J. Microbiol. Biotechnol. (2006), **16**(12), 1841–1848

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Transcription Analysis of Daptomycin Biosynthetic Genes in *Streptomyces roseosporus*

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Received: October 29, 2005
Accepted: December 30, 2005

Abstract Insights into gene expression have the potential for improvement of antibiotic yield and the development of robust production hosts for use in recombinant biomolecule production. Cubicin™ (daptomycin for injection) is a recently approved antibiotic active against many Gram(+) pathogens, including those resistant to methicillin, vancomycin, and fluoroquinolones. Daptomycin is produced as a secondary metabolite by *Streptomyces roseosporus*. A 128 kb region of DNA including the daptomycin biosynthetic gene cluster (*dpt*) has been cloned and sequenced. Using a selected array of nucleic acid probes representing this region, we compared the expression levels of the *dpt* genes between *S. roseosporus* wild-type (WT) and derived *S. roseosporus* high-producer of daptomycin (HP). We observed that the majority of the biosynthetic genes were upregulated in HP compared with WT; a total of 12 genes, including those encoding daptomycin synthetase, showed consistently and significantly higher expression levels, at least 5-fold, in HP compared with WT. In contrast, some genes, flanking the *dpt* cluster, were expressed at higher levels in the WT strain. The expression of housekeeping genes such as *S. roseosporus rpsL*, *rpsG*, and 16S (positive controls) and presumptive intergenic regions in the *dpt* cluster (negative control) were identical in the two strains. In addition, we compared transcription during the early, mid-log, and early-stationary phases of growth in the HP strain. The same set of genes was upregulated and downregulated under all conditions examined; housekeeping genes showed no relative change in expression level over the periods of growth tested. Analyses of this type would be of value in studies of strain improvement and also for the identification of gene regulation processes that are important for secondary metabolite production.

Key words: *Streptomyces roseosporus*, daptomycin, biosynthetic gne cluster, DNA microarray techniques, calcium-dependent lipopeptide antibiotic (CDA)

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Daptomycin is a novel cyclic lipopeptide antibiotic that is a potent inhibitor of Gram-positive bacteria, including strict anaerobes, enterococci, staphylococci, and streptococci (Fig. 1). Daptomycin is active against antibiotic-resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis*, penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant enterococci (VRE) [1, 4, 9, 17, 18, 22, 23]. The antibiotic is produced by *S. roseosporus* as a member of the A21978C lipopeptide factor complex [6, 7]. The biosynthetic gene cluster responsible for producing this complex (the *dpt* cluster) was cloned from *S. roseosporus* as part of a 128-kb region, including up to 64 open reading frames (ORFs) [17]. They include three genes encoding the multimodular subunits of the nonribosomal peptide synthetase (*dptA*, *dptBC*, and *dptD*), accessory genes proposed to have roles in biosynthesis (*dptE*, *dptF*, *dptG*, *dptH*, and *dptI*) or other aspects of A21978C and daptomycin metabolism (*dptP*, *dptR1*, *dptR2*, and *dptR3*), as well as a large number of ORFs encoding ABC transporter systems and ORFs with no significant orthologs in public databases and whose function in secondary metabolism is unknown.

As with most antibiotic-producing strains, the wild isolate and its earliest derivatives (WT) produced insufficient quantities of the active compound to support extensive clinical trials and commercial product production. Traditionally, an extensive strain improvement program for the organism requires optimization of bioreactor parameters (media formulation, fermentation conditions), together with genetic modification of the producing strain involving the use of a variety of mutagenic processes. For any given antibiotic producing strain, thousands of mutants derived from successive steps of mutagenesis may be required [8]. Antibiotics are often made as groups of related compounds in the producing strain, and each one needs to be isolated and tested to arrive at the most effective molecule. Frequently, the basic structure is modified by feeding

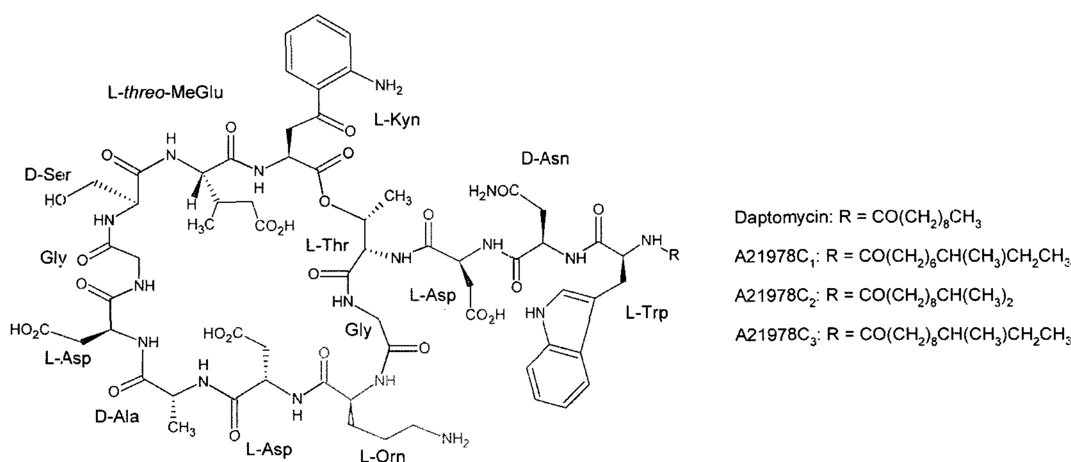

Fig. 1. Structure of A21978C factors produced by *S. roseosporus*.

precursor or by semisynthesis to produce analogs with more favorable therapeutic properties. Although the concept of developing methods or strategies for strain improvement has always tended to change according to strain and production necessities, the usual available approach is to use classical strain improvements as described above [11, 13, 16, 29]. There are several genetic and molecular genetic methods useful for increasing the yields of microbial secondary metabolites. These include random chemical mutagenesis, rational selection of spontaneous or induced mutations, transposition mutagenesis, targeted deletions, and duplications and genetic recombination by protoplast fusion. Among the various approaches used with daptomycin-producing *S. roseosporus*, a study of transposition mutagenesis indicated that such induced mutations may result in enhanced daptomycin production [18, 19]. DNA microarray techniques provide a powerful approach to measure genome-wide mRNA expression under diverse biological conditions and could provide a better understanding of modulation of gene expression associated with antibiotic production [2, 3, 5, 12, 14, 15, 21, 27].

A comparative transcriptome analysis of UA031 (=NRRL 11379), an early strain of *S. roseosporus* producing low amounts of daptomycin (referred to as "WT" in this work), and UA343, a high-producing derivative ("HP"), was conducted to examine the transcription kinetics of the daptomycin biosynthetic gene cluster at different phases of cell growth. A DNA microarray with 119 "70-mers," including all the ORFs associated with the cloned *dpt* region, together with housekeeping genes from *S. roseosporus* and a related streptomycete, *Streptomyces coelicolor*, was used in transcriptional comparisons of HP and WT. The identification of specific over- or under-expression patterns would provide a guide to future strain improvement initiatives and the analysis of heterologous biosynthetic gene expression.

MATERIALS AND METHODS

Bacterial Strains, Media, and Culture Conditions

S. roseosporus UA031 (=NRRL 11379), a wild-type (WT), and UA343 (HP), a high-producing strain, were used for expression analysis studies (provided by Cubist Pharmaceuticals, Inc., Lexington, U.S.A.) [17–19]. Compositions for media A355 and PM1 have been published elsewhere [17]. Aerobic cultures were grown with agitation in 125-ml baffled flasks at 30°C in 20 ml of Trypticase soy broth for 48 h. For seed cultures, 1% of this culture was transferred to a 125-ml baffled flask containing 20 ml of seed medium A355, and shaken at 200 rpm for 48 h at 30°C. Triplicate production cultures were generated by transferring 4% of the seed culture to 125-ml baffled flasks containing 50 ml of production medium PM1, shaken at 200 rpm for up to 10 days at 30°C. 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, mid-log, and early-stationary phases. The mid-logarithmic phase was defined as the mid-point of the linear range of exponential growth (usually 48 h), and the early stationary phase was the time when growth slowed to zero (approximately 72 h).

RNA Extraction

Early-log, mid-log, late-log, and early-stationary phases samples of *S. roseosporus* cells were pelleted by centrifugation at 4°C and 8,000 ×*g* for 10 min, and the supernatants were removed for measurement of antibiotic production by HPLC as described previously [17]. 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.). Ten % volume of RNA Protect Bacteria Reagent was added to each culture broth and kept at room temperature (RT) 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 and mixed vigorously (64°C for 1 min period, 10 times), and the liquid phase was transferred to a new tube and RNA was purified again by phenol-chloroform extraction. The quality of RNA was evaluated by agarose gel electrophoresis, and residual DNA was removed with DNase I treatment [24]. 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}.

Generation of cDNA Probe 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.) with 0.25 µg of cDNA labeling random primer along with oligo dT (Invitrogen, Carlsbad, U.S.A.) used as the positive control. Aminoallyl dUTP was then incorporated into the purified cDNA by addition of 5 µl of 5× buffer, 5 µl of 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 incubated for 2 h at 42°C. Free amine was removed using Microcon YM30 (Millipore, Bedford, MA, U.S.A.) columns as per the manufacturer's instructions and the concentrated samples were dried under vacuum. The probe was labeled by the addition of 1/16 of one 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 RT in the dark. The probe was quantified using an image scanner (Amersham Pharmacia Biotech, U.S.A.) prior to hybridization. The Cy3 (WT RNA at early-stationary phase) and Cy5 (HP RNA at early-log, mid-log, or early-stationary 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, heated for 5 min at 95°C, 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 by using a Gene Pix 400A scanner and the Gene Pix 3.0 software (Axon Instrument, U.S.A.).

DNA microarrays were prepared by spotting 133 DNA 70-mers (representing 115 *S. roseosporus* and 18 *S. coelicolor* genes). In addition, the array included cyanine dye labeled GFP (position marker), and 70-mers for *S. roseosporus glnA* (GenBank accession U58138), *whiR* and *whiG* (U58281), *rpsL* and *rpsG* (U60191), and *16S* (unpublished) (positive controls), *Rhodococcus* RHA1 genes (25, bphA1, bphC, bphD2 *Rhodococcus* control) (negative controls), and *Arabidopsis* genes (*Arabidopsis thaliana* control 249A17T7_ferredoxin [2Fe_2S] precursor) (spike control). 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 [20, 28, 30]. These manipulations were carried out in the laboratory of Dr. Colleen Nelson, Prostate Centre, Jack Bell Research Centre, UBC, BC, Canada.

Data Analysis

Microarray images were quantified using ImaGene (version 5.5, BioDiscovery, U.S.A.) and RSP (R Script Program, written by Doug Hoffart and Jochen Brumm, Microarray Facilities, Prostate Centre, Vancouver, BC, Canada) for background correction of all values. Data processing (normalization, quality control, and statistical analysis) was done using GeneSpring (version 6.0, Silicon Genetics). To ascertain the reproducibility of growth-phase-dependent changes, the data obtained from each time point were assessed separately, and only the genes that showed consistent expression patterns in two experiments (early-

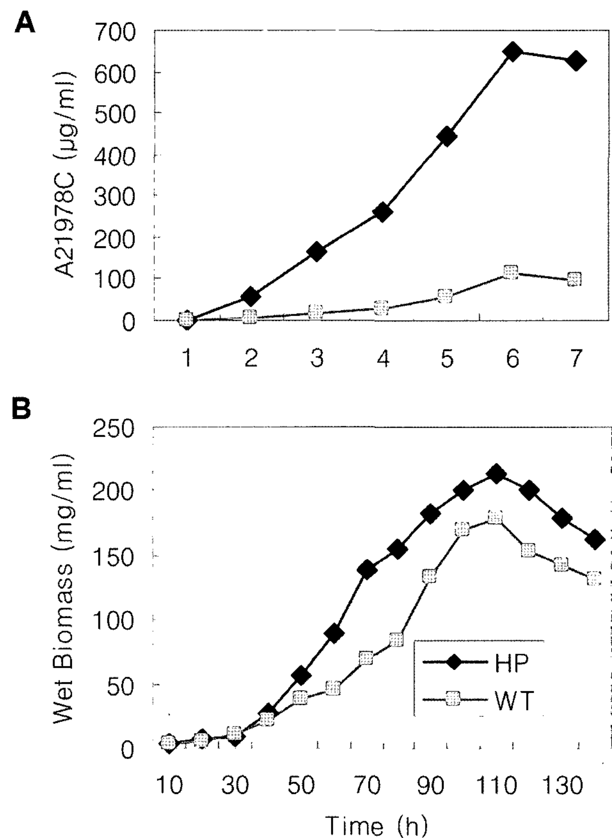

Fig. 2. Production of A21978C (**A**) and growth curve (**B**) of *S. roseosporus* WT and HP strains.

log vs. mid-log phase, and early-log vs. early-stationary phase) are reported.

RESULTS AND DISCUSSION

Comparison of *dpt* Gene Expression in Early-Stationary Phase Cultures of *S. roseosporus* WT and HP Strains

In large fermentation scale, production of A21978C was increased up to 30-fold greater in HP than WT, inspite of similar overall growth profiles. Figure 2 illustrates the growth-phase dependence of production, as indicated by measurement of total A21978C lipopeptides produced by the two strains over time. According to this growth phase, RNA samples were taken in early-stationary phase and hybridized to DNA microarrays, and the results showed upregulation of 12 genes and downregulation of 5 genes (Table 1) (from 2- to 7-fold) in the HP compared with the WT; the major *dpt* genes exhibited statistically significant changes in expression (P≤0.05 or P≤0.01). Figure 3 shows a scatterplot of the expression of all genes analyzed. Among the upregulated genes, the 3 NRPS encoding *dpt* genes, *dptA*, *dptBC*, and *dptD* represented by multiple oligonucleotide probes, showed more than 4-fold increases. Nine other genes (including *dptE*, *dptF*, *dptG*, *dptH*; ORF 35, the periplasmic component of an ABC transporter system; ORF 53, ORF 54, and ORF 55, components of another presumptive ABC transporter system; and ORF 49, a putative phosphatase) showed more than 2-fold increase in the HP. The 4 downregulated genes in *S. roseosporus* were an oxidoreductase, a hypothetical protein, monooxygenase, and a dehydrogenase, and these genes are not components of the identified *dpt* biosynthetic cluster. The *S. roseosporus* housekeeping genes tested (*rpsL*, *rpsG*, and *16S* RNA) exhibited similar gene expression levels in both WT and HP strains, and 18 conserved housekeeping genes, represented by orthologs from the *S. coelicolor* genome (data not shown), showed similar patterns of expression in

Table 1. Microarray expression analysis of *S. roseosporus* genes in high-producer (HP) versus wild-type (WT) strains during the early-stationary phase.[a]

Gene/ORF	Probe Description	Probe No.	HP vs. WT	
			Fold activation[b]	P value
dptA	NRPS A domain (Trp1 module)	70	5.0	3.8E-02*
dptA	NRPS A domain (Asn2 module)	71	5.7	4.1E-02*
dptA	NRPS A domain (Asp3 module)	72	5.6	6E-03**
dptA	NRPS A domain (Thr4 module)	73	6.3	7.7E-03**
dptA	NRPS A domain (Gly5 module)	74	6.0	7.3E-02*
dptBC	NRPS A domain (Orn6 module)	75	4.1	5.0E-04**
dptBC	NRPS A domain (Asp7 module)	76	5.2	3.5E-01
dptBC	NRPS A domain (Ala8 module)	77	4.5	7.4E-04**
dptBC	NRPS A domain (Asp9 module)	78	3.6	6.0E-02*
dptBC	NRPS A domain (Gly10 module)	79	4.7	1.2E-03**
dptBC	NRPS A domain (Ser11 module)	80	4.9	2.0E-03**
dptD	NRPS A domain (mGlu12 module)	81	6.5	1.3E-01
dptD	NRPS A domain (Kyn13 module)	82	6.6	1.9E-02*
dptE	Accessory gene (acyl CoA ligase)	36	2.5	2.7E-02*
dptF	Accessory gene (acyl carrier protein)	37	1.9	5.1E-02*
dptG	Accessory gene (esterase)	31	1.9	8.0E-02*
dptH	Accessory gene (thioesterase)	32	2.6	2.4E-01
ORF 19	Oxidoreductase	9	-2.1	2.0E-03*
ORF 20	Monooxygenase	10	-2.8	4.9E-01
ORF 22	Hypothetical protein	13	-2.3	1.0E-03**
ORF 27	Hypothetical protein	17	-1.3	3.3E-04*
ORF 35	ABC Transporter	25	3.1	2.7E-02*
ORF 49	Phosphatase	35	2.9	3.4E-03*
ORF 53	ABC transporter periplasmic component	41	3.6	1.9E-03*
ORF 54	ABC transporter	42	2.3	4.1E-02*
ORF 55	ABC transporter	43	1.9	9.1E-03*
16S	Housekeeping gene (rRNA)	122	1.0	3.5E-02*
rpsL	Housekeeping gene (ribosomal protein)	118	1.0	5.1E-04**
rpsG	Housekeeping gene (ribosomal protein)	119	1.0	7.7E-03**
IG_{17-18}	Intergenic region	111	1.1	7.8E-02*

[a]Values represent higher (positive) or lower (negative) expression in the *S. roseosporus* high-producer than in the *S. roseosporus* wild-type. *, P≤0.05; **, P<0.01.

[b]The fold change value is determined by dividing the average HP (value of signal intensity) by the average WT (value of signal intensity).

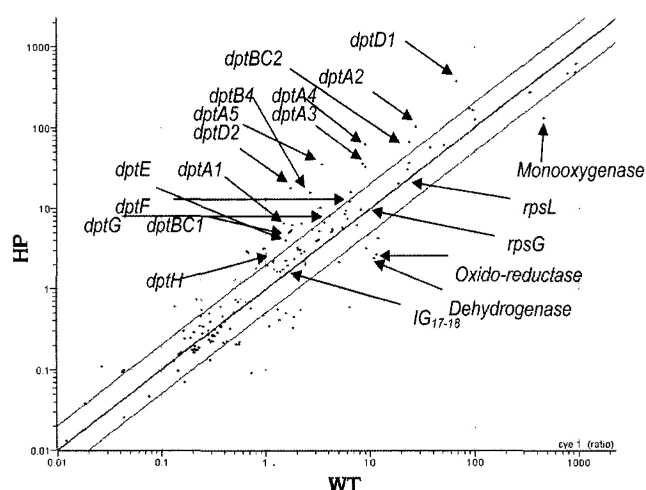

Fig. 3. Scatterplot of genes expressed in different strains (HP versus WT), derived from the GeneSpring software after background correction.
The upper region indicates upregulated genes and the lower region downregulated genes.

both strains. The 8 *S. roseosporus* intergenic regions showed no change in signal level, as expected. Table 1 summarizes the expression values for the differentially regulated genes. In our studies, the high-producing strain HP, which produced 30-fold more daptomycin than an earlier unimproved strain, was derived through repeated mutagenesis steps (γ ray and UV). The nature of the multiple genetic changes that occur during such improvement schemes is not known, and we cannot offer any comment on the mutational differences between *S. roseosporus* HP and WT.

DNA microarray analyses provide a convenient means of identifying differences in gene expression associated with the onset of antibiotic biosynthesis. DNA microarray analysis of growth-related functions in streptomycetes has been described [26]. Huang *et al.* [10] studied growth-phase responsive gene expression in *S. coelicolor*, which produces a calcium-dependent lipopeptide antibiotic (CDA) similar to daptomycin. They found that the abundance of *cda* genes increased at the start of the "transition" phase in

Table 2. Growth-phase-dependent gene expression during daptomycin production.[a]

Gene/ORF	Probe description	Probe No.	EL vs. ML (ML/EL)		EL vs. ES (ES/EL)	
			Fold activation[b]	P value	Fold activation	P value
dptA	NRPS A domains	70	6.6	2.2E-02*	5.4	1.9E-02*
dptA	NRPS A domains	71	6.3	2.7E-02*	4.9	3.4E-02*
dptA	NRPS A domains	72	6.2	2.6E-02*	5.2	4.7E-02*
dptA	NRPS A domains	73	6.8	5.1E-02*	4.7	4.4E-02*
dptA	NRPS A domains	74	6.4	7.3E-02*	4.7	7.3E-02*
dptBC	NRPS A domains	75	4.7	2.5E-02*	4.7	2.6E-02*
dptBC	NRPS A domains	76	5.2	3.5E-02*	4.5	2.8E-01
dptBC	NRPS A domains	77	5.6	2.4E-02*	4.9	7.4E-03**
dptBC	NRPS A domains	78	5.0	3.0E-02*	4.4	4.5E-02*
dptBC	NRPS A domains	79	4.6	1.2E-01	4.1	1.2E-03*
dptBC	NRPS A domains	80	5.0	2.5E-02*	4.1	2.0E-02*
dptD	NRPS A domains	81	6.1	2.9E-01	4.0	7.0E-02*
dptD	NRPS A domains	82	6.0	3.3E-02*	5.2	5.9E-02
dptE	Accessory genes	36	4.5	6.E-02*	2.5	1.9E-01
dptF	Accessory genes	37	2.6	2.9E-02*	1.4	7.3E-02*
dptG	Accessory genes	31	2.7	1.3E-03**	1.9	6.0E-03**
dptH	Accessory genes	32	4.7	4.0E-02*	2.7	2.4E-01
ORF 14	Oxidoreductase	10	-1.8	1.0E-02*	-2.9	2.8E-01
ORF 19	Oxidoreductase	9	-2.2	3.8E-03**	-2.5	8.0E-03**
ORF 22	Hypothetical protein	13	-2.5	4.1E-01	-1.5	4.3E-02*
ORF 27	Hypothetical protein	17	-2.1	4.1E-01	-1.5	4.3E-02*
ORF 35	ABC transporter	25	2.8	9.0E-01	2.4	2.6E-03**
ORF 49	Phosphatase	35	2.9	1.4E-02*	2.6	5.2E-02*
ORF 53	ABC solute	41	3.6	2.2E-02*	3.1	1.1E-02*
ORF 54	ABC transporter	42	3.1	2.9E-02*	2.1	7.1E-02*
ORF 55	ABC transporter	43	2.8	3.3E-03**	2.1	4.5E-02*
16S	Housekeeping gene	122	1.0	7.5E-02*	1.0	5.5E-02*
rpsL	Housekeeping gene	118	1.0	6.3E-01	1.0	1.1E-01
rpsG	Housekeeping gene	119	1.0	8.0E-02*	1.0	4.9E-02*
IG_{17-18}	Intergenic region	111	1.0	5.5E-03**	1.3	2.8E-03**

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

mid-logarithmic growth, before antibiotic was found in the culture medium. Likewise, we compared gene expression in *S. roseosporus* WT and HP strains and showed that a number of daptomycin biosynthetic genes were expressed at significantly higher levels in HP than in WT, as might be expected.

Expression of *dpt* Genes as a Function of Growth Phase in *S. roseosporus* HP

Antibiotics (often referred to as secondary metabolites) are usually produced in the late phase of growth (i.e., transition or stationary phase) of their producing organisms, similar to daptomycin production in *S. roseosporus* (see Fig. 2).

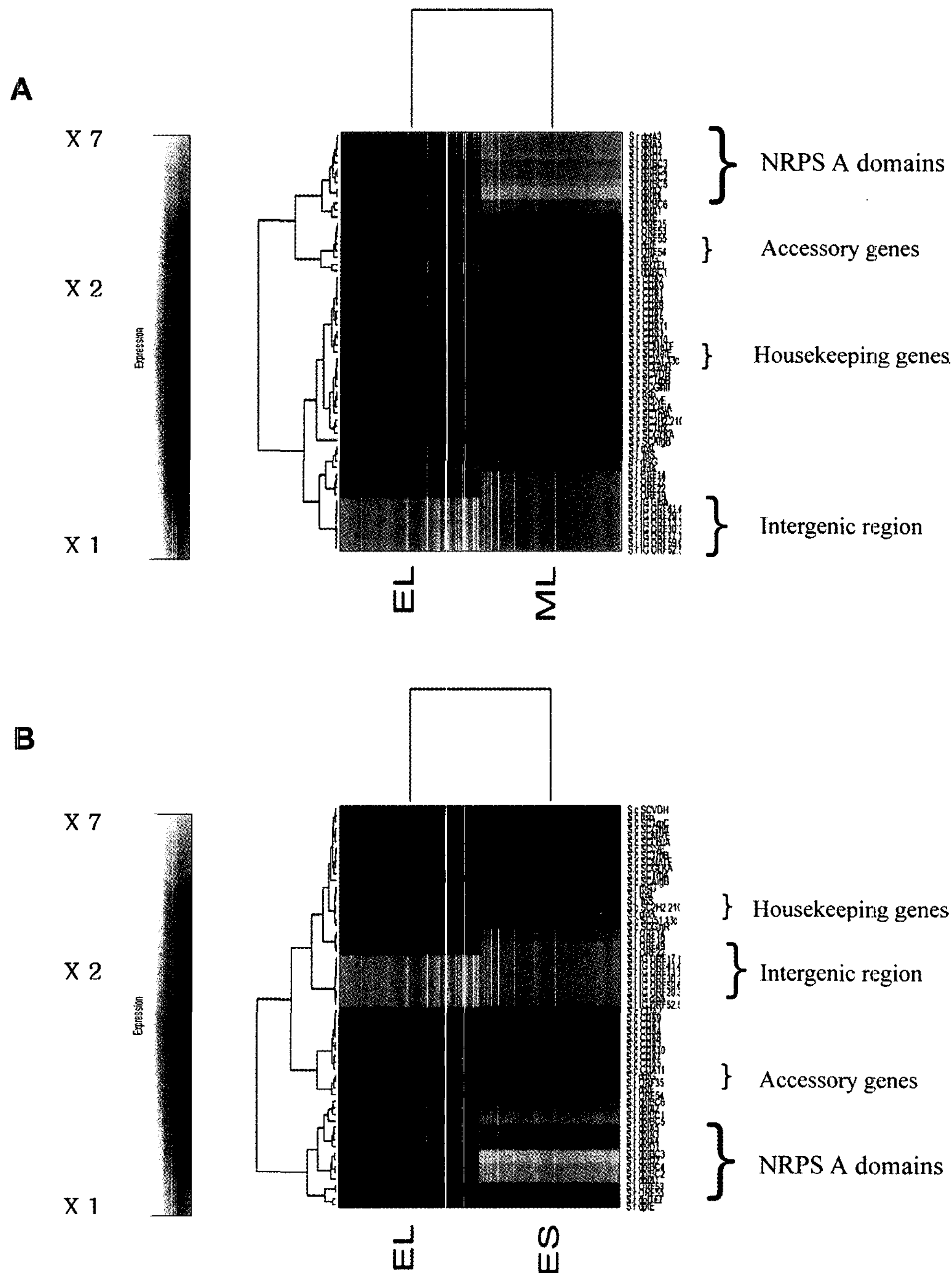

Fig. 4. Expression profiles of genes differentially expressed during growth of *S. roseosporus*.
A. EL: early-log phase vs. ML: mid-log phase; **B.** EL: early-log phase vs. ES: early-stationary phase. Profiles indicate the extent of gene expression relative to different time point. Consistency in the amount of cDNA used for analysis; 90% of the genes analyzed showed no change in expression during cell growth, whereas the remaining 10% was divided into upregulated and downregulated genes. Blue represents an increase, and red-shaded a decrease, in hybridizing cDNA. Light red indicates no detectable change in transcript level.

Therefore, it was of interest to examine the transcription of the *dpt* genes during growth and production. Of the genes examined on the DNA microarrays described in Table 1, we determined that 10 genes were upregulated (from 4-fold to 7-fold) and 4 genes were downregulated at the mid-log phase (compared with the early-log phase). Figure 4 (A and B) shows the expression profiles of major genes differentially expressed during the time course. The upregulated genes included the biosynthetic genes; *dptA*, *dptBC*, *dptD*, *dptE*, *dptF*, *dptG*, *dptH*, and 3 ABC transporters. In the early-stationary phase (compared with the early-log phase), the same 12 genes were upregulated, and one other gene was downregulated. Housekeeping genes showed no relative changes of expression levels over the period of growth examined. The importance of nutrient supply during daptomycin production has been studied, but more information is needed. How do variations (in nature and concentration) of carbon, phosphorus, and nitrogen sources influence biosynthetic gene expression? DNA microarray approaches can be of value in providing quantitative information on gene expression under bioreactor conditions, with the objective of maximizing secondary metabolite production. More detailed genetic studies of *S. roseosporus* should focus on identifying the regulatory elements involved in daptomycin production and its functional relationship with primary metabolism.

At present, the complete nucleotide sequence of the genome of *S. roseosporus* has not yet been determined. However, to achieve rational schemes for production of small molecules by improvement of bacterial or fungal strains, complete sets of genes must be included in microarray studies. The same principles would apply to the development of engineered strains for increased enzyme production or biotransformation processes on the industrial scale.

Acknowledgments

This research was funded by Cubist Pharmaceuticals, Inc. We thank Dr. David Kau for conducting the A21978C determinations, Dr. Hirofumi Hara for advice concerning many aspects of microarray analysis, and Dr. Colleen Nelson for providing facilities for DNA microarray printing. We also thank Dr. R. Baltz for his encouragement and review of a draft of this manuscript.

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