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Functional Dissection of Sigma-like Domain in Antibiotic Regulatory Gene, *afsR2* in *Streptomyces lividans*

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Abstract The 63-amino-acid-encoding *afsR2* is a global antibiotics-stimulating regulatory gene identified from the chromosome of *Streptomyces lividans*. To dissect a putative functional domain in *afsR2*, several *afsR2*-derivative deletion constructs were generated and screened for the loss of actinorhodin-stimulating capability. The *afsR2*-derivative construct missing a 50-bp C-terminal region significantly lost its actinorhodin-stimulating capability in *S. lividans*. In addition, site-directed mutagenesis on amino acid positions of #57–#61 in a 50-bp C-terminal region, some of which are conserved among known Sigma 70 family proteins, significantly changed the AfsR2's activity. These results imply that the C-terminal region of AfsR2 is functionally important for antibiotics-stimulating capability and the regulatory mechanism might be somehow related to the sigma-like domain present in the C-terminal of AfsR2.

Key words: *afsR2*, *Streptomyces*, antibiotic regulation, sigma factor

The bacterial genus *Streptomyces* is widely known for its ability to produce a variety of secondary metabolites including medically important products such as antibiotics, antitumor agents, immunosuppressors, and enzyme inhibitors [3, 7, 17, 18, 21, 23]. It has been well documented that antibiotic production generally occurs during the stationary phase of growth of *Streptomyces* spp. cells and correlates temporally with the formation of aerial mycelium in cultures grown on the surface of solid media [1, 3, 7]. Several pleiotropic genes that govern antibiotic production have been identified; some of these affect only antibiotic production whereas others affect both antibiotic production and morphological differentiation, suggesting that the two processes share elements of genetic control [1, 2].

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Among several known regulatory genes affecting antibiotic biosynthetic pathways in *Streptomyces* spp. is the *afs* genes family including *afsR, afsK,* and *afsR2* [5, 10, 11, 20, 22]. The AfsK and AfsR proteins are a Ser/Thr two-component system transducing the environmental signal to the initiation of *afsR2* transcription, resulting in stimulation of antibiotic biosynthesis in *S. coelicolor* and *S. lividans* [4, 6, 9, 12, 16]. The *afsR2* in *S. lividans*, also known as *afsS* in *S. coelicolor*, [16] is located immediately 3' to *afsR*, and encodes a 63-amino-acids protein of which the functional mechanism still remains unknown. Multiple copies of *afsR2* stimulate overproduction of actinorhodin and undecylprodigiosin in both *S. lividans* and *S. coelicolor*, as was observed also for *afsR* [16, 22]. However, whereas a single copy of the *afsR2* gene normally does not lead to significant actinorhodin production in *S. lividans* [18, 22], one chromosomal copy of the identical gene results in extensive biosynthesis of actinorhodin pigment in *S. coelicolor* [16], giving *S. coelicolor* colonies the deep-blue color responsible for its species name. Recently, it was reported that the expression of *afsR2* in *S. lividans* is physiologically regulated and that *afsR2* mRNA synthesis from a single chromosomal *afsR2* gene can be stimulated by specific growth conditions to yield a *S. coelicolor*-like level of actinorhodin biosynthesis [14]. It also indicated that the requirement for multiple copies of *afsR2* to promote actinorhodin production in *S. lividans* is conditional, rather than absolute, and also demonstrated the existence in *S. lividans* of both *afsR2*-dependent and -independent mechanisms of actinorhodin synthesis [14]. Nonetheless, the functional domain and regulatory mechanism of this small-sized *afsR2* gene still remains unknown. Here, we report that a 50-bp C-terminal region in *afsR2*, which shows some homologies to the region 3 of known Sigma 70 family proteins, is functionally important for antibiotics-stimulating capability in *S. lividans*. In addition, three alternating aspartates located in the middle of the 50-bp C-terminal region of AfsR2 should play a critical functional role as a positive regulatory protein.

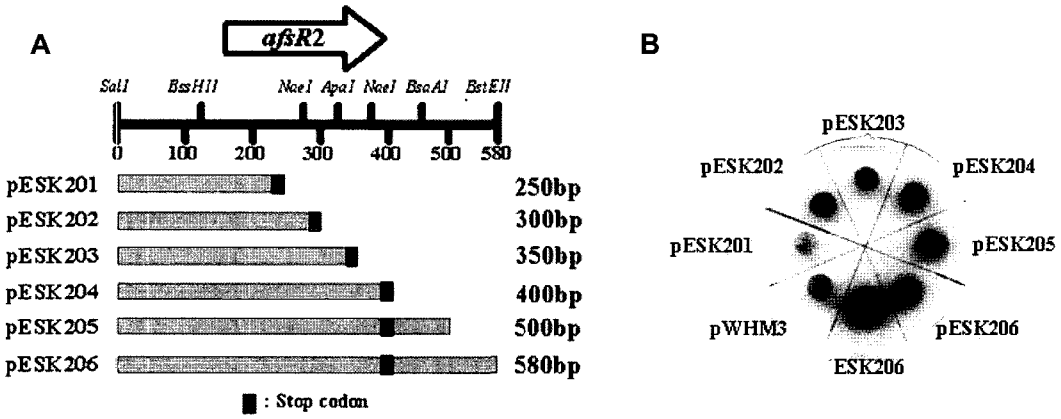

Fig. 1. A. Construction map of various *afsR2* derivatives with C-terminal deletions. **B.** Actinorhodin production by *S. lividans* transformants containing various constructs of *afsR2* derivatives (pESK201–pESK206) and *afsR2*-integrated *S. lividans* [13].

It has been previously suggested that the C-terminal region of *afsR2* might be functionally important because the central-region-deleted AfsR2 retained a higher activity than the C-terminal-region-deleted one in terms of actinorhodin stimulation in *S. lividans* [22]. To further dissect a putative functional domain in *afsR2*, several *afsR2*-derivative constructs missing various-sized C-terminal regions were generated, followed by the screening for the loss of actinorhodin-stimulating capability. The six *afsR2*-derivative constructs were generated using PCR with one common BamHI-containing forward primer (forward primer: 5'-GGATCC-GTCGACCGGTGGCCGGG-3') and six different PstI-containing reverse primers (reverse primer #1: 5'-CTGCA-GTCATTGTCC TGCGGTGTGGC-3', #2: 5'-CTGCAGT-CATGTCGAGCGTCGTGACG-3', #3: 5'-CTGCAGTCA-GTTGTCCATCGTGGTGA-3', #4: 5'-CTGCAGGTCTAC-TTGCC GTCGCCGTC-3', #5: 5'-CTGCAGCGGAGCG-GTGACGGAGCCGG-3', #6: 5'-CTGCAGGGTCACCGT-CCCCGCGG ACG-3'). The artificial stop codon sequence was also included in three reverse primers (#1–3) to ensure the proper translational termination (Fig. 1A). The PCR was performed for 30 cycles with a Rapid Thermocycler (Idaho Technology, U.S.A.) using a routinely used high G+C DNA amplification program; denature at 96°C for 30 sec, annealing at 40°C for 30 sec, and extension at 72°C for 35 sec [13, 19]. The PCR-amplified six BamHI-PstI fragments with the sizes of 250, 300, 350, 400, 500, and 580-bp, were individually cloned into the pGEM-T plasmid (Promega, U.S.A.), followed by the complete sequencing confirmation. Six of each BamHI-PstI fragment was then subcloned into a streptomycetes-*E. coli* shuttle vector pWHM3 [8] in an opposite direction of the thiostrepton promoter (named pESK201, pESK202, pESK203, pESK204, pESK205, and pESK206, respectively) (Fig. 1A). These constructs were then transformed into *S. lividans* TK21 through a PEG-assisted protoplast transformation, followed by the thiostrepton antibiotic selection [8].

Each spore of *S. lividans* transformants containing the plasmid was spotted and cultured on minimal medium plate at 30°C for 5 days, followed by the ammonium fuming of the colonies to visualize the actinorhodin production. As shown in Fig. 1B, the *S. lividans* containing pESK204, pESK205, or pESK206 showed a similar actinorhodin-overproducing phenotype, indicating that the complete ORF of *afsR2* is sufficient to function as a global regulatory gene. However, the *S. lividans* containing pESK203, pESK202, or pESK201 failed to stimulate the actinorhodin production and only exhibited a very low basal level of actinorhodin production, which is similar to the level shown in the strain containing the cloning vector, pWHM3 (Fig. 1B). Because there is a significant difference in terms of actinorhodin stimulation between pESK203 (the C-terminal 50-bp-deleted *afsR2* construct) and pESK204 (complete *afsR2* ORF), the final 50 bp present in the C-terminal of *afsR2* should play a critical functional role as a global regulatory gene in *S. lividans*.

A sequence database search with the *afsR2* suggests that there are some limited homologies between the C-terminal of AfsR2 and the region 3 of several known bacterial Sigma-factor proteins (Fig. 2). Specifically, the region including three alternating aspartates (amino acid positions of #57, 59, and 61) found in the C-terminal 50 bp of *afsR2* was found to be relatively well-conserved among several Sigma 70 family proteins (Fig. 2). To verify the biological significance of this Sigma-like domain in AfsR2, a couple of site-directed mutagenesis was performed. The first mutated construct replaced three aspartates at amino acid positions of #57, 59, and 61 with three alanines using PCR with a common forward primer described above and a reserve primer (5'-TTGCATGCCTGCAGGTCTACTTG-CCGGCGCCGGCCAGGGCCAGGG CGGGC-3'). The third aspartate at amino acid position #61 was found to be highly conserved among all the Sigma 70 family proteins (Fig. 2). The second mutated construct replaced aspartate

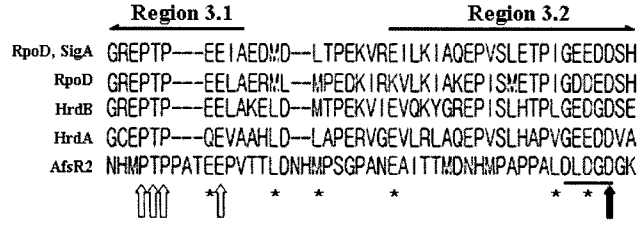

Fig. 2. Amino acid sequence alignment between AfsR2 and Region 3 of Sigma 70 family proteins.

The AfsR2 amino acids identical to some of the Sigma 70 proteins are indicated by asterisks, and the conserved amino acids in both AfsR2 and Sigma 70 proteins are indicated by the open arrows. The amino acids located at the positions of #57–#61 in AfsR2 are indicated by the underline and the conserved aspartic acid at #61 was indicated by the closed arrow.

and leucine at amino acid positions of #57 and 58 with glycine and glutamate, respectively, using PCR with the same forward primer and reverse primer (5'-TTGCATCCTG-CAGGTCTACTTGCCGTCGCCGTCCTCGCCCA GGG-GGGGC-3'). Both the PCR constructs were confirmed by complete sequencing, and then cloned into pWHM3 resulting in pESK204-4 and pESK204-5, respectively. These two *afsR2*-derivative constructs were individually transformed into *S. lividans*, followed by the observation of actinorhodin production. As shown in Fig. 3, the *S. lividans* containing pESK204-4 failed to stimulate the actinorhodin production, of which the amount is similar to the vector-containing *S. lividans* control strain, indicating that the three alternating aspartates in the C-terminal region of *afsR2* is functionally critical as a global antibiotics regulatory gene. However, the *S. lividans* containing pESK204-5 showed slightly higher actinorhodin production compared with those containing

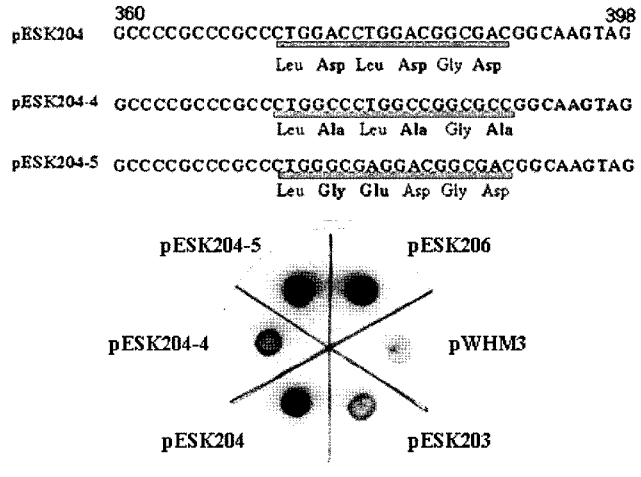

Fig. 3. Actinorhodin production by *S. lividans* transformants containing various constructs with site-directed mutagenesis of C-terminal region in *afsR2*.

Three aspartates located at the positions of #57, 59, and 61 in AfsR2 were replaced by three alanines in pESK204-4, and the aspartate-leucine at #57–58 were replaced by glycine-glutamate in pESK204-5.

the full-length wild-type sequence of *afsR2* (Fig. 3). Because the glycine and glutamate at amino acid positions #57 and #58 in pESK204-5 are the same as the conserved amino acids in the *S. coelicolor* housekeeping Sigma HrdB protein, the antibiotics-stimulating mechanism of AfsR2 might be related to the Sigma-like domain located in the C-terminal region. This is the first experimental evidence suggesting the biological significance of the Sigma-like domain of AfsR2, of which the detailed regulatory mechanism remains to be further revealed.

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