

**Possible Negative Regulatory Effects of Antibiotic Production
in a Heterologous System by Sare_4854, a Novel Member of
Streptomyces Antibiotic Regulatory Protein Family (SARP)
from a Marine Microorganism *Salinispora arenicola*
CNH643 DSM 44819**

Xiao Shu^{1#}, Wei Zhang^{1#}, Jinlong Yu^{1#}, Shaowen Wu^{1#}, Yanling Ma¹,
Minzhe Zhu¹, Sisi Xia¹, Shichao Xia¹, Hao Zhang¹, Aiying Li^{1,2*} and Chao Qi^{1*}

¹Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Sciences,
Huazhong Normal University, Wuhan 430079, China.

²School of Life Sciences, State Key Laboratory of Microbial Technology,
Shandong University, Jinan - 250 100, China.

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Streptomyces antibiotic regulatory proteins (SARP) normally play positive regulatory roles during streptomyces antibiotic biosynthesis. sare_4854, a gene in *Salinispora arenicola* CNH643 DSM 44819 was proposed to encode a SARP. Here, sare_4854 was expressed in a heterologous system for functional characterization. However, the antibiotic production in the resultant strain was repressed significantly. Bioinformatic analysis showed that Sare_4854 has an additional nucleoside triphosphate hydrolases (NTPase) domain at the C-terminus, besides a SARP-like domain (DBD: DNA binding domain and BTAD: bacterial transcriptional activation domain) at the N-terminus conserved in all SARPs. The possible mechanism of Sare_4854 in antibiotic synthesis in this abnormal phenomenon was discussed here.

Key words: SARP, Sare_4854, antibiotic synthesis, Streptomyces.

Marine actinobacteria are emerging sources of bioactive compounds, enzymes, fungicides, and have been designated as a biologically active compound's factory in recent years. Because of their extremely specialized marine habitats, most of the compounds isolated from these marine actinobacteria were found to have more unique actions and structures than those from terrestrial actinobacteria. Many active marine sources show anti-tumor, anti-cancer, anti-

microtubule, anti-proliferative, cytotoxic, photo protective, antibiotic and antifouling properties¹⁻⁴.

The accumulation of secondary metabolites in actinomycetes is a result of cooperation of many genes located in a gene cluster. The regulation of these compounds' biosynthesis are controlled by a series of regulatory genes, including the SARP family. SARPs from different actinomycetes constitute a large family and are known as pathway-specific activators in the biosynthesis of these secondary metabolites⁵⁻⁶.

SARPs are characterized by a conserved DNA-binding domain (DBD) and by an accompanying bacterial transcriptional activation domain (BTAD)⁷. The crystal structures of SARPs have shown that DNA-binding domain consists of three α -helices packed against two antiparallel β -sheets, forming a winged helix-turn-helix⁸, and

* To whom all correspondence should be addressed.
Tel.: + 86 27 67862703; + 86 18502742910
E-mail: Aiying Li: aiyli@126.com
Chao Qi: qichao@mail.ccnu.edu.cn

the BTAD consists of seven α -helices¹⁶. It was explained that several SARPs from streptomyces could activate transcription of their target genes by recruiting RNA polymerase (RNAP) to the promoter region, where a DNA–SARP–RNAP complex (competent for transcriptional initiation) is formed⁷.

Sare_4854 in the *Salinispora arenicola* CNH643 DSM 44819 was presumed to encode a SARP and function as an activator in the antibiotic biosynthesis because it has conserved domains existing in all SARPs. However, none of SARPs from marine *Salinispora* has been investigated for their role and mechanism. To test the function of Sare_4854, in the present study, we performed a heterologous expression of Sare_4854 in a streptomyces strain producing antitumor and antibacterial polyketide antibiotic medermycin (MED) and discussed its possible mechanism.

MATERIALS AND METHODS

Bacterial Strains, Plasmids

Streptomyces sp. AM-7161 is a producer of MED⁹, *Salinispora arenicola* CNH643 DSM 44819 is a marine actinomycete¹⁰ and *Escherichia coli* strain DH5 \pm was used as the host for general DNA manipulation. The plasmid pWHM4* was an auto-replicative expression vector in streptomyces¹¹. The pMD18-T vector acts as a cloning vector.

DNA Manipulations and Strain Cultivation

The Sare_4854 was amplified by standard PCR method from *Salinispora arenicola* CNH643 DSM 44819 with primers A sequence and B sequence. After this was completed, the Sare_4854 was constructed into the plasmid pWHM4* to give a recombinant expression plasmid. The cultivation of *Streptomyces* and *E. coli* were performed in LA and R4 medium as previously described¹². Thiostrepton (25 μ g/ml as the working concentration) and neomycin (10 μ g/mL) were used in R4 media of *Streptomyces* cultures. When necessary, ampicillin (100 μ g/mL) was used in *E. coli* cultures. Other general manipulations follow standard protocols as outlined in the references¹³.

Bioinformatic analysis

DNA sequence alignments of SARPs were performed with DNAMAN and updated BLAST programs online. Secondary structure

prediction and proteolysis of SARPs were conducted with the program provided by <http://bioinf.cs.ucl.ac.uk/psipred/>, <http://www.predictprotein.org> and <http://ffas.ljcrf.edu/ffas-cgi>.

Heterologous Expression of Sare_4854

The recombinant plasmid pWHM4* (pWHM4*/Sare_4854) containing Sare_4854 was introduced into medermycin producing *Streptomyces sp.* AM-7161 to get a transformant *Streptomyces* strain. The transformant was incubated at 30 °C for 7 days in liquid R4 and solid R4 cultures respectively, while the wild *Streptomyces sp.* AM-7161 strain were kept under the same conditions as the negative control. The MED of the transformant and wild *Streptomyces sp.* AM-7161 were extracted with ethyl acetate as described in a recent study¹⁴.

RESULTS

Construction of Expression Plasmid

The PCR product amplified from *Salinispora arenicola* CNH643 DSM 44819 showed a similar size (2300bp) to the reported Sare_4854 size (Fig 1.B). To construct the expression recombinant plasmid, the PCR product was firstly subcloned onto a pMD18-T vector, then transferred into pWHM4*. The expression of Sare_4854 on the resultant recombinant plasmid pWHM4*-Sare_4854 was controlled by a strong constitutive promoter (PerME). The recombinant plasmid was identified by restrict digestion and PCR amplification (Fig. 1), and then further confirmed by sequencing.

Comparative Analysis of Proposed SARP Genes

SARPs are featured with two conserved domains (a DBD domain and an accompanying BTAD), while the unique roles of different SARPs are distinguished by the domains at the C-terminus of the proteins. The deduced product of Sare_4854 is a protein of 771 amino acid that shares conserved SARP-like domains (DBD and BTAD) at N-terminus similar to other SARPs (Fig 1.B), proposing that Sare_4854 is a member of the SARP family. Meanwhile, an additional NTPase domain exists at C-terminal of the Sare_4854 (Fig. 2).

Effect of Sare_4854 on MED Production

To characterize the roles of Sare_4854 for the production of antibiotics in MED-producing

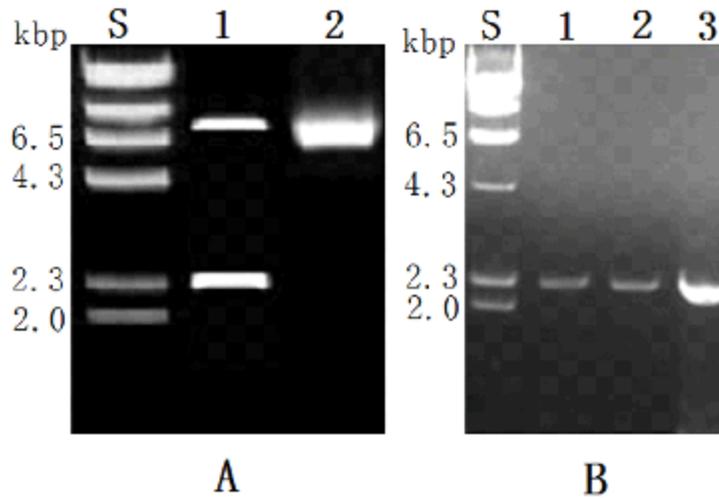


Fig. 1. Construction of the expression plasmid pWHM4*- sare_4854 A): The recombinant plasmid pWHM4-sare_4854 was confirmed by restrict digestion (lane S: standard molecular mass of DNA; lane 1: digestion product of plasmid pWHM4/sare_4854; lane 2: the recombinant plasmid pWHM4/sare_4854.). B): The recombinant plasmid pWHM4/sare_4854 was identified by PCR amplification. (lane S: standard molecular mass of DNA; lane 1 and 2: the product of PCR with plasmid pWHM4/sare_4854 as a template; lane 3: the product of PCR with *Salinispora arenicola* CNH643 DZM 44819 as a template.)

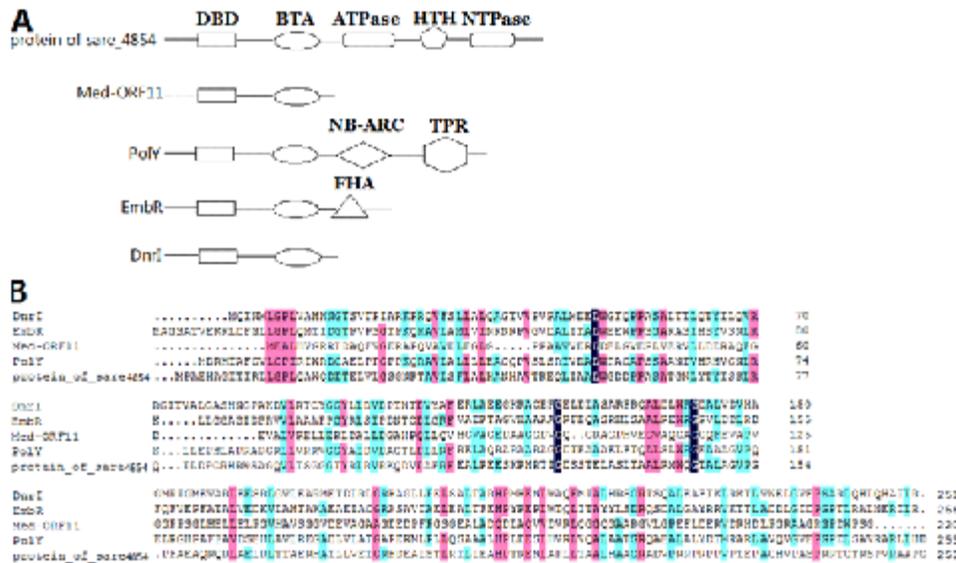


Fig. 2. Comparison between SARPs Domain structure and amino acid alignment of parts of SARPs: A): The domain structures of different SARPs (DBD: DNA-binding domain; BTA: bacterial transcriptional activation domain; HTH: helix-turn-helix domains; FHA: forkhead-associated domain; TRP: tetratricopeptide repeat domain; NB-ARC: a nucleotide-binding adaptor shared by APAF-1, certain R gene products and CED-4). B): Alignment of the SARP-like domain of SARPs (the SARP-like domain contains DBD and BTA)

Streptomyces sp. AM-7161, the transformant and native of *Streptomyces* sp. AM-7161 were cultured under conditions. The production of antibiotics in culture broths and sporulation of streptomycetes was compared in Fig.3A and Fig.3B. A higher yield

antibiotics indicated by a brown colony color and better sporulation than the transformant harboring the pWHM4-Sare_4854 plasmid could be observed in the native strain.

To further characterize the function of

Sare_4854 in the MED biosynthesis of *Streptomyces* sp. AM-7161, MED produced by the transformant and native *Streptomyces* sp. AM-7161 in the same volume liquid R4 medium were

extracted with EtoEc. The results showed that the native *Streptomyces* sp. AM-7161 produced more MED than the transformant (Fig.3C and Fig.3D).

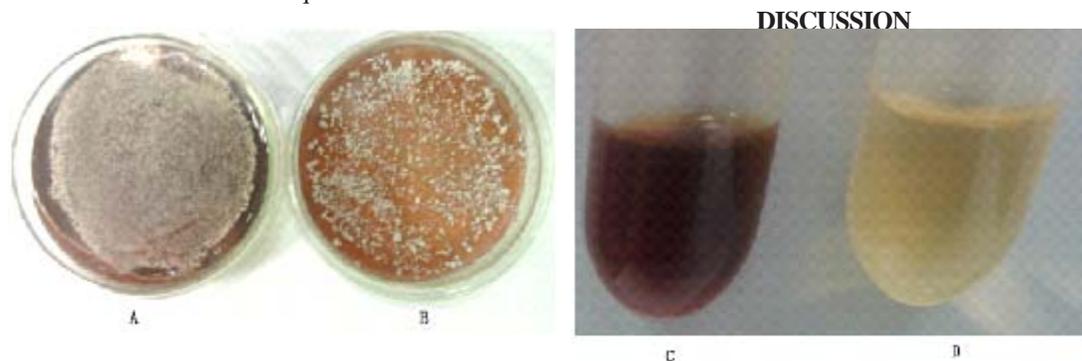


Fig. 3. Antibiotics production of the transformant and native of *Streptomyces* sp. AM-7161: A): Antibiotics and spores productions of the transformant of *Streptomyces* sp. AM-7161 in the solid R4 medium; B): Antibiotics and spores production of the native *Streptomyces* sp. AM-7161 in the solid R4 medium; C): MED production of the transformant of *Streptomyces* sp. AM-7161 in liquid R4 medium were extracted with 1,4-dioxane; D) MED production of native *Streptomyces* sp. AM-7161 in liquid R4 medium were extracted with 1,4-dioxane

SARPs encoding genes were distributed widely on a high number of antibiotic biosynthetic gene clusters, including those for actinorhodin, lankamycin, MED, auricin, tylosin and so on [5, 6, 15-18]. Generally, SARPs were reported as activators in the biosynthesis of these secondary metabolites. In this study, Sare_4854 proposed to encode a SARP protein was amplified from *Salinispora arenicola* CNH643 DZM 44819 by PCR and expressed in MED-producing streptomycetes. Surprisingly, it seemed to repress both MED production and morphological differentiation.

Salinispora arenicola CNH643 DSM 44819 is a rare strain isolated from the ocean and has many unique genes on its genome, probably used for adaptation to marine environment¹⁹. Such a repressing effect of SARP homology marine Sare_4854 might have different roles than typical SARP members. Besides DBD and bacterial BTAD, existence of an additional NTPase domain at the C-terminal of the protein allowed us to suggest it make a contribution to an unexpected role in MED production.

Generally, SARPs initiate the transcription of target genes and morphological differentiation through the binding between DBD and activation roles by BTAD in different cellular states, when additional domains at the C-terminus

of SARPs could presumably enhance the binding between DNA and SARPs in different cellular states²⁰⁻²². However, the effect of additional domains of SARPs is dependent upon background genotype and the growth medium used^{23,24}. We predicted that the NTPase domain at the C-terminal of the protein of Sare_4854 would produce an amount of (p)ppGpp, probably not required for MED biosynthesis in *Streptomyces* sp. AM-7161. A negative role for (p)ppGpp has been reported in some studies^{24,25}, so the amount of (p)ppGpp produced by Sare_4854 may also repress the antibiotics biosynthesis and morphological differentiation in *Streptomyces* sp. AM-7161.

On the other hand, the correct localization of the SARP-binding site with respect to the “10 element is critical for the formation of the stable DNA-SARP-RNAP complex. Because of this, the different DNA-SARP-RNAP complexes could in essence, compete with each other for antibiotic-transcription initiation⁷. So, we speculate that the localization of Sare_4854 may not form an efficient functional DNA-(Sare_4854)-RNAP complex on the Med gene cluster, but might block the formation of DNA-(other SARP)-RNAP in *Streptomyces* sp. AM-7161. We found only one SARP-homology med-ORF11 in the MED gene cluster and have recently proved its positive role during MED biosynthesis (data not shown). In all probability,

Sare_4854 repressed MED production by interfering with the interaction between Med-ORF11 and its target promoter DNA. The precise mechanism of this phenomenon will be further investigated in the near future.

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