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Disruption of negative regulators (*SP_nsdA* and *SP_nsdB*) in *Streptomyces peucetius* causes doxorubicin overproduction

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Pathway-specific regulatory genes generally participate in the secondary metabolites-related biosynthesis process. The genes *nsdA* and *nsdB* were reported to have a negative effect on the production of actinorhodin, prodigiosin and calcium-dependent antibiotic from *Streptomyces coelicolor*. In this study, we searched for similar genes in the *Streptomyces peucetius* genome, the doxorubicin producer. Amino acid sequence similarity between SC_NsdA and SP4635 (SP_NsdA) was 88.1%, and between SC_NsdB and SP1750 (SP_NsdB) was 78.4%. High performance liquid chromatography (HPLC) analysis revealed that the disruption of *SP_nsdA* and *SP_nsdB* significantly increased doxorubicin production by 2.07 and 1.74-fold, respectively. The *SP_nsdA* and *SP_nsdB* disruption mutants produced more yellow pigment and early aerial mycelium than did the original wild-type strain. These results show that *SP_nsdA* and *SP_nsdB* negatively affected doxorubicin production and morphological differentiation in *S. peucetius*.

Key words: Doxorubicin, gene disruption, negative regulator, secondary metabolite, *Streptomyces peucetius*.

INTRODUCTION

Streptomycetes are Gram-positive, soil-dwelling bacteria, and are widely recognized as producers of various secondary metabolites. Streptomycetes produce about 75% of commercially and medically useful antibiotics, and their antibiotic biosynthesis is mediated by several types of pathway-specific regulators (Champness, 2000).

Regulatory genes that are required for antibiotic biosynthesis act as positive and/or negative elements in antibiotic production. Identification and inactivation of repressor genes have proven effective in overproduction

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of clinically important drugs. Secondary metabolites with clinical value have been overproduced in significantly higher amount through the combined application of genetic engineering and strain improvement techniques. Pathway-specific regulatory genes like *actII-orf4*, *redD*, *cdaR* and *mmyR* regulate the antibiotic-related biosynthetic genes in *S. coelicolor* (Bibb, 1996), and other global regulators such as *bldA* (Fernandez-Moreno et al., 1991), *bldB* (Eccleston et al., 2002), *bldD* (Elliot et al., 1998) and *bldG* (Bignell et al., 2000) perform the highest-level of regulation and affect both morphological and physiological differentiation (Chater, 1993, 2001). In other cases, some regulatory genes containing *absA1-absA1* (Anderson et al., 2001; Ryding et al., 2002), *cutS-cutR* (Champness et al., 1992), *phoR-phoP* (Sola-Landa et al., 2003) and *tcrA* (Liu and Yang, 2006) are pathway-specific repressors that regulate antibiotic production in a negative way since their mutation or deletion results in the overproduction of antibiotics. *S. coelicolor* is a genetically well-characterized strain that can produce four types of antibiotics such as actinorhodin (Act), undecylprodigiosin (Red), calcium-dependent antibiotic (CDA), and methylenomycin (Mmy). Among the various regulatory genes, especially SCO5582 (*nsdA*) and SCO7252 (*nsdB*) in *S. coelicolor* were identified by gene disruption as a gene negatively affecting antibiotic production and sporulation (Li et al., 2006; Wang et al., 2009; Zhang et al., 2007).

Doxorubicin (DXR) was first isolated from *Streptomyces peucetius* subsp. *caesica* ATCC27952, a mutant strain derived from *S. peucetius* ATCC29050 (Arcamone et al., 1969) and it is commonly used in the treatment of a wide range of cancers including bladder, breast, stomach, lung, ovaries, thyroid, soft tissue sarcoma, multiple myeloma and others (Alfaro et al., 2013). Increasing DXR production is very important because the chemical synthesis of DXR is a tedious process and also it is chemically labile. On the other hand, multistep reactions requiring electrophilic bromination limited yield of DXR during its semi-synthetic production using daunorubicin (DNR) (Lown, 1993). Although a number of organisms produce DXR, *S. peucetius* ATCC27952 is the only organism reported to produce DXR (Grein, 1987). Therefore, the generation of industrial strain of *S. peucetius* ATCC27952 for DXR production is important. In this study, we found two genes, SP4635 and SP1750, in *S. peucetius* ATCC27952 which are orthologous genes of *nsdA* and *nsdB* in *S. coelicolor*, respectively. We studied the level of DXR production and morphological differentiation by gene disruption in *S. peucetius*.

MATERIALS AND METHODS

Bacterial strains, culture conditions and vectors

Escherichia coli were grown in Luria-Bertani (LB) broth (Difco) and maintained on LB agar medium at 37°C. *S. peucetius* were grown in R2YE (50 ml, 5% sucrose, 0.02% potassium sulfate, 1% magnesium chloride, 1% glucose, 0.5% yeast extract, and 0.01% Difco casamino acid) and maintained on R2YE agar medium at 28°C. DNA manipulation was carried out in *E. coli* XL1-Blue (Stratagene). pGEM-T easy vector (Promega, USA) was the routinely used cloning vector for DNA manipulation, and pKC1139 was used as *E.coli-Streptomyces* shuttle vector for gene inactivation.

DNA manipulation

We compared the genome of *S. peucetius*, and found two genes (SP4635 and SP1750) which have high sequence similarity with *nsdA* and *nsdB* in *S. coelicolor*. For disruption of SP4635 (*SP_nsdA*) and SP1750 (*SP_nsdB*) in *S. peucetius*, the upstream and downstream fragments (SP4635U, SP4635D, SP1750U and SP1750D) were amplified by polymerase chain reaction (PCR). The primer sequences were as follows: SP4635UF (5'-GTC GAG CTG GGC CTC GAT GAG GTC-3'), SP4635UR (5'-TCT TCT AGA ACC GGA GGG TCA GAC-3'), SP4635DF (5'-GGT TCT ACT CGT ACG ACC GGT TCG-3'), SP4635DR (5'-GCG GAA TTC GAC GAT CCG CAT TCC-3'), SP1750UF (5'-ACA AGC TTC TGC AG ATA CGC CC CA-3'), SP1750UR (5'-AAT CTA GAC GGC CGG ACT CAT CGA-3'), SP1750DF (5'-GTT CTA GAA ACC CGC CTC TTC GAG-3') and SP1750DR (5'-GGG AAT TCG GCT CGA GGG-3'). PCR was carried out under the following conditions: denaturation at 94°C for 7 min, and in each cycle, annealing at 55-65°C for 1 min and polymerization at 72°C, denaturation at 94°C for 1 min, for total of 30 cycles and finally gap filling at 72°C for 7 min.

Construction of recombinants

Amplified DNA fragments of *SP_nsdA* and *SP_nsdB* were cloned into the pGEM-T easy vector and then transformed into *E. coli* XL1-Blue. The upstream fragment of SP4635U was digested with *Hin*dIII and *Xba*I, and the downstream fragment of SP4635D was digested with *Xba*I and *Eco*RI; then they were cloned into pKC1139. The upstream fragment of SP1750U and the downstream fragment of SP1750D were digested and cloned as mentioned above. For the final construct, *SP_nsdA* and *SP_nsdB* recombinants were digested with *Xba*I and then ligated with the fragment of the thiostrepton resistance gene (1.0 kb) obtained from pIBR25 (Thuy et al., 2005), thereby resulting in pOJH0117 and pOMK1228. All these plasmid were confirmed by enzyme digestion and PCR sequencing. pOJH0117 and pOMK1228 were transformed into the *E. coli* ET12567 used as demethylation host, and then finally transformed into the wild type strain of *S. peucetius* ATCC 27952 (Flett et al., 1997).

DNA sequence accession number

The nucleotide sequences of *SP_nsdA* and *SP_nsdB* reported in this paper have been deposited in the NCBI nucleotide sequence database under accession numbers KF500401 and KF500402, respectively.

Transformation into *S. peucetius*

The protoplast transformation and the selection of thiostrepton-resistant transformants were performed using previously described methods (Jnawali et al., 2011). Wild-type *S. peucetius* ATCC 27952 strain was cultured in a 50-ml R2YE medium for 36 h at 28°C. The culture broth was transferred to a 50-ml tube and washed with 10.3% sucrose. The protoplasts were generated by incubating the mycelia at 37°C for 55 min with the addition of 3 ml of lysozyme (5 mg/ml). The recombinant DNA was transformed into *S. peucetius*, and the

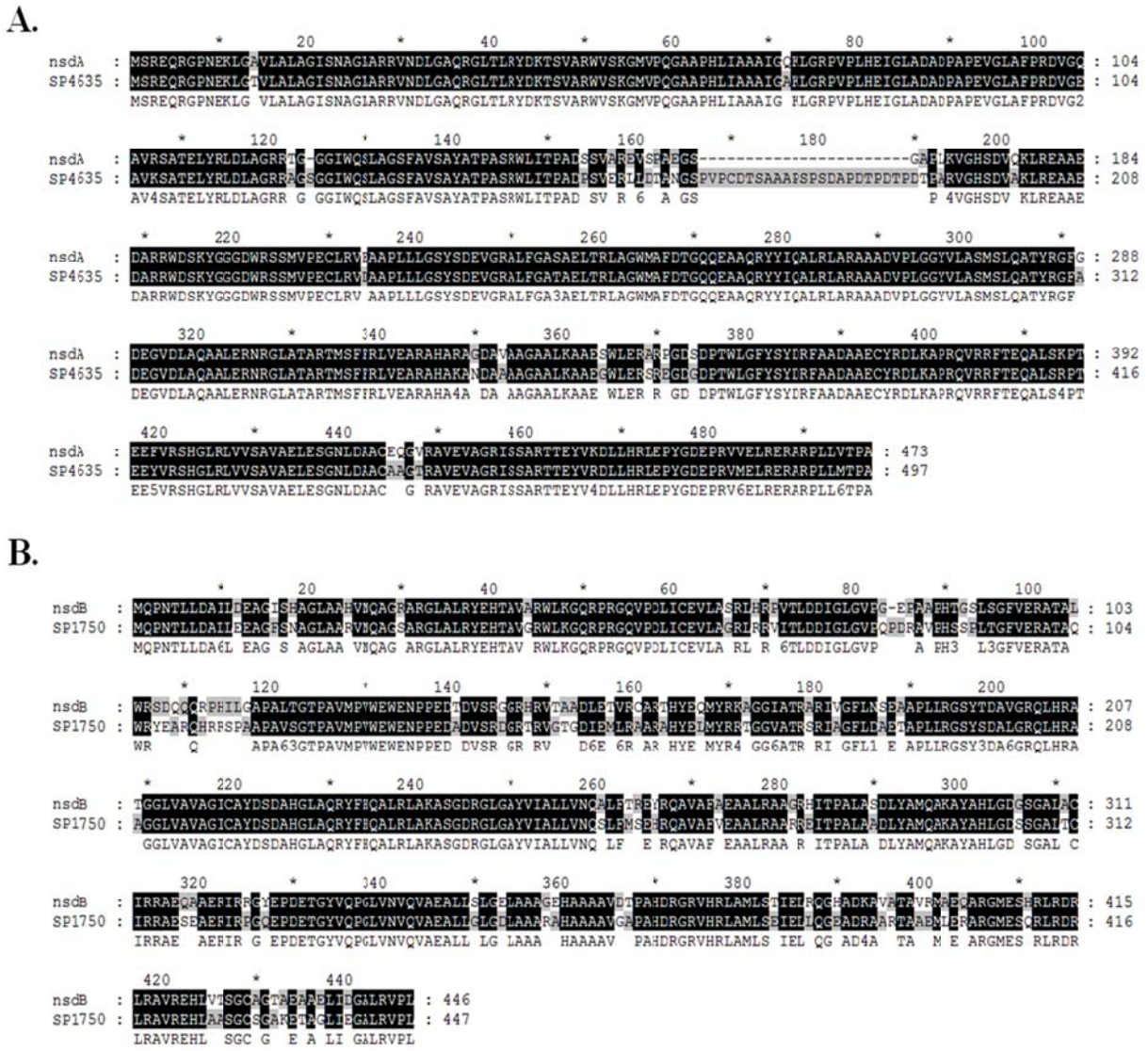

Figure 1. Multiple sequence analysis of SP4635 and SP1750. A. NsdA from *S. coelicolor* and SP4635 (SP_NsdA) from *S. peucetius*; B. NsdB from *S. coelicolor* and SP1750 (SP_NsdB) from *S. peucetius*.

protoplasts were mixed with plasmid DNA. 200 µl of 40% (w/v) polyethylene glycol 1,000 (PEG, Merck-Shuchardt) solution was promptly added, followed by brief centrifugation to remove PEG; the protoplasts were resuspended in protoplast buffer, and each plate was overlaid with 3 ml of soft agar (0.4%) containing 40 ug/ml thiostrepton. All the transformants were confirmed by PCR sequencing.

HPLC

The *S. peucetius* wild type and mutant strains were extracted with two volumes of $CHCl_3$:CH_3OH (9:1). The extract was dried under reduced pressure using a rotary evaporator and reconstituted in 1.5 ml of methanol. A 15-µl aliquot of the extract was analyzed by HPLC using a reversed-phase C18 column with a mixture of 100% acetonitrile (solvent B), distilled water (solvent A, pH 2.34 by trifluoroacetic acid) and sodium sulfate (1.327 g/L) for 71 min, with a flow rate of 1 ml/min by the following method: 0-50 min (0-100% B), 50-60 min (100% B), and 60-70 min (100% A). Peaks were monitored using a UV absorbance detector at 254 nm. DXR was used as a control.

RESULTS

Sequencing analysis

'*nsdA*' and '*nsdB*' are known as the negative transcriptional regulatory genes in *S. coelicolor*. According to the multiple sequence alignment result, SP4635 (SP_NsdA) showed 88.1% amino acid identity with 'NsdA' of *S. coelicolor*, and SP1750 (SP_NsdB) showed 78.4% amino acid identity with 'NsdB' of *S. coelicolor* (Figure 1). We proposed these genes have a negative effect on the production of DXR in *S. peucetius*, and designed primers for functional characterization of these genes.

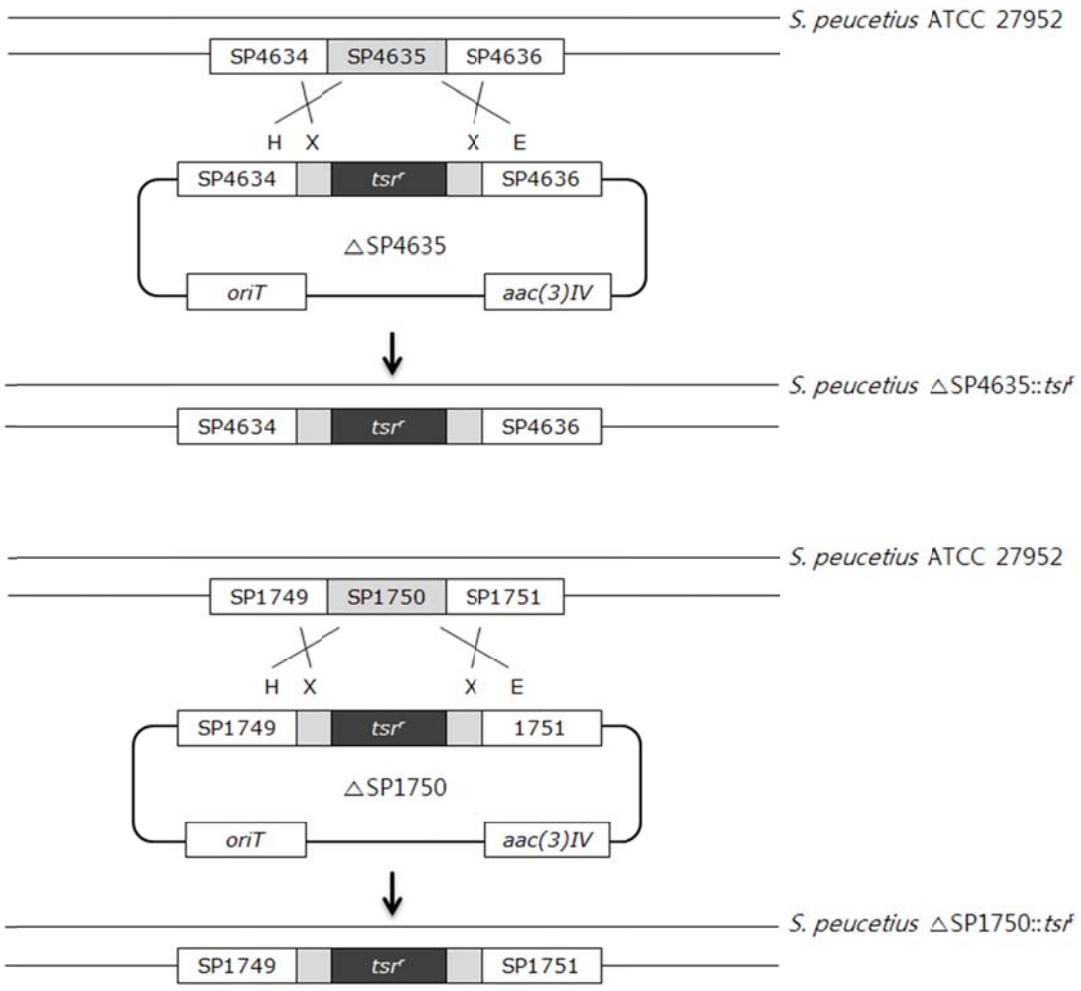

Figure 2. Scheme for gene disruption plasmid of pOJH0117 and pOMK1228. An internal DNA fragment of SP4635 upstream was inserted into the *Hin*dIII and *Xba*I sites of pKC1139 and that of SP4635 downstream was inserted into the *Xba*I and *Eco*RI of pKC1139. Thiostrepton resistance gene was inserted at the center of SP4635 upstream and SP4635 downstream, resulting in recombinant plasmid pOJH0117. pOMK1228 was also constructed similarly.

Construction of pOJH0117 and pOMK1228 for gene disruption

For disruption of the genes, SP4635 and SP1750, a total of 4 oligonucleotides were used, and PCR was performed as shown in materials and methods section. Finally, obtained PCR products such as SP4635U (1.3 kb), SP4635D (1.1 kb), SP1750U (1.7 kb), and SP1750D (1.6 kb) were cloned into a T-vector. All cloning was confirmed by restriction enzyme reaction and PCR sequencing (data not shown), then *E. coli* transformation was performed. After the PCR product was purified from the T-vector clone, it was again cloned into pKC1139 used as *E. coli-Streptomyces* shuttle vector. For the selection of final transformant, thiostrepton resistance gene (*tsr*^{r}) obtained from pIBR25 (Thuy et al., 2005) was inserted between upstream and downstream fragment as shown in Figure 2. Finally, pOJH0117 (*SP_nsdA* knock-out plasmid) and pOMK1228 (*SP_nsdB* knock-out plasmid) were constructed, and all these constructions were confirmed by PCR sequencing (data not shown).

Phenotype of *SP_nsdA* and *SP_nsdB* disruption transformants

After pOJH0117 and pOMK1228 were transformed into *S. peucetius*, we carried out plate assay to investigate the morphological differentiation between the wild-type and mutant strains (*S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750). Both mutants were different from the wild-type strain with respect to the growth rate and extent of mycelium. When cultured on an R2YE medium at 28°C, both the mutants began to grow aerial mycelium about 1 day earlier than the wild-type strain, and after 2 days, they also produced a yellow pigment, which is the color of

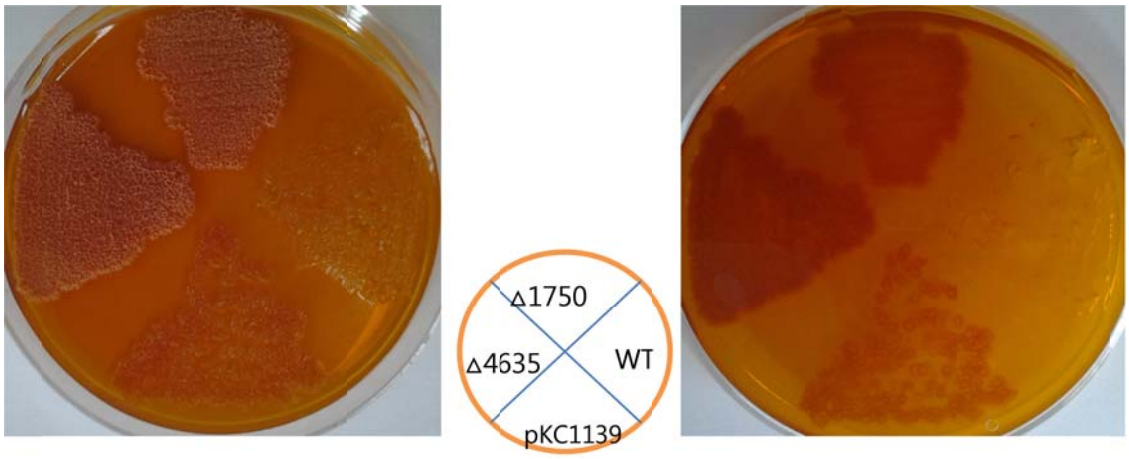

Figure 3. Morphological differenciation between wild-type and mutants strains. Incubation on R2YE agar plate (Apr^r, Tsr^r) for 3 days.

DXR. As expected, wild-type strain did not grow on this plate because it does not have resistance to apramycin and thiostrepton (Figure 3). On the other hand, the wild-type strain produced DXR upon longer incubation, but, both mutants rapidly produced more amount of DXR than the other mutant that only had a vector (Figure 4B). These results indicate that SP4635 and SP1750 play a negative role in morphological differentiation and production of DXR in *S. peucetius*.

Analysis of the enhanced DXR production

HPLC analyses were carried out to assess the quantitative change in DXR production. At first, we investigated the growth pattern of *S. peucetius.* For studying the growth rate of *S. peucetius*, it was grown on R2YE liquid medium at 28°C. After 3 days, the OD value of *S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750 dropped, while that of the wild-type strain and the mutant that only had a vector kept on increasing (data not shown). This result also indicated that SP4635 and SP1750 negatively regulate DXR production because DXR can inhibit cell growth due to its toxicity. In other words, *S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750 produced more amount of DXR and inhibited cell growth as described below. After this, we extracted DXR from the wild-type and mutants strains. We carried out HPLC analysis using extracted samples (Figure 4A). Standard DXR was detected at about 9.5 min and also desired peaks were detected at the same time from mutant and wild-type extracts. Both *S. peucetius* ΔSP4635 and *S. peucetius* Δ1750 mutants produced DXR about 2.07 and 1.74 times, respectively as compared to *S. peucetius* wild-type (Figure 4B). Using the complementary experiments, same wild-type level of DXR production was recovered from both mutants (data not shown). These results showed that SP4635 and SP1750 have a negative effect on DXR production in *S. peucetius*.

DISCUSSION

In this paper, *SP_nsdA* and *SP_nsdB* were identified in *S. peucetius*, and they shared a high degree of sequence identity with the well-known proteins, NsdA (SCO5582) and NsdB (SCO7252), which negatively control the secondary metabolite production and morphological differentiation in *S. coelicolor* (Li et al., 2006; Uguru et al., 2005; Zhang et al., 2007). Wang et al. (2009) also reported that disruption of *nsdA* increased milbemycin A4 and nanchangmycin about 1.5- and 9-fold in *S. binchengensis*. SP_NsdA/SP_NsdB belong to a protein family containing a domain of unknown function, DUF921, which has so far been found in *S. coelicolor* and *S. griseus* (Marchler-Bauer et al., 2003), and they also have a tetratricopeptide repeat (TPR)-like domain, which may mediate protein-protein interactions (D'Andrea and Regan, 2003). The TPR-like motifs are degenerate 34 amino acid sequences identified in common streptomyces, which are Gram-positive bacteria that can produce many secondary metabolites. However, they do not contain additional DNA binding domains, and it is speculated that they possibly contain undefined domains that play a role in DNA binding. Alternatively, they might also interact with certain transcriptional regulators to control production of secondary metabolites.

The disruption of *SP_nsdA* and *SP_nsdB* resulted in higher production of DXR in *S. peucetius*, suggesting their negative effect on DXR biosynthesis. In general, DXR production is limited because DNR, the precursor of DXR, is produced commercially by semi-synthesis rather than by purification from *S. peucetius*. Therefore, this study could be helpful for industrial production of DXR.

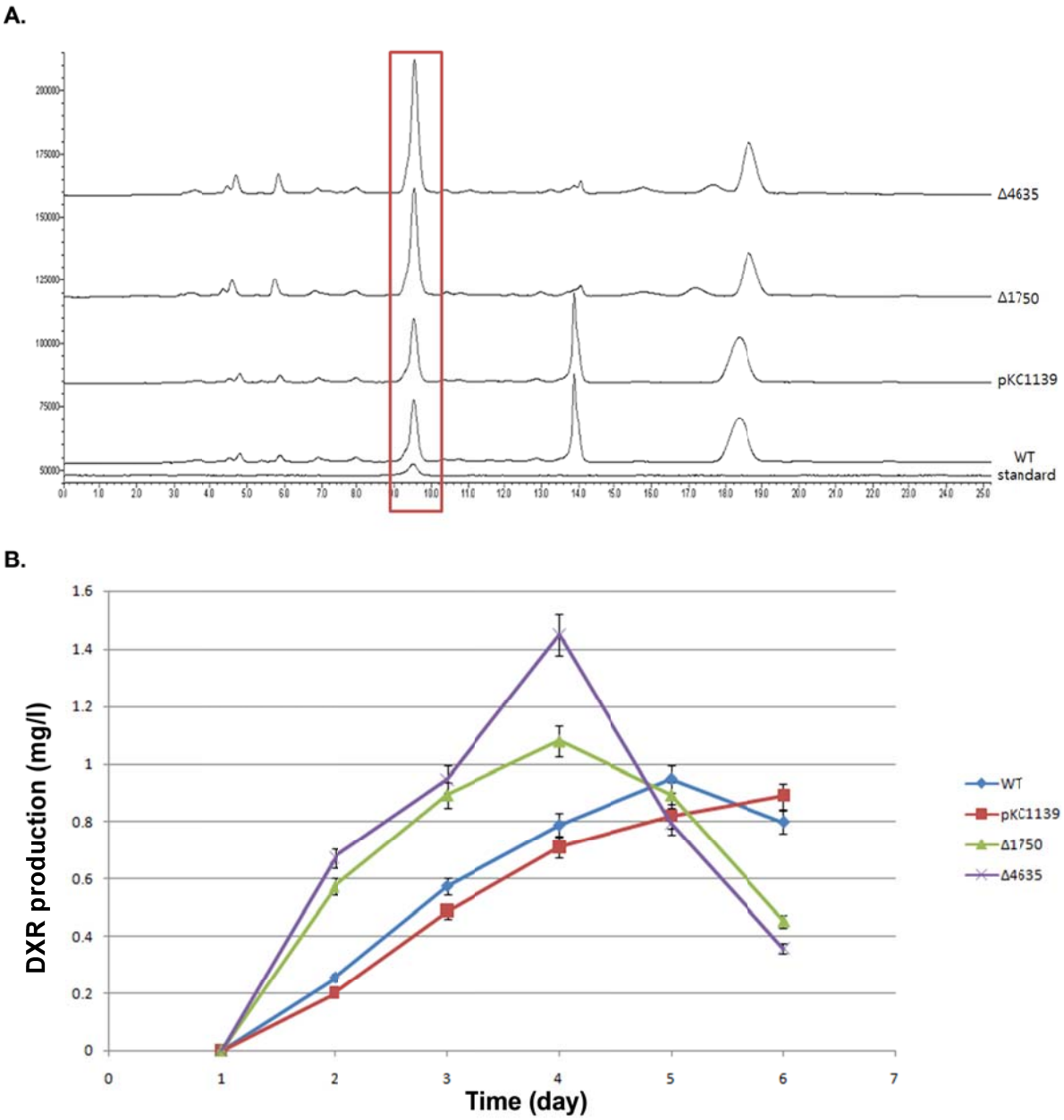

Figure 4. Comparison of DXR production. A. HPLC analysis of DXR production between the wild-type and mutants strains. B. Comparison of DXR production during 6 days. After 4 days, DXR yield from *S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750 was higher than that from wild-type and pKC1139-inserted mutant.

The fact that our group could secure the genome of *S. peucetius* indicates that new transcription factors, including pathway specific regulators and their corresponding factors for DXR biosynthesis, will be identified, which could be potential in enhancing the yield of DXR.

Conflict of Interest

The author(s) have not declared any conflict of interests.

ACKNOWLEDGMENT

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