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Proteomics-driven Identification of Putative AfsR2-target Proteins Stimulating Antibiotic Biosynthesis in *Streptomyces lividans*

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Abstract AfsR2, originally identified from *Streptomyces lividans,* is a global regulatory protein which stimulates antibiotic biosynthesis. Through its stable chromosomal integration, the high level of gene expression of *afsR2* significantly induced antibiotic production as well as the sporulation of *S. lividans*, implying the presence of yet-uncharacterized AfsR2-target proteins. To identify and evaluate the putative AfsR2-target proteins involved in antibiotic regulation, the proteomics-driven approach was applied to the wild-type *S. lividans* and the *afsR2*-integrated actinorhodin overproducing strain. The 2D gel-electrophoresis gave approximately 340 protein spots showing different protein expression patterns between these two *S. lividans* strains. Further MALDI-TOF analysis revealed several AfsR2-target proteins, including glyceraldehyde-3-phosphate dehydrogenase, putative phosphate transport system regulator, guanosine pentaphosphate synthetase/polyribonucleotide nucleotidyltransferase, and superoxide dismutase, which suggests that the AfsR2 should be a pleiotropic regulatory protein which controls differential expressions of various kinds of genes in *Streptomyces* species.

Keywords: AfsR2, proteomics, antibiotic regulation, *Streptomyces*

INTRODUCTION

The bacterial genus *Streptomyces* is widely known for its ability to produce a variety of secondary metabolites, including products of medical importance such as antibiotics, antitumor agents, immunosuppressors, and enzyme inhibitors [1-3]. Production of most secondary metabolites by *Streptomyces* generally occurs during the stationary phase of cell growth with complicated mechanisms, and correlates temporally with the formation of aerial mycelium in cultures grown on the surface of solid media [1,2,4-6]. Thus far, this complex *Streptomyces* regulatory network has been partially identified. Several key regulatory genes have been revealed; some of these affect only secondary metabolite production, while others pleiotropically affect both metabolite production and morphological differentiation, suggesting the presence of multiple regulatory systems [4,7].

Among several previously-reported regulatory genes affecting the antibiotic biosynthetic pathways in *Streptomyces* spp. is the *afs*-gene family, which includes *afsR*, *afsK*, and *afsR2* [8-12]. The *afsR2* in *S. lividans*, also known as *afsS* in *S. coelicolor* [13] is located immediately 3′ to *afsR*, and encodes a 63-amino-acid protein of which the function and mechanism might be related to the sigma factor protein [Kim *et al.*, unpublished data]. It was reported that the *afsR2* expression in *S. lividans* is physiologically regulated, and mRNA synthesis from a single chromosomal *afsR2* gene can be stimulated by specific growth conditions [14]. Recently, the wild-type *S. lividans*, which does not produces actinorhodin under a typical growth condition, was successfully transformed into the actinorhodin overproducing strain through a single chromosomal integration of *afsR2* [15]. Although it has been clearly demonstrated that *afsR2* overexpression is a major factor which stimulates antibiotic biosynthesis in *S. lividans*, the AfsR2-target proteins and their signal transduction regulatory mechanisms remain unknown. In this brief communication, the proteomics-driven approach using 2D gel-electrophoresis and the MALDI-TOF analysis revealed several previously-unknown AfsR2-target proteins involved in antibiotic regulation, suggesting that AfsR2 should be a key pleiotropic regulatory protein controlling a variety of gene expressions in *Streptomyces* species.

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MATERIALS AND METHODS

Chemicals and Reagents

All chemicals for the experiments including urea, thio-

urea, 3-[(3-cholamidopropyl) dimethyammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), benzamidine, Bradford solution, acrylamide, iodoacetamide, bis-acrylamide, sodium dodecyl sulfate (SDS), acetonitrile, trifluoroacetic acid and α-cyano-4-hydroxycinnamic acid were purchased as electrophoresis grade or ACS reagents from Sigma-Aldrich (MO, USA). Pharmalyte (pH 3.5~10), IPG DryStrips (pH 4-10 NL, 23 cm), and modified porcine trypsin (sequencing grade) were purchased from Amersham Biosciences (NJ, USA), Genomine Inc. (Pohang, Korea), and Promega (WI, USA), respectively.

Protein Sample Preparation and 2D Gel-electrophoresis

The *S. lividans* cell pellets isolated at various culture time points were harvested and washed twice with ice-cold PBS (molecular cloning grade), followed by motor-driven homogenization (PowerGen125, Fisher Scientific, NH, USA) in sample buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (v/v) pharmalyte containing 1 mM benzamidine. The proteins were extracted for 1 h at room temperature with a vortex, and then centrifuged at 15,000 × g for 1 h at 15°C. The insoluble materials in the pellet were discarded, and only the soluble fraction normalized in protein concentration was used for 2D gel-electrophoresis. The IPG dry strips were equilibrated for 12~16 h with sample buffer (7 M urea, 2 M thiourea containing 2% CHAPS, 1% DTT, and 1% pharmalyte), after which they were loaded with 200 μg of each sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences, NJ, USA) following the instructions of the manufacturer. For IEF, the voltage was linearly increased from 150 to 3,500 V over the course of 3 h for sample entry, followed by a constant 3,500 V and completed focusing after 96 kVh. Prior to the second dimension, strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first round with 1% DTT and second with 2.5% iodoacetamide. The equilibrated strips were inserted onto SDS-PAGE gels (20~24 cm, 10~16%). The SDS-PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences, NJ, USA) following the instructions of the manufacturer. The 2D gels were run at 20°C for 1,700 Vh, and were then silver-stained as described by Oakley *et al.* [16] without fixing and sensitization steps.

Image Analysis and In-gel Enzymatic Digestion

Quantitative analysis of digitized images was carried out using the PDQuest software (version 7.0, BioRad, CA, USA) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for the significant expression variations which deviated over two-fold in expression level compared with control or normal sample. Protein spots were enzymatically in-gel digested using modified porcine trypsin in a manner similar to that previously described by Shevchenko *et al.* [17]. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain. The dried gel pieces were rehydrated with trypsin (8~10 ng/μL) and incubated for 8~10 h at 37℃. The proteolytic reaction was terminated by the addition of 5 μL of 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration, the peptide mixture was desalted using C_{18}ZipTips (Millipore) and eluted in 1~5 μL of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1 μL of mixture was spotted onto a target plate.

MALDI-TOF Analysis and Database Search

Protein analyses were performed using an Ettan MALDI-TOF (Amersham Biosciences, CA, USA). The peptides were evaporated with an N_2 laser at 337 nm, using a delayed extraction approach. They were accelerated with 20 KV injection pulses for time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identifycation by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak *m/z* (842. 510 and 2,211.1046) as internal standards.

RESULTS AND DISCUSSION

To identify and evaluate the biological significance of AfsR2 protein, two *S. lividans* strains were selected; the actinorhodin less-producing wild-type TK21 strain and the actinorhodin over-producing ESK206 strain (Fig. 1A and 1B). The ESK206 strain is a stable transformant, previously constructed *via* homologous chromosomal integration of *afsR2* [15]. Because the only genetic difference between these two strains is copy number of afsR2; the single copy in the TK21 and the double copies in the ESK206, these two strains should be a perfect pair for comparative proteomics to evaluate the putative AfsR2-target proteins involved in antibiotic regulation. These two *S. lividans* strains were cultured for 8 days. Samples were harvested every 24 h to determine cell growth and actinorhodin production (Fig. 1C and 1D). The 8-day cultures with the highest actinorhodin production were harvested and applied to the 2D gel electrophoresis. Based on two independent 2D gel electrophoresis experiments with ranges of pH 4~10 and pH 4~7, approximately 10,000 protein spots with 340 spots showing intensity differences of more than 2-fold were identified (Figs. 2 and 3). Considering that the single gene overexpression led to the change of numerous protein expressions, the AfsR2 effect for antibiotic stimulation is a very complicated process involved in the upstream pathway of the regulatory mechanism. Among 340 protein spots, the most noticeable 16 protein spots showing an expression

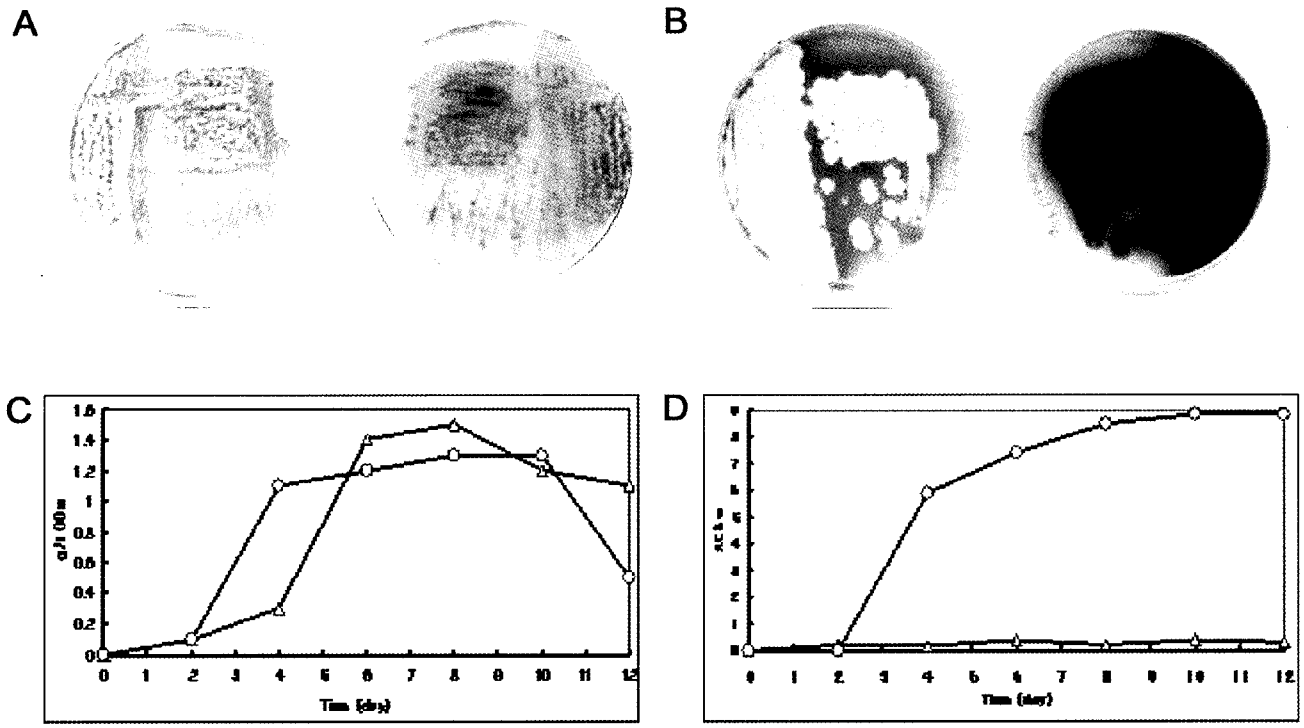

Fig. 1. Actinorhodin production by the wild-type *S. lividans* TK21 (A) and the *S. lividans* ESK206 (B). The same plate was photographed twice; colony-side-up (left) and colony-side-down (right). Measurement of dry cell weight (C) and actinorhodin production (D) in R2YE liquid culture by pWHM3-containing *S. lividans* TK21 (triangle) and *S. lividans* ESK206 (circle).

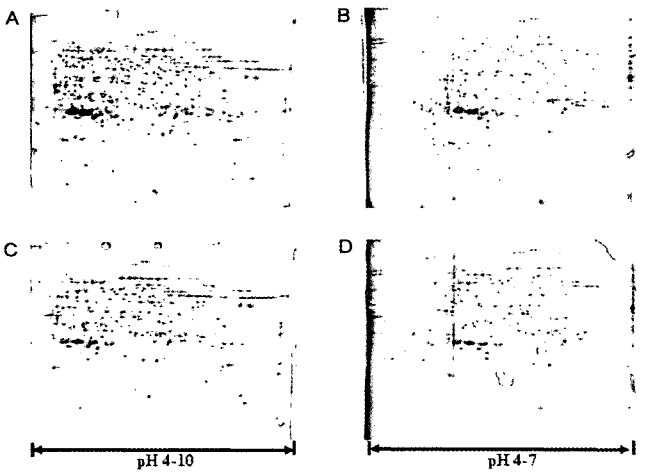

Fig. 2. 2D-gel electrophoresis using 23 cm large gel system (10~16% gradient). A, *S. lividans* TK21 with pH 4~10 gradient, B, *S. lividans* TK21 with pH 4~7 gradient, C, *S. lividans* ESK206 with pH 4~10 gradient, D, *S. lividans* ESK206 with pH 4~7 gradient.

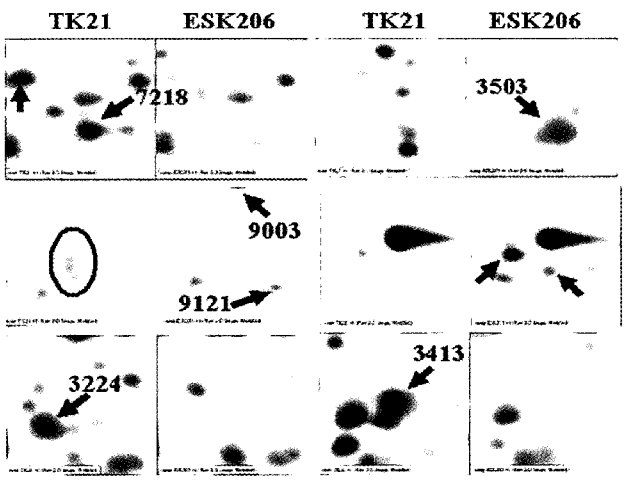

Fig. 3. Protein spot images showing different intensities on 2D-gel electrophoresis between *S. lividans* TK21 and *S. lividans* ESK206. The identifications of each protein numbered in this figure were listed in Table 1.

difference of more than 10-fold were further characterized using MALDI-TOF (Table 1). The identification of all of the proteins analyzed by MALDI-TOF was successfully determined due to the complete genome sequence information of the physiologically-related *S. coelicolor* (Table 1). Upon *afsR2* expression, some of the down-regulated proteins were putative secreted solute binding protein (SCO 6569), putative secreted protein (SCO 1860), glyceraldehyde-3-phosphate dehydrogenase (SCO 1947), and putative nucleotide-binding protein (SCO 5249). Among the down-regulated proteins, glyceraldehyde-

Table 1. List of proteins and their pI and MW values identified using MALDI-TOF. A., Down-regulated proteins *via afsR2*-overexpression in *S. lividans*. B, Up-regulated proteins *via afsR2*-overexpression in *S. lividans*.

	Spot ID	PI	M. W.	Identification	SCO number
A.	1302	4.4	37.8	Putative secreted solute binding protein	6569
	3224	5.0	28.8	Putative secreted protein	1860
	3413	4.9	46.7	Glyceraldehyde-3-phosphate dehydrogenase	1947
	7202 7218	6.0 6.2	30.7 28.2	Putative nucleotide-binding protein	5249
B.	2123	4.7	23.9	Putative regulatory protein	0512
	2526	4.9	50.6	Guanosine pentaphosphate synthetase/ polyribonucleotide nucleotidyltransferase	5737
	3114	5.0	26.4	Putative dehydrogenase	5262
	3322	4.9	31.6	Hypothetical protein	7800
	4322	5.2	39.8	Putative fructose 1,6-bisphosphate aldolase	3649
	4214 5207 8008	5.2 5.4 6.8	28.3 28.0 16.0	Putative phosphate transport system regulatory protein	4228
	4706	5.1	69.7	Probable 3-hydroxyacyl-CoA dehydrogenase	3834
	4710	5.2	87.9	Delta-1-pyrroline-5-carboxylate dehydrogenase	5520
	5414	5.6	40.0	Probable anti-sigma factor	3548
	5301	5.4	30.7	Putative hydrolase	0267
	9003	7.8	13.0	Superoxide dismutase	5254
	9121	7.9	27.0	Putative oxidoreductase	4352

3-phosphate dehydrogenase (SCO 1947) is known to be the key enzyme required for the energy generation involved in primary metabolism [18,19]. The AfsR2-induced down regulation of glyceraldehyde-3-phosphate dehydrogenase in ESK206 implies that AfsR2 expression initiates the secondary metabolism including early production of actinorhodin and the healthy sporulation (Fig. 1B). The most-significantly up-regulated proteins were guanosine pentaphosphate synthetase/polyribonucleotide nucleotidyltransferase (SCO 5737), putative dehydrogenase (SCO 5262), putative fructose 1,6-bisphosphate aldolase (SCO 3649), putative phosphate transport system regulator (SCO 4228), superoxide dismutase (SCO 5254), and putative oxidoreductase (SCO 4352). It has been well documented that the phosphate concentration plays an important role in *Streptomyces* antibiotic production [20,21]. The AfsR2-induced up-regulation of the putative phosphate transport system regulator (SCO 4228) in ESK206 strongly implies that the many phosphate-dependent kinases, previously-reported antibiotic regulatory systems, could be the putative AfsR2-target proteins. Interestingly, the *S. lividans* DNA microarray data with the total ESK206 RNAs also revealed significant transcript increase of the putative phosphate transport system regulator (SCO 4228) (Kim *et al.*, unpublished data). The superoxide dismutase (SCO 5254) has been previously proposed as the major defense protein against various oxidative stresses during the stationary phase [22, 23]. Therefore, AfsR2 may not directly induce the expression of superoxide dismutase, but may indirectly stimulate stationary metabolism. The most plausible pleiotropic target protein for AfsR2 identified with comparative proteomics is believed to be the guanosine pentaphosphate synthetase/polyribonucleotide nucleotidyltransferase (SCO 5737). This protein was originally suggested to be the enzyme involved in the biosynthesis of ppGpp, one of the well-known bacterial molecules, which initiates the stringent response [24,25]. In experiments, the increase of ppGpp concentration has been confirmed to coincide with antibiotic biosynthesis and morphological differentiation in various *Streptomyces* species [26,27]. Additionally, ppGpp was suggested to induce the RpoS sigma factor involved in the expression of more than 50 genes, as well as several antibiotic pathway-specific regulatory genes [28-30]. Recently, this SCO 5737 protein was identified to contain the RNA polyA polymerase activity, which might control the overall RNA stability in *Streptomyces* species [31]. Therefore, the guanosine pentaphosphate synthetase/polyribonucleotide nucleotidyltransferase (SCO 5737) should be the key pleiotropic target protein induced by AfsR2, of which the mechanism will be further pursued.

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