$, and $\Delta bldD$ were grown in 30 ml R5 liquid medium in 250 ml baffled flasks for 6 days at 30 °C. 5 μ l fermentation supernatant from these cultures was added to LB agar plates, which was 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 scoring the growth-inhibition zones. Furthermore, erythromycin A produced by these cultures were quantitatively analyzed by high performance liquid chromatograph (HPLC) as described previously [14]. Erythromycin was isolated from the fermentation culture, and quantified by a standard curve [15].

Results and Discussion

Characterization of the *SACE_0012* Gene Deletion Mutant

Given a key role of the TetR-family regulator in morphological differentiation in actinomycetes [16], by gene inactivation and phenotype observation, we have identified several TetR-family regulators related to morphogenesis in *S. erythraea*, including the *SACE_7040* gene previously reported [7] and the *SACE_0012* gene currently studied. Bioinformatic analysis shows that the *SACE_0012* gene has a full-length of 690 bp (GenBank Accession No. NC-009142.114,813–115,502 nt) and is a member of the TetR-family regulators that consists of 229 amino acids with a molecular mass of 25 kDa. To investigate its function, *SACE_0012* was inactivated by replacing the 690 bp gene with a thiostrepton resistance cassette in *S. erythraea* A226 by the linearized fragment homologous recombination. A thiostrepton resistant mutant $\Delta SACE_0012$ was formed and confirmed by PCR analysis (Fig. 1a, b).

When grown on R3M medium, the mutant $\Delta SACE_0012$ formed aerial hypha earlier than original strain A226 in a three-day assay (Fig. 3b). When complemented with a cloned *SACE_0012* under the control of the *PermE** constitutive promoter (pZMW-0012), the $\Delta SACE_0012/pZMW-0012$ strain had restored the timing of aerial mycelium on R3M agar medium (data not shown). After a longer cultivation to the sixth day, no significant phenotypic difference was observed

between the wild-type strain A226, mutant $\Delta SACE_0012$, and $\Delta SACE_0012/pZME-0012$ (data not shown), revealing that *SACE_0012* was responsible for the early aerial hypha formation of *S. erythraea*. Moreover, $\Delta SACE_0012$ and A226 strains had comparable inhibition activity for *B. subtilis*, and produced similar amount of erythromycin A by HPLC analysis of fermentation products (Fig. S1A–B), confirming that *SACE_0012* was specifically involved in the morphological differentiation of *S. erythraea*.

Effect of *SACE_0012* Disruption on Transcription of the Genes for Morphogenesis and Erythromycin Biosynthesis

To test the effect of *SACE_0012* disruption on the expression of morphogenesis and erythromycin biosynthesis genes, we compared A226 and mutant $\Delta SACE_0012$ for the transcriptional change to sporulation genes (*whi* and *bldD*), an aerial mycelium formation gene *amfC* [17], and the erythromycin structure gene *eryA* (Table S1). The homologous genes to *whiA*, *whiB*, *whiG* involved in the regulation of sporulation in *Streptomyces* [18] (*SACE_2141*, *SACE_6464*, *SACE_6040*, respectively) were examined by qRT-PCR. *SACE_2141*, *SACE_6464*, *SACE_6040* transcriptions were slightly increased but not statistically different in mutant $\Delta SACE_0012$ over strain A226. *bldD* and *eryA* were also not differentially expressed. However, the transcriptional levels of the *amfC* homolog *SACE_7115* (Table S1), an aerial mycelium formation gene conserved presented in *Streptomyces* [17], were approximately 3.0-fold higher in the mutant $\Delta SACE_0012$ (Fig. 2).

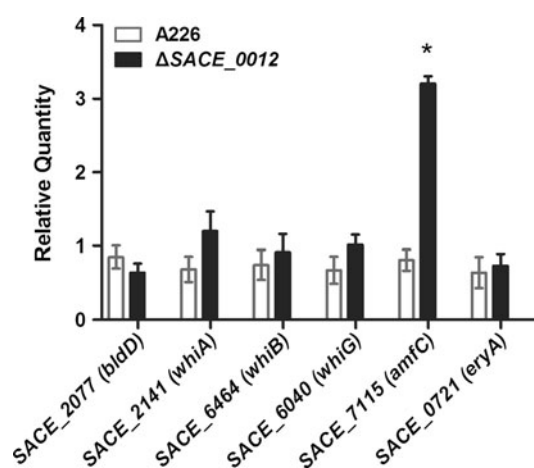
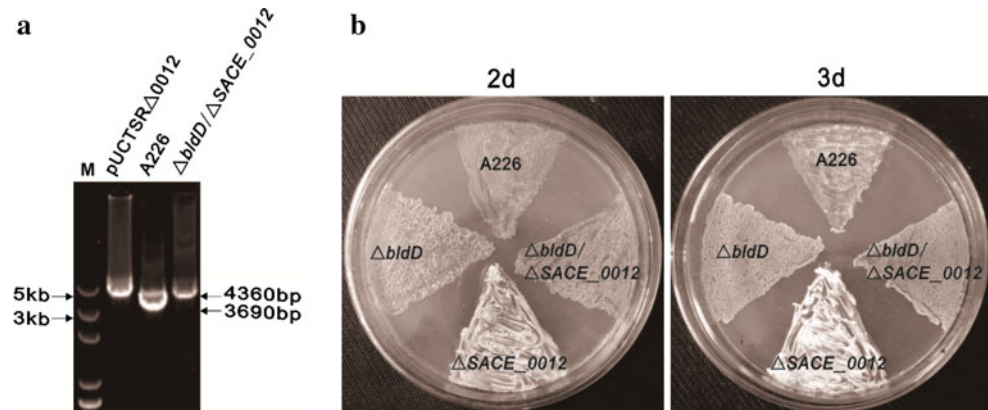


Fig. 2 Transcriptional analysis of the genes for morphogenesis and erythromycin biosynthesis in the *SACE_0012* mutant. Mean values of three independent experiments are shown, with the standard deviation indicated by error bars. Statistical significance (* $P < 0.05$) compared to culture of wild-type strain A226

Fig. 3 Confirmation and phenotype of the $\Delta bldD/\Delta SACE_0012$ double mutant. **a** Confirmation of $\Delta bldD/\Delta SACE_0012$ mutant by PCR analysis with the primer pair P1/P4. **b** Phenotype of the $\Delta bldD/\Delta SACE_0012$ mutant



SACE_0012 Disruption Failed to Restore the Defect in the Mycelium Formation of a *bldD* Mutant

bldD is required for development differentiation in *S. erythraea* [1]. To examine the relationship of *SACE_0012* and *bldD*, *SACE_0012* was disrupted in the *bldD* mutant to form a double knockout mutant strain, $\Delta bldD/\Delta SACE_0012$ (Fig. 3a). No significant phenotypic difference was observed between the mutant $\Delta bldD/\Delta SACE_0012$ and $\Delta bldD$ (Fig. 3b), indicating that *SACE_0012* disruption could not restore the defect of aerial hyphae in *bldD* mutant. qRT-PCR analysis showed that *SACE_0012* transcriptions were slightly decreased but not obviously different in mutant $\Delta bldD$ (Fig. 5). These indicate that *SACE_0012*, although influencing morphological differentiation, seems to have no connection with the BldD regulatory system.

SACE_0012 Disruption Did Not Further Accelerate the Mycelium Formation of *SACE_7040* Mutant

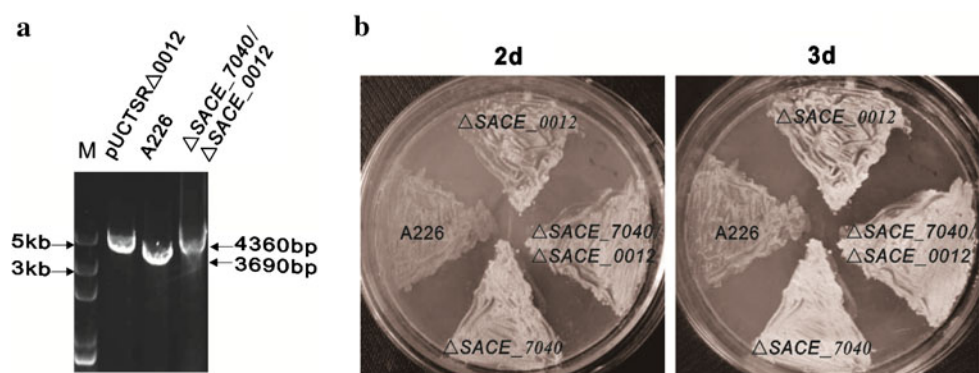
We identified a TetR-family regulator *SACE_7040* negatively involving in the morphological differentiation of *S. erythraea*, in which an interplay with the *bldD* gene was previously established [7]. Therefore, we inactivated the *SACE_0012* gene in the *SACE_7040* mutant to study combined effect upon the morphogenesis (Fig. 4a). It appears that the mycelium formation of *SACE_7040* mutant was earlier than *SACE_0012* mutant, but no obvious change of

aerial mycelia was detected in the double knockout mutant $\Delta SACE_7040/\Delta SACE_0012$ relative to the *SACE_7040* mutant (Fig. 4b). Likewise, *SACE_0012* transcriptions were slightly decreased but not statistically different in mutant $\Delta SACE_7040$ (Fig. 5).

In conclusion, these results indicated that compared with original strain A226, aerial hypha formation initiates earlier in *SACE_0012* mutant. The likely cause of an early aerial hypha formation is the higher transcriptional level of *amfC*. Previous genetic evidences revealed that *amfC* positively controlled aerial mycelium formation in *Streptomyces coelicolor* and *Streptomyces griseus*, and was distributed widely in this genus [17], implying that *amfC* might be under the control of *SACE_0012* to affect the early aerial hypha formation of *S. erythraea*. In addition, we found that *SACE_0012* disruption could not restore the defect of aerial development in a *bldD* mutant, and also could not further accelerate the mycelium formation in a mutant of *SACE_7040* gene. Further qRT-PCR analysis showed that *SACE_0012* transcriptions were slightly decreased but not obviously different in mutant $\Delta bldD$ and $\Delta SACE_7040$ relative to A226. Thus, *SACE_0012* was likely independent of the BldD regulatory system for controlling *S. erythraea* morphogenesis, distinct from the TetR-family regulator *SACE_7040* previously reported [7].

With structural and sequence conserved analysis [19], homologous of *SACE_0012* are predominantly distributed in rare actinomycetes, such as *Amir_6428* from *Actinosynnema mirum* (identities 50 %), *BN6_77090* from *Saccharothrix*

Fig. 4 Confirmation and phenotype of the $\Delta SACE_7040/\Delta SACE_0012$ double mutant. **a** Confirmation of $\Delta SACE_7040/\Delta SACE_0012$ mutant by PCR analysis with the primer pair P1/P4. **b** Phenotype of the $\Delta SACE_7040/\Delta SACE_0012$ mutant



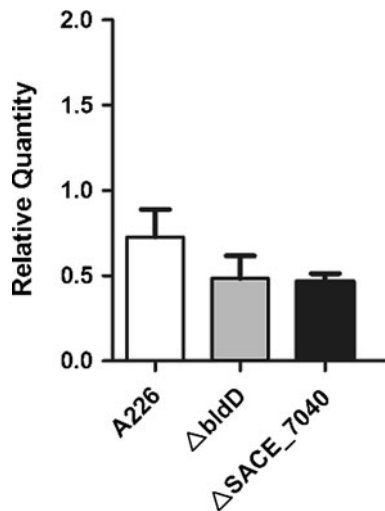


Fig. 5 Relative transcriptional levels of *SACE_0012* in the deletion mutants of *bldD* and *SACE_7040* over A226. Mean values of three independent experiments are shown, with the standard deviation indicated by error bars

espanaensis (identities 50 %), *AMED_0889* from *Amycolatopsis mediterranei* (identities 48 %), etc. (Fig. S3). However, functional analysis of the TetR-family regulator has been never reported, signifying a new regulatory mechanism for mycelial formation in actinomycetes, such as how it works with its ligand and target [10]. Therefore, these findings provide novel insights into *S. erythraea* developmental biology. In the future, more detailed regulatory mechanism of the *SACE_0012* gene will likely be valuable to deepening the understanding of the modulation of *S. erythraea* morphogenesis.

Acknowledgments We are grateful to Professor Yiguang Wang (Chinese Academy of Medical Sciences, Beijing, China) for providing *S. erythraea* A226 and Professor David T. Weaver (Anhui University, Hefei, China) for reviewing the manuscript. This study was supported by Grants from the National Basic Research Program (973) (Grant No. 2013CB734001), the National Natural Science Foundation of China (Grant Nos. 30870069) and The Natural Science Foundation of Anhui Province (Grant No. 1208085MC46).

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