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Proteomics-Driven Identification of SCO4677-Dependent Proteins in *Streptomyces lividans* and *Streptomyces coelicolor*

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AfsR2 is a global regulatory protein that stimulates antibiotic biosynthesis in both *Streptomyces lividans* and *S. coelicolor*. Previously, various *afsR2*-dependent genes including a putative *abaA*-like regulatory gene, SCO4677, were identified through comparative DNA microarray analysis. To further identify the putative SCO4677-dependent proteins, the comparative proteomics-driven approach was applied to the SCO4677-overexpressing strains of *S. lividans* and *S. coelicolor* along with the wild-type strains. The 2D gel electrophoresis gave approximately 277 protein spots for *S. lividans* and 207 protein spots for *S. coelicolor*, showing different protein expression patterns between the SCO4677-overexpressing strains and the wild-type strains. Further MALDI–TOF analysis revealed that only 18 proteins exhibited similar expression patterns in both *S. lividans* and *S. coelicolor*, suggesting that the SCO4677 could encode an *abaA*-like regulator that controls a few cross-species common proteins as well as many species-specific proteins in *Streptomyces* species.

Keywords: SCO4677, proteomics, cross-species regulation, *Streptomyces*

The bacterial genus *Streptomyces* is well known for its ability to produce a variety of secondary metabolites, including medically important products such as antibiotics, antitumor agents, immunosuppressors, and enzyme inhibitors [1, 6, 17]. Production of most secondary metabolites produced 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, 3, 6, 13, 16]. 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, whereas others pleiotropically affect both metabolite production and morphological differentiation, suggesting the presence of multiple regulatory systems [2, 3].

Among several previously reported regulatory genes affecting the antibiotic biosynthetic pathways in *Streptomyces* species is the *afs* gene family, which includes *afsR*, *afsK*, and *afsR2* [5, 7, 8, 24, 25]. The *afsR2* in *S. lividans*, also known as *afsS* in *S. coelicolor* [17], 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 [11]. Previously, the wild-type *S. lividans*, which does not produce actinorhodin under a typical growth condition, was successfully transformed into the actinorhodin overproducing strain through a single chromosomal integration of *afsR2* [12]. Then, the transcriptomics-driven comparative DNA microarray analysis was applied to the wild-type *S. lividans* and the *afsR2*-expressing actinorhodin overproducing strain. Among several *afsR2*-dependent genes, an *abaA*-like putative regulatory gene, SCO4677, was identified as one of the most-significantly upregulated potential target gene [9]. An *abaA* was previously reported to encode an important regulatory factor for antibiotic production in *S. coelicolor* [4]. Here, we report the identification of several previously unknown SCO4677-dependent proteins both in S. *lividans* TK21 and *S. coelicolor* M145, using 2D gel electrophoresis and MALDI–TOF analysis.

The SCO4677 was cloned *via* PCR amplification with a *Bam*HI-containing forward primer and *Xba*I-containing reverse primer [forward primer: GGATCCtgagtgacttctcacggtac; reverse primer: GGAACCcacgttctgaggttaagctt]. The PCR was performed using a routinely used high G+C DNA amplification program. PCR-amplified target genes included the putative upstream ribosome binding site, start codon, and stop codon sequences. The PCR-amplified 0.43-kb product was cloned into a pGEM-T easy plasmid (Promega,

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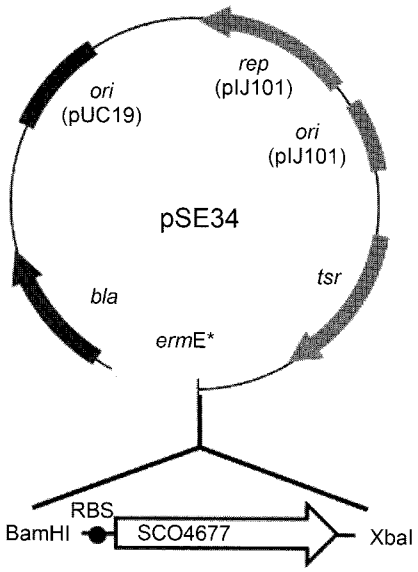

Fig. 1. Schematic map of the plasmid containing SCO4677 in the high-copy *Streptomyces* expression vector pSE34.

WI, U.S.A) and confirmed by sequencing. The *Bam*HI–*Xba*I fragment was subcloned into *Streptomyces–E. coli* shuttle vector pSE34 (Fig. 1). The plasmid was introduced into *S. coelicolor* M145 or *S. lividans* TK21 using the polyethylene glycol (PEG)-mediated protoplast transformation method, followed by the thiostrepton (*tsr*) selection method [11]. These four transformants, *S. lividans*/pSE34, *S. lividans*/p4677, *S. coelicolor*/pSE34, and *S. coelicolor*/p4677, were individually cultured in R2YE liquid cultures for 8 days, and the samples were harvested every 24 h to determine cell growth and actinorhodin production. Unlike *afsR2*, there was no significant blue antibiotic actinorhodin production stimulated by the SCO4677 overexpression both in *S. coelicolor* and *S. lividans* (data not shown). The 8-day cultures were then harvested and washed twice with ice-cold PBS, followed by motor-driven homogenization (PowerGen125, Fisher Scientific, NH, U.S.A.) in sample buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, and 2% (v/v) pharmalyte] containing 1 mM benzamidine. The protein samples were extracted for 1 h at room temperature with a vortex, and then centrifuged at 15,000 $\times g$ for 1 h at 15°C. The insoluble materials that remained 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% (w/v) CHAPS, 1% (w/v) DTT, and 1% (w/v) 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, U.S.A.) following the instructions of the manufacturer. For IEF analysis, 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), the first round with 1% DTT and the second with 2.5% iodoacetamide. The equilibrated strips were inserted onto SDS–PAGE gels (23 cm, 10–16%). The SDS–PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences, NJ, U.S.A.) 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.* [19] without fixing and sensitization steps.

Quantitative analysis of digitized images was carried out using the PDQuest software (version 7.0; BioRad, CA, U.S.A.) 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 2-fold in expression level compared with the control sample. Protein spots were enzymatically in-gel digested using modified porcine trypsin in a manner similar to that previously described by Shevchenko *et al.* [20]. 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°C. 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

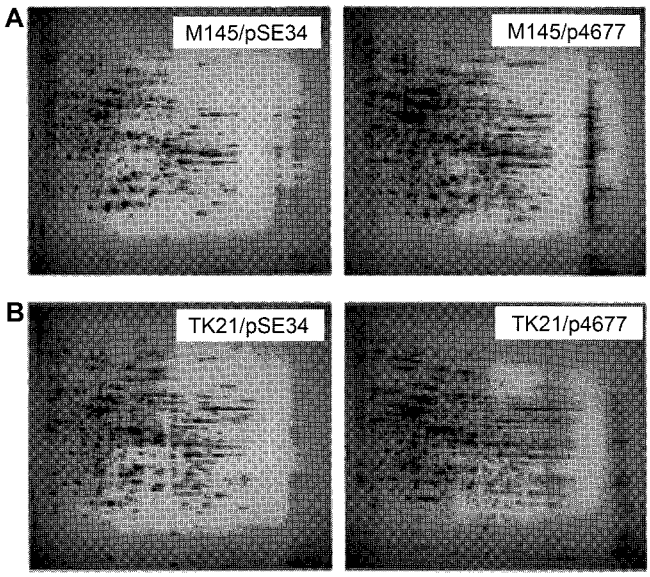

Fig. 2. 2D gel electrophoresis using a 23 cm large gel system (10–16% gradient).
A. Empty-vector-containing *S. coelicolor* M145 (left) and SCO4677-containing *S. coelicolor* (right). **B.** Empty-vector-containing *S. lividans* TK21 (left) and SCO4677-containing *S. lividans* TK21 (right).

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. Based on two independent 2D gel electrophoresis experiments with ranges of pH 4–10 and pH 4–7, approximately 1,600 protein spots with 207 spots in *S. coelicolor* and 277 spots in *S. lividans* showing intensity differences of approximately more than 1.5-fold were identified, respectively (Fig. 2 and 3).

Among protein spots identified above, the most noticeable 18 protein spots exhibiting similar expression patterns by both *S. coelicolor* and *S. lividans* were further characterized using MALDI–TOF. Protein analyses were performed using an Ettan MALDI–TOF (Amersham Biosciences, CA, U.S.A.). The peptides were evaporated with a 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 identification by peptide mass fingerprinting. Spectra were calibrated with trypsin autodigestion ion peak *m/z* (842.510 and 2,211.1046) as internal standards. The identification of all of the proteins analyzed by MALDI–TOF was successfully determined owing to the complete genome sequence information of *S. coelicolor* (Table 1). Among 18 proteins identified by MALDI–TOF analysis, all turned out to be SCO4677-dependent overexpressed proteins both in *S. lividans* TK21 and *S. coelicolor* M145, except for the SCO0546-encoding pyruvate carboxylase (Fig. 3). Although the biological significance of these SCO4677-dependent proteins needs to be further pursued, one of the most interesting observations described here in proteomics-driven identification is that the relatively small portions (18 out of over 200 proteins) were similarly regulated in both *S. lividans* and *S. coelicolor* species. In conclusion, an *abaA*-like regulatory gene, SCO4677, could control a few cross-species common proteins as well as many species-specific proteins in *Streptomyces* species, implying that *Streptomyces* comparative

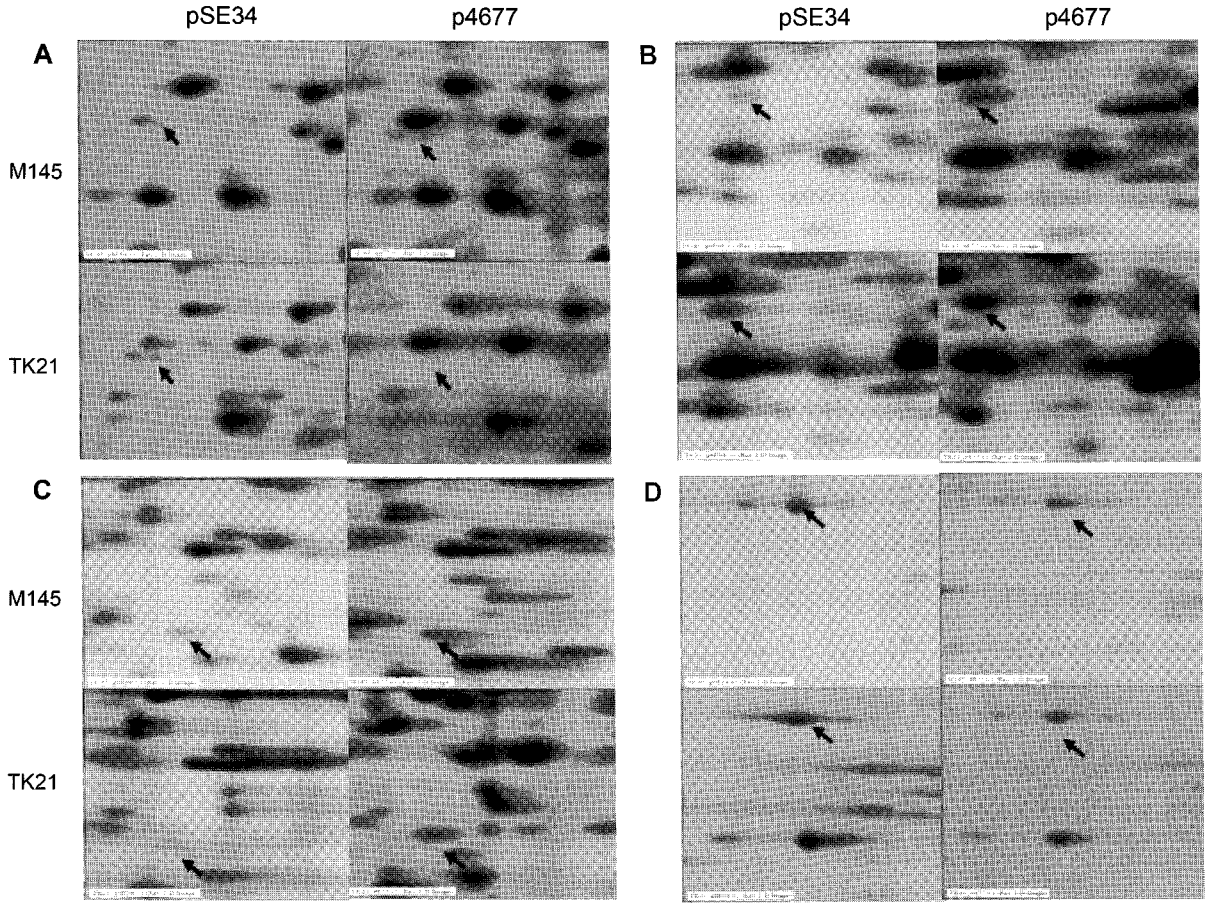

Fig. 3. Protein spot images showing different intensities on 2D gel electrophoresis between empty-vector-containing *S. coelicolor* M145 (or *S. lividans* TK21) and SCO4677-containing *S. coelicolor* (or *S. lividans* TK21).
A; 1501 (SCO1089), **B**; 4314 (SCO7511), **C**; 4529 (SCO3052), **D**; 4818 (SCO0546).

Table 1. List of SCO4677-dependent overexpressed proteins both in *S. lividans* TK21 and *S. coelicolor* M145, identified by MALDI-TOF analysis.

Spot ID	PI	M.W	Identification	Fold of increase	SCO number
209	4.20	30.46	Hypothetical protein	1.9	SCO4506
214	4.29	30.90	6-Phosphogluconate dehydrogenase	1.6	SCO3877
1501	4.30	47.55	Hypothetical protein	3.5	SCO1089
2406	4.62	41.87	Zinc-containing dehydrogenase	2.4	SCO0179
3104	4.83	28.90	Triosephosphate isomerase	1.5	SCO1945
3304	4.81	35.82	Malate dehydrogenase	1.6	SCO4827
3509	4.91	44.49	3-Phosphoshikimate 1-carboxyvinyltransferase	1.6	SCO5212
4314	5.13	38.06	Glyceraldehyde-3-phosphate dehydrogenase	1.5	SCO7511
4404	5.05	42.36	Acyl-coA dehydrogenase	3.1	SCO1198
4520	5.19	45.37	UDP-glucose-6-dehydrogenase	3.3	SCO3052
[a]4813	5.11	88.75	Pyruvate carboxylase	0.4	SCO0546
6006	5.48	14.29	Ribose-5-phosphate isomerase B	1.3	SCO2627
6507	5.49	46.95	Secreted protein	3.1	SCO6276
6623	5.63	48.31	Protoporphyrinogen oxidase	1.4	SCO6041
6701	5.44	60.27	Succinate dehydrogenase	4.0	SCO0923
6714	5.75	55.98	Uroporphyrin-III C-methyltransferase/uroporphyrinogen-III synthase	2.8	SCO3317
7302	5.86	38.23	Hypothetical protein	1.9	SCO6660
7521	6.57	44.56	Serine hydroxymethyl transferase	1.7	SCO5470

[a]Spot ID 4813, SCO0546-encoding pyruvate carboxylase was identified as a SCO4677-dependent less-expressed gene product in both *S. lividans* and *S. coelicolor*.

proteomics should be a valuable method for identification of previously unidentified proteins involved in *Streptomyces* regulatory cascade systems.

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