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Full Length Research Paper

DNA microarray analysis of expression of growth phase-responsive genes in antibiotic-producing *Streptomyces* sp. KH29

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Screening of new compounds and elucidation of biosynthetic pathways for secondary metabolites are very important fields of biology; however, most of the currently identified compounds have already been well-researched. Here, we set out to identify genes related to secondary metabolite production, specifically antibiotic production, by *Streptomyces* sp. KH29, using known DNA chip data for *S. coelicolor*. Our results indicate that the relationship between the growth-phase–dependent production of antibiotic against multi-drug resistant *A. baumannii* and the expression profiles of antibiotic biosynthetic gene expression profiles could lead to a rational strategy for improvement of actinomycetes genetic strains. Furthermore, genome-wide screening using cDNA microarrays containing sequences from the *S. coelicolor* genome, together with industrial strains that over-produce antibiotics and are related to the Streptomycetes, and may be an efficient approach for the discovery of regulatory genes affected by unidentified mutations in the industrial strains.

Key words: *Streptomyces* sp. KH29, biosynthetic genes, DNA microarray.

INTRODUCTION

The ability of *Streptomyces* species to produce multiple antibiotics or related secondary metabolites has attracted great interest among researchers (Adamidis and Champness, 1992). This ability is generally ascribed to the existence of a gene cluster found in *Streptomyces* strains, which encodes enzymes for a number of secondary metabolite pathways. *Streptomyces* produces many kinds of secondary metabolites, including antibiotics (Beppu, 1992; Brian et al., 1996; Fernandez-Moreno et al., 1992). Screening of new compounds and elucidation of biosynthetic pathways for the secondary metabolites are very important fields of biology; however, most of the currently identified compounds have already been well-researched (Gramajo et al., 1993; Hara et al., 1983; Holt et al., 1992; Hong et al., 1991). To solve this problem, a microarray system that is based on data related to the genes involved in the biosynthesis of secondary metabolites has been designed (Horinoushi et al., 1990; Ishizuka et al., 1992).

Mycelial bacteria of the genus *Streptomyces* and related bacteria are of great medical and commercial importance, through their production of approximately 70% of clinically useful antibiotics (Jones, 1985; Kelly et al., 1991; Kim et al., 2001; Lee et al., 2000). Among the *Streptomyces* species producing various antibiotics and natural products, the genome sequence of *S. coelicolor* was published in 2002 (Bentely et al., 2002). Using this genomic data, the first report in which a DNA chip of *S. coelicolor* was implemented was published in 2001 (Huang and Chang, 2005), and many studies subsequently followed using complementary DNA chips (Huang

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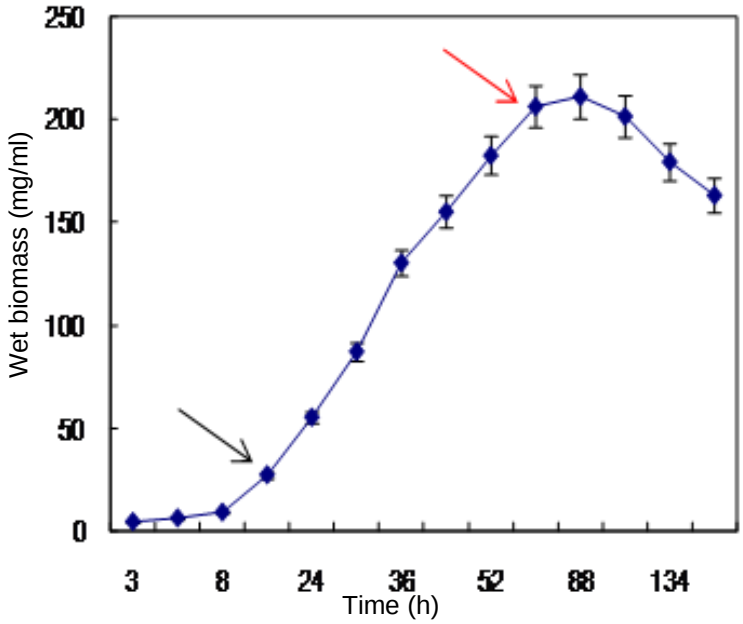

Figure 1. Growth curve of *Streptomyces* sp. KH29. Black arrow indicates the EL phase, and red arrow indicates the ES phase.

and Chang, 2005; Lee et al., 2010). In *Streptomyces*, DNA chip analyses have mostly focused on the analysis of gene expression data of known genes, due to the difficulties of analysis caused by many unknown and hypothetical genes (Horinoushi et al., 1990; Huang and Chang, 2005; Kang et al., 2007).

We had previously isolated *Streptomyces* sp. KH29, which produces an antibiotic effective against multi-drug resistant *Acinetobacter baumannii* and KH29 was identified as a *Streptomyces cinnamonensis* (Lee et al., 2010). Here, we aimed to identify genes involved in production of secondary metabolites, particularly, in antibiotic production, by *Streptomyces* sp. KH29, using known DNA chip data for *S. coelicolor*.

MATERIALS AND METHODS

Bacterial strains, media and culture condition

KH29 aerobic cultures were grown in shake culture, using 20 ml of Trypticase soy broth in 125 ml baffled flasks at 30°C for 48 h. For seed cultures, 1% of this culture was transferred to a 125 ml baffled flask containing 20 ml of seed medium KH29; these cultures were grown with shaking at 200 rpm for 48 h at 30°C. Triplicate production cultures were generated by transferring 4% of the seed culture to baffled 125 ml flasks containing 50 ml production medium; cultures were grown at 30°C with shaking at 200 rpm for up to 10 days. Growth curves were determined by weighing wet biomass at intervals, starting at 3 h, and continuing at intervals as the cultures progressed through early log (EL), mid-log, and early stationary (ES) phase. The mid-log phase was defined as the mid-point of the linear range of exponential growth (usually 48 h), and the ES phase was defined as the time when growth slowed to zero (approximately 72 h) (Figure 1). EL and ES samples of *Streptomyces* sp. KH29 cells were pelleted by centrifugation at 8,000 × *g,* at 4°C for 10 min, and the supernatant was removed for measurement of antibiotic production by the paper disk method described previously (Lee et al., 2010).

RNA extraction

Total RNA was isolated with the RNA Protect Bacteria Reagent (Qiagen, U.S.A.) and acid phenol: chloroform (1:1, vol/vol) (Ambion, U.S.A.). RNA Protect Bacteria Reagent was added to 10% of the volume of each culture; these mixtures were kept at room temperature for 5 min. After centrifugation (5,000 × *g*, 15 min), the supernatant was extracted with phenol. An equal volume of glass beads (3 mm) was added to the pellet, together with 10% acid phenol-chloroform, followed by vigorous mixing at 64°C for a 1 min period; this was repeated 10 times. The liquid phase was transferred to a new centrifuge tube and the RNA purified again by phenol-chloroform extraction. RNA quality was evaluated by agarose gel electrophoresis, and residual DNA was removed by DNase I treatment. An RNeasy® Clean-up Kit (Qiagen, U.S.A.) was used according to the manufacturer's instructions to obtain highly purified RNA, which was quantified by OD_{260}.

cDNA prove generation and microarray processing

cDNA was synthesized from 3 µg of total RNA in a reverse transcriptase reaction using Superscript II (-) (Invitrogen, Carlsbad, U.S.A.) and an oligo-dT primer (Invitrogen, Carlsbad, U.S.A.), with 0.25 µg of cDNA labeling random primer used as a positive control. Aminoallyl-dUTP was then incorporated into the purified cDNA by addition of 5 µl of 5× buffer, 5 µl dNTP-dUTP mix (0.5 mM each of dGTP, dATP, dCTP; 0.2 mM aminoallyl-dUTP; and 0.3 mM dTTP), and the reaction mixture was subsequently incubated for 2 h at

42°C. Free amines were removed using Microcon YM30 columns (Millipore, Bedford, M.A., U.S.A.) as per the manufacturer's instructions, and the concentrated samples were vacuum dried. The probe was labeled by the addition of 1/16 of a reaction tube of FluoroLink Cy5 (emission: 635 nm, red) or Cy3 (emission: 532 nm, green) monofunctional dye (Amersham Pharmacia Biotech, U.S.A.) in 0.05 M sodium bicarbonate (pH 9.0) and incubated for 1 h at room temperature in the dark. The probe was quantified using an image scanner (Amersham Pharmacia Biotech, U.S.A.) prior to hybridization. The Cy3- (RNA at EL phase) and Cy5- (RNA at ES phase) reaction mixtures were combined and unincorporated dye was removed (Qia-Quick PCR purification column, Qiagen, U.S.A.). For hybridization, SlideHyb#1 hybridization solution (Ambion, U.S.A.) was added to the labeled probe; samples were heated for 5 min at 95°C, then cooled to 68°C and applied to the hybridization system (GeneTec, Germany), for 18 h at 42°C. Washes were performed according to the manufacturer's instructions (GeneTec, Germany). The hybridized slides were scanned and analyzed using a Gene Pix 4000B scanner and the Gene Pix 3.0 software (Axon Instrument, U.S.A.). DNA microarrays were prepared by spotting the 150 *S. coelicolor* genes (DNA 70-mers), which is used in cDNA microarrays and which contains 97% of the predicted genes from *S. coelicolor,* on glass slides, as described previously (Huang and Chang, 2005; Kang et al., 2007; Rhee and Davis, 2006). The *Streptomyces* GeneChip arrays were prepared according to the manufacturer's published protocol (Affymetrix). The 70-mer oligos (Qiagen, U.S.A.) were arrayed on SuperAmine slides, according to the manufacturer's protocol, suspended in 20 µl of 3× SSC, and printed on aminosilane-coated ArrayIt® SuperAmine slides (Telechem International Inc., U.S.A.). The slides were spotted (600 pM) using a MicroGrid II robot from Biorobotics (Wang et al., 2003). Microarray images were quantified using ImaGene software (version 5.5, BioDiscovery, U.S.A.) for background correction of all values. Data processing (normalization, quality control, and statistical analysis) was done using GeneSpring software (version 7.0, Silicon Genetics). To ascertain reproducible growth phase-dependent changes, the data obtained from each time-point were assessed separately, and only the genes which showed consistent expression patterns in the EL vs. ES phase are reported.

Microarray validation: qRT-PCR and data analysis

Differential expression of specific genes was also examined by quantitative real-time PCR (qRT-PCR) using a DNA Engine Chromo4™ PTC-200 Peltier Thermal Cycler (MJ Research, Reno. NV. USA) with probes and primers designed with the Primer Express software (Applied Biosystems, Foster, CA, USA) in the custom mode, using sequences from the *S. coelicolor* database. The primers used in this study are those previously shown in Rhee et al., 2006. To generate a standard curve, we used colony PCR. A single colony from each of three independent cultures of *E. coli* (DH 10B, pUZ8002 with inserted *dpt* cluster) was denaturated in a thermocycler (MJ Research) at 100°C for 10 min using 50 µL HPLC grade water in 0.2 mL thin-walled PCR tubes. The cell debris was pelleted by a brief centrifugation and 10 µL of the supernatant was used for PCR. The amplification reaction was performed in a final volume of 20 µL containing iQ™SYBR® Green Supermix (100mM KCl, 40mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP, BIO-RAD, Hercules, CA, USA), iTaq DNA polymerase (50unitsmL^{-1}), 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers. The final concentrations of forward and reverse primers, and probe were 200 nM for all genes. To reduce variability between replicates, PCR premixes that contained all reagents except for the sample cDNA were prepared in 1.5 mL microcentrifuge tubes. The amplification conditions were 10 min at 95°C, and 40 cycles of 30 s at 95°C, 30 s at 61°C, 30 s at 72°C, and finally, 10 min at 72°C, followed by a melting curve analysis (ranging from 65 to 95°C). The Opticon® 2 software (MJ Research) was used to analyze dissociation profiles and calculate cycle threshold (C_T) values (cycle number at which the ΔRn crossed the baseline). The amplification efficiencies were determined by serial dilution and calculated as y = -ax + b, r^2= c; y = amplification efficiency, a is the slope of the dilution curve and r is the correlation coefficiency. For each assay, a standard curve was prepared by serial dilution of a known amount of the PCR products. Samples were quantified using the C_T and interpolation from the standard curve to yield a copy number for all cDNA samples examined. Calculations were performed with Excel version 10.1 (Microsoft, USA). The results are shown as mean ± standard deviation (SD) for three replicate PCR reactions. Reproducibility between assays was determined by regression analysis.

RESULTS AND DISCUSSION

Major antibiotic biosynthetic genes in *Streptomyces* sp. KH29 were upregulated from 2- to 4-fold in the ES phase compared to the EL phase. Of these genes, expression of the six biosynthetic genes (*absB*, *abaA*, *afsK*, *sigE*, *bldN*, and *whiK*), represented by multiple oligonucleotide probes, consistently increased by more than 3-fold, while expression of another gene (*absA*) also increased by more than 2-fold. Four downregulated genes were identified in *S. coelicolor*, viz. those encoding a glucose kinase, a sporulation factor, a sigma factor, and an ArpA-like protein. Interestingly, the *afsR* gene exhibited similar gene expression levels in both ES and EL growth phase (Table 1, Figure 2). Table 1 summarizes the expression values for the genes that were differentially regulated between the EL and ES growth phases in *Streptomyces* sp. KH29 transformants. Most of the antibiotic biosynthetic genes were highly expressed in *Streptomyces* sp. KH29 in the ES phase, but were not detected in the EL phase. These results indicate that the antibiotic cluster in *Streptomyces* sp. KH29 could be used to produce many kinds of antibiotics against multi-drug resistant *A. baumannii*.

The RNA expression pattern of putative biosynthetic genes from *Streptomyces* sp. KH29 was determined, and transcripts that exhibited significantly different expression ($P \leq 0.05$) between the two differential growth phases were identified, such as *absA*, *absB*, and *abaA,* which are involved in the regulation of biosynthesis of secondary metabolites. This analysis demonstrated the upregulation of seven genes and the downregulation of four major biosynthesis-associated genes (from 3- to 4- fold) in the ES compared to the EL phase. Antibiotic production in *Streptomyces* spp. is generally dependent on the growth phase and involves the expression of physically clustered regulatory and biosynthetic genes. Our data demonstrate that antibiotic pathway genes are regulated at the level of transcription during *Streptomyces* sp. KH29 growth; this mode of regulation has been used to identify biosynthetic genes in other *Streptomyces* species.

Table 1. Growth phase-dependent genes expression of *Streptomyces* sp. KH29[a].

Gene Name	Putative function	El vs. ES (ES/EL)	
		Fold activation[b]	P value
absA	Histidine kinase	2.8	1.9E-02*
absB	Leaky phenotype	3.2	2.2E-03**
abaA	Unknown	3.7	2.1E-01*
afsK	Ser/Thr kinase	2.5	2.4E-02*
sigE	Sigma factor	4.2	1.1E-02*
bldN	Sigma factor	3.4	2.0E-01*
whiK	Two-component regulator	3.1	1.2E-03**
afsR	DNA-binding protein	1.1	2.2E-01*
glkA	Glucose kinase	-2.9	2.4E-01*
whiB	Sporulation	-3.3	1.1E-02*
afsQ2	Sigma factor	-3.2	2.1E-01*
cprB	ArpA-like protein	-2.2	2.2E-02*

[a] Values represent higher (positive) or lower (negative) expression in the early stationary (ES) phase than in the early log (EL) phase in *Streptomyces* sp. KH29. *, P < 0.05; **, P < 0.01. [b] The fold change value is determined by dividing the average (value of signal intensity).

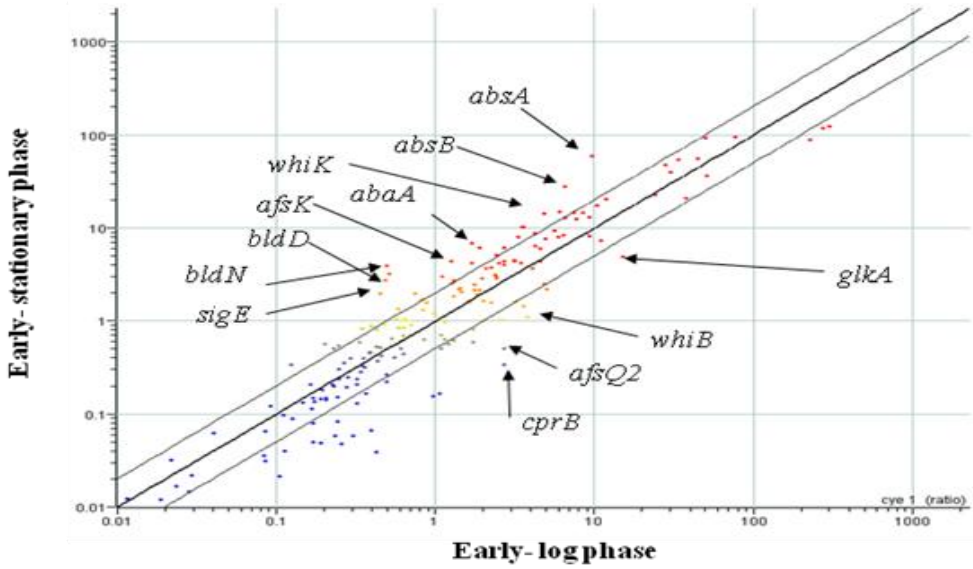

Figure 2. Scatter plot of genes expressed in differential growth phases (ES vs. EL phase). The plot was generated using GeneSpring software after background correction. The upper region of the plot indicates upregulated genes, and the lower region indicates downregulated genes.

To confirm the patterns of differential gene expression obtained by DNA microarray analysis of mRNA isolated from the *Streptomyces* sp. KH29 strain, we analyzed the expression of the putative up-regulated and down-regulated genes by real-time quantitative RT-PCR (qRT-PCR). We first evaluated real-time qRT-PCR for quantitation of *absA*, *absB*, *sigE*, *glkA*, *cprB* and housekeeping gene (*afsR)* expression from *Streptomyces* sp. KH29 strain. All values are presented as mean ± standard deviation (SD). Table 2 shows that the average of all transcripts values is given as a copy number, ranging from 581.27.60±20.21 to 11451.33±90.16. Thus, in comparing levels of EL versus ES transcripts, the *absA*, *absB* and *sigE* genes were up-regulated 3.08, 3.31 and 4.63 fold, respectively. However, comparing levels of EL versus ES in *glkA* and *cprB* genes, copy numbers ranged in comparing levels of EL

Table 2. Growth phase-dependent genes expression of *Streptomyces* sp. KH29 by qRT - PCR.

Gene name	EL vs. ES (ES/EL)[a]		Gene name	EL vs. ES (ES/EL)[a]	
	C_T	Number of copies[b]		C_T	Number of copies
absA (ES)	25.33	2234.88	*absB* (ES)	22.76	1974.32
	25.03	2165.65		24.02	1875.05
	25.28	2018.57		23.93	1945.82
Average		2139.7±306.25			1932.06±34.18
absA (EL)			*absB* (EL)		
	30.04	761.74		27.32	547.33
	30.11	675.46		26.97	597.03
	30.55	643.6		26.41	599.45
Average		693.60±61.12			581.27.60±20.21
Fold change[c]		**3.08**			**3.31**
sigE (ES)	14.39	2692.22	*afsR* (ES)	22.68	11084.32
	14.55	2520.53		21.74	12176.75
	14.62	2498.31		22.06	10755.94
		2570.35±106.12			11451.33±90.16
sigE (EL)	17.52	556.32	*afsR* (EL)	24.83	11124.48
	17.35	594.05		27.39	10071.21
	17.77	512.97		26.45	11069.49
Average		554.44±40.57			10754.06±474.26
Fold change		**4.63**			**1.06**
glkA (ES)	25.93	6301.95	*cprB* (ES)	20.91	1773.29
	26.04	6012.51		21.11	1293.4
	25.99	6181.72		20.75	1285.05
Average		6165.39±145.40			1450.24±129.01
glkA (EL)	29.02	18041.71	*cprB* (EL)	25.7	3954.29
	29.22	17962.38		24.36	3393.41
	29.31	19013.19		24.17	3124.33
Average		18338.09±39.99			3490.34±221.41
Fold change		**−2.91**			**−2.40**

[a] *Streptomyces* sp. KH29. [b] The number of copies of all transcripts are calculated by extrapolation of the C_T values using the equation to the respective lines generated by the standard curves (data not shown). [c] The fold change value is determined by dividing the average (number of copies) of growth-dependent, and early-stationary (ES) phase and early-log phase (EL) of each strains.

versus ES transcripts, the *absA*, *absB* and *sigE* genes were up-regulated 3.08, 3.31 and 4.63 fold, respectively. However, comparing levels of EL versus ES in *glkA* and *cprB* genes, copy numbers ranged from 6165.39 ± 145.40 to 18338.09 ± 39.99 and 1450.24 ± 129.01 to 3490.34 ± 221.419. These results show that expression of associated genes in the qRT-PCR analysis of the *Streptomyces* sp. KH29 strain was similarly measured by the microarray analysis (ranging from 2 to 4 fold change) (Table 1). In the qRT-PCR data, the average copy number of the housekeeping gene (*afsR*) ranged from 10754.06 ± 474.26 to 11451.33 ± 90.16 and no significant differences in expression were detected between ES and EL fold change as a 1.06. However, the overall agreement between the qRT-PCR data and the microarray data was approximately 90%. In general, the differential expression trends were similar in both the microarray and qRT-PCR analysis.

These results indicate that the relationship between the growth phase-dependent production of an antibiotic against multi-drug resistant *A. baumannii* and the expression profiles of the antibiotic's biosynthetic gene expression profiles could lead to a rational strategy for improvement of actinomycetes genetic strains. The functionally

related, physically contiguous *Streptomyces* genes enabled us to use microarray expression profiles to discover and delineate groupings of genes that have potentially related functions, and to more precisely delineate previously identified biosynthetic pathways. Furthermore, these results demonstrate a means by which the important genes for the biosynthesis of aminoglycosides, polyene antimycotics, enediynes, alpha-glucosidase inhibitors, glycopeptides, and orthosomycins could be studied. Our results now suggest that genome-wide screening using cDNA microarrays containing sequences from the *S. coelicolor* genome, together with industrial strains that over-produce antibiotics and are related to the Streptomycetes, may be an efficient approach for the discovery of regulatory genes affected by unidentified mutations in the industrial strains. Moreover, to devise rational strategies for small-molecule production by improvement of bacterial or fungal strains, complete sets of genes must be included in microarray studies. The same principles apply to the development of engineered strains for increased enzyme production or for biotransformation processes on an industrial scale.

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