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Effect of Antibiotic Down-Regulatory Gene *wblA* Ortholog on Antifungal Polyene Production in Rare Actinomycetes *Pseudonocardia autotrophica*

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The rare actinomycete *Pseudonocardia autotrophica* was previously shown to produce a solubility-improved toxicity-reduced novel polyene compound named Nystatin-like Pseudonocardia Polyene (NPP). The low productivity of NPP in *P. autotrophica* implies that its biosynthetic pathway is tightly regulated. In this study, *wblA*$_{pau}$ was isolated and identified as a novel negative regulatory gene for NPP production in *P. autotrophica*, which showed approximately 49% amino acid identity with a global antibiotic down-regulatory gene, *wblA*, identified from various *Streptomycetes* species. Although no significant difference in NPP production was observed between *P. autotrophica* harboring empty vector and the *S. coelicolor wblA* under its native promoter, approximately 12% less NPP was produced in *P. autotrophica* expressing the *wblA* gene under the strong constitutive *ermE** promoter. Furthermore, disruption of the *wblA*$_{pau}$ gene from *P. autotrophica* resulted in an approximately 80% increase in NPP productivity. These results strongly suggest that identification and inactivation of the global antibiotic down-regulatory gene *wblA* ortholog are a critical strategy for improving secondary metabolite overproduction in not only *Streptomyces* but also non-*Streptomyces* rare actinomycete species.

Keywords: *wblA*, antifungal polyene macrolide, rare actinomycetes, *Pseudonocardia autotrophica*

Introduction

Polyene macrolides, a large family of polyketides with antifungal activities, are structurally characterized by polyhydroxylated macrocyclic lactones composed of 20–40 carbons with three to eight conjugated double bonds [3, 15]. These compounds include antibiotics such as nystatin A1, amphotericins A and B, pimaricin, candicidin/FR-008, and CE-108/rimocidin [9]. Despite the recent introduction of new antifungal drugs such as next-generation azoles and echinocandins, polyene macrolides continue to be the most potent broad-spectrum antifungal agents available for clinical use, which mostly covers life-threatening fungal infections, particularly in patients who have undergone organ transplantation, received aggressive chemotherapy, or have AIDS [13]. The disadvantages of polyene macrolides are their relatively high toxicity, especially toward kidney cells, and poor distributions in tissues owing to low water solubility. In order to improve the therapeutic efficacy and reduce the toxicity of polyenes, several strategies, including combination therapy, structural modifications, and altering the physical state of the therapeutic agent in the drug delivery system, have been employed [2, 12, 14].

Using a polyene cytochrome P450 hydroxylase-specific genome screening strategy, the gram-positive rare actinomycete *Pseudonocardia autotrophica* KCTC9441 was previously determined to contain genes related to polyene biosynthesis. This *P. autotrophica* gene cluster specifies the biosynthesis of nystatin-like *Pseudonocardia* polyene (NPP) [8]. The complete NPP structure includes an aglycone identical to nystatin with a unique di-sugar moiety, mycosamine (α1-4)-*N*-acetyl-glucosamine. Remarkably, when disaccharide-containing NPP was compared with mycosamine-containing nystatin, the former exhibited approximately 300-fold higher water solubility as well as 10-fold reduced hemolytic activity while retaining about 50% of its antifungal activity. These results suggest that NPP is a promising antifungal agent with improved cytotoxicity and water solubility [10]. Unfortunately,

the NPP biosynthetic pathway is likely to be tightly regulated based on the very low NPP productivity observed under most *P. autotrophica* culture conditions [6].

A *whiB*-like putative transcription factor gene named *wblA* was originally proposed as a global negative regulatory gene involved in polyketide biosynthesis in *S. coelicolor* [7, 11]. Overexpression of *wblA* inhibited actinorhodin biosynthesis in *S. coelicolor*, and the transcript encoded by an actinorhodin-specific activator gene was reduced in *wblA*-overexpressing *S. coelicolor*, suggesting that *wblA* is a broadly functioning down-regulatory gene for polyketide biosynthesis in *Streptomyces* species [7, 11]. Subsequently, several *wblA* orthologs showing more than 90% amino acid identities, such as $wblA_{spe}$ in *S. peucetius* and $wblA_{tmc}$ in *Streptomyces* sp. CK4412, were also proved to down-regulate the production of doxorubicin and tautomycetin, respectively. Here, we report that there is a less homologous *wblA* ortholog present in the rare actinomycete *P. autotrophica*, and its deletion also stimulated the intrinsically low level of NPP production. This is a first report on the identification and application of a *wblA* ortholog in a non-*Streptomyces* rare actinomycete strain.

Materials and Methods

Bacterial Strains, Plasmids, and Media

The rare actinomycete *Pseudonocardia autotrophica* KCTC9441, purchased from the Korean Collection for Type Cultures, was used as an NPP-producing strain. The *P. autotrophica* strains were grown in ISP medium 2 (glucose 0.4%, yeast extract 0.4%, malt extract 1%, and agar 2%) at 30°C for sporulation and YEME liquid medium for NPP production [5, 8]. *E. coli* DH5α and the *Streptomyces* integrative plasmid *ermE**pSET152 were used for the cloning and expression of target genes [5]. Methylation-deficient *E. coli* ET12567/pUZ8002 was used as a host for intergeneric *E. coli*-*Streptomyces* conjugations [1]. All *E. coli* strains were cultured at 37°C in Luria broth or on Luria agar and supplemented with the appropriate antibiotics when needed [5].

Identification of *wblA* Ortholog from *P. autotrophica* Genomic Library

The *P. autotrophica* whole genome, with a total size of approximately 10 Mbp and containing more than 10,000 ORFs, was analyzed. *In silico* analysis of amino acid sequences was performed by using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A cosmid library was prepared using the commercially available Supercos-1 cosmid system (Stratagene, USA). The cosmid library was screened by polymerase chain reaction (PCR) using forward primer 5′- GGATCCCGATGACAG CGCCGACCG -3′ and reverse primer 5′- TCTAGAAGTAGCGAC GACCGGAGC-3′. PCR was performed in a final volume of 20 µl containing 0.4 µM of each primer as well as 0.25 mM of each of the four dNTPs (Roche, Switzerland), 1 µl of extracted DNA, 1 U of Ex Taq polymerase (TaKaRa, Japan) within its recommended reaction buffer, and 10% dimethyl sulfoxide (DMSO). Amplifications were performed in a Thermal Cycler (BioRad, USA) according to the following profile: 30 cycles of 60 sec at 95°C, 30 sec at 62°C, and 40 sec at 75°C. Amplified products were analyzed by electrophoresis in 1% (w/v) agarose gels and verified by sequencing using a T7 promoter primer/T3 primer in a pSupercos-1 vector.

Inactivation of *wblA* Ortholog in *P. autotrophica*

The *wblA* ortholog ($wblA_{pau}$) identified from *P. autotrophica* was inactivated using a PCR-targeted gene disruption system [4]. An apramycin resistance gene/*ori T* cassette for replacement of $wblA_{pau}$ was amplified using the following primers: disF 5′-ACAAGAACCCCGAGCTCTTCTTCCCCGTCGGAAGCGACGATT CCGGGGATCCGTCGACC-3′ and disR 5′- AGGGCGTAGGCC AGACAGTGCTCTCGGACCGGGCAGCGATGTAGGCTGGAGCTG CTTC-3′. This cassette was introduced into *E. coli* BW25113/pIJ790 containing the $wblA_{pau}$ gene, which also contained the *P. autotrophica* cosmid. Gene replacement in $wblA_{pau}$ was confirmed by PCR analysis of the mutated ($\Delta wblA_{pau}$) cosmid. The mutated ($\Delta wblA_{pau}$) cosmid was introduced into *P. autotrophica* by conjugation from *E. coli* ET12567/pUZ8002. After incubation at 30°C for 16 h, each plate was flooded with 1 ml of sterile water containing apramycin at a final concentration of 50 mg/ml and nalidixic acid at a final concentration of 25 mg/ml. Incubation continued at 30°C until conjugants appeared. The double-crossover recombinants were first selected with apramycin resistance (apr^R) and kanamycin sensitivity (kan^S), followed by PCR confirmation.

Cloning and Functional Expression of *S. coelicolor wblA* (SCO3579)

A 603 bp PCR-amplified *wblA* (SCO3579) included the putative promoter from *S. coelicolor*, a ribosome-binding site, start codon, and stop codon sequences. Otherwise, a 398 bp fragment encompassing SCO3579 with a ribosome-binding site, except the promoter, was amplified. The PCR product was cloned into the RBC T&A cloning vector, analyzed by sequencing, and ligated in the *Streptomyces* integrative plasmid pSET152, yielding plasmid p3579 (containing *S. coelicolor* promoter) and pE3579 (containing *ermE** promoter).

High-Performance Liquid Chromatography (HPLC) Assay for NPP

The *P. autotrophica* culture was extracted with an equal volume of butanol, followed by concentration and dissolving in methanol. For HPLC analysis of these extracts, a Shimadzu SPD M10A (Shimadzu, Japan) with a reversed-phase C-18 column (5 µm particles, 4.6 × 150 mm; Agilent) was used. The column was equilibrated with 50% solvent A (0.05 M ammonium acetate, pH 6.5) and 50% solvent B (methanol), followed by development using the following gradient:50% B (0 min), 75% B (3 min), 100% B (30 min), 50% B (33 min), and 50% B (40 min) at a flow rate of 1.0 ml/min. UV/Vis detection followed at 305 nm.

Results

Identification of *wblA* ortholog *wblA$_{pau}$* in *P. autotrophica*

Since the whole genomic information of *P. autotrophica* was analyzed (unpublished data), the putative *wblA* ortholog could be identified through a BLASTp search using the amino acid sequence of WblA (SCO3579) from *S. coelicolor* with the GenoTech Prokaryotic Genome Automatic Annotation System. Using database-assisted *in silico* analysis, *P. autotrophica* genomic DNA was shown to contain the *wblA* ortholog gene, named *wblA$_{pau}$*. *S. coelicolor* WblA is believed to be functionally responsible as a WhiB family transcriptional regulator and contains highly conserved regions (α-helix and Cys-X-X-Cys motif) found in all *wblA* ortholog genes from *Streptomyces* species. Unlike previously reported *Stretpomyces wblA* orthologs showing more than 90% amino acid identities, the 82-amino-acid-containing *wblA$_{pau}$* showed a relatively low degree of amino acid identity (approximately 49%) with the translated products of *wblA* genes from *S. coelicolor*, *S. avermitilis* MA-4680, *S. griseus* NBRC 13350, *Streptomyces* CK4412, and *S. peucetius* ATCC27952, as well as 39% amino acid identity with the *wblA* gene from *Corynebacterium glutamicum* ATCC13032 (Fig. 1 and Table 1).

Functional Expression of *wblA* (SCO3579) in Rare Actinomycete *P. autotrophica*

To demonstrate that *S. coelicolor wblA* (SCO3579) is responsible for regulation of NPP biosynthesis, this gene was cloned from its promoter to the stop codon, using the *Streptomyces* integrative plasmid pSET152, after which it was introduced into the NPP-producing strain *P. autotrophica*. The level of NPP production was not significantly different compared with that of *P. autotrophica* harboring empty vector alone (Fig. 2). To ascertain whether additional expression of *wblA* (SCO3579) is not responsible for NPP production or whether the *S. coelicolor* promoter is not working in *P. autotrophica*, *wblA* (SCO3579) was expressed under the strong constitutive *ermE** promoter. Comparison of NPP production between wild type and *wblA* (SCO3579)-containing exconjugants revealed that expression of *wblA*

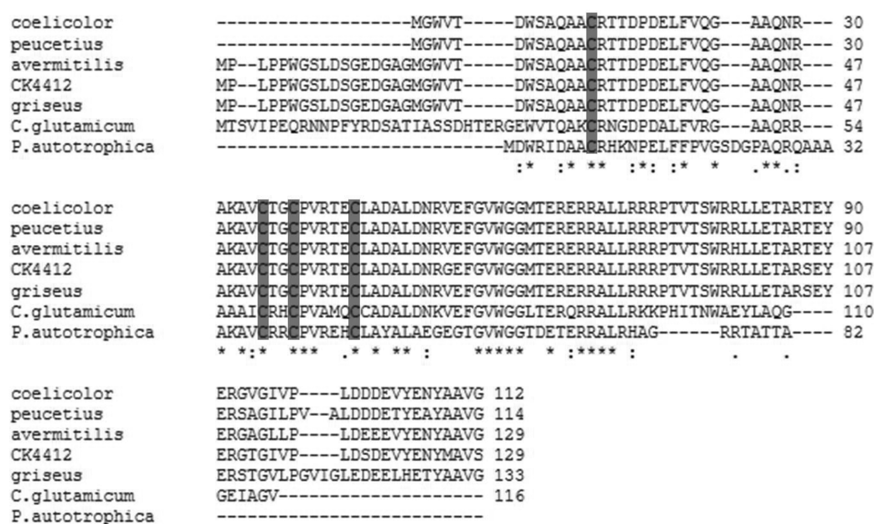

Fig. 1. Amino acid sequence alignments between *wblA* ortholog (*wblA$_{pau}$*) from *P. autotrophica* and various *Streptomyces* WblAs.
Conserved (asterisk) and homologous (colon) amino acids are marked. Cysteine conserved regions (gray highlight) were identified in the WblAs.

Table 1. List of putative WhiB-family transcriptional regulator in various strains.

Genes	Strains	Identities	Similarity	E-value
Putative WhiB-family transcriptional regulator (SCO3579)	*Streptomyces coelicolor* A3(2)	49%	54%	4e-14
Putative WhiB-family transcriptional regulator (WblA$_{spe}$)	*Streptomyces peucetius* ATCC27952	49%	54%	3e-14
Putative WhiB-family transcriptional regulator (SAV4584)	*Streptomyces avermitilis* MA-4680	49%	54%	9e-14
Putative WhiB-family transcriptional regulator (WblA$_{tmc}$)	*Streptomyces* sp. CK4412	49%	54%	7e-14
Putative WhiB-family transcriptional regulator (SGR3340)	*Streptomyces griseus* NBRC13350	49%	54%	7e-14
Putative WhiB-family transcriptional regulator (WhcA)	*Corynebacterium glutamicum* ATCC13032	39%	47%	1e-12

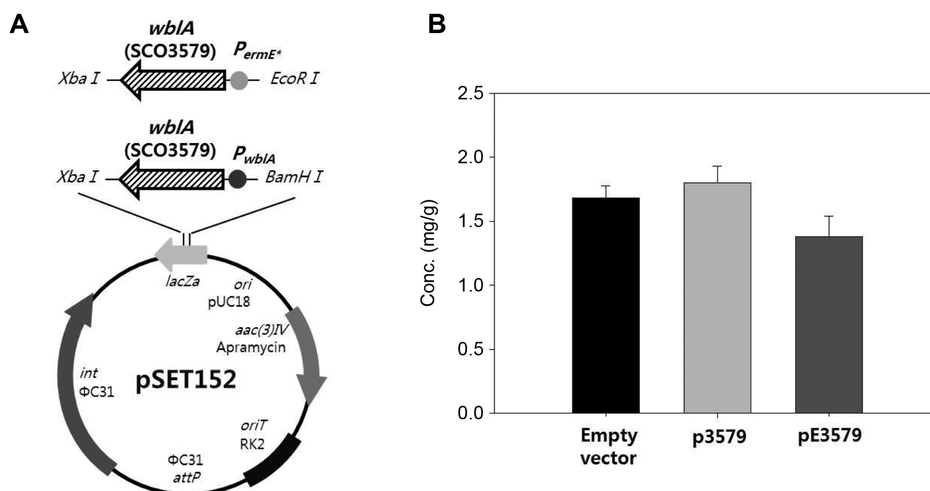

Fig. 2. Functional expression of the *wblA* (SCO3579) gene in *P. autotrophica*.

(**A**) Plasmid map of the *Streptomyces* integrative vector for expression of *wblA*. (**B**) NPP production yield (average of triplicate) in *P. autotrophica* harboring the empty vector *ermE**pSET152 (black); p3579, expressing *wblA* (SCO3579) under *S. coelicolor* promoter in *P. autotrophica* (light gray); pE3579, expressing *wblA* (SCO3579) under strong constitutive *ermE** promoter in *P. autotrophica* (dark gray) was analyzed by quantitative HPLC assay.

(SCO3579) reduced NPP production by approximately 12% in *P. autotrophica* (Fig. 2).

Stimulated NPP Production *via* $wblA_{pau}$ Disruption from *P. autotrophica*

The *wblA* ortholog gene from *P. autotrophica* ($wblA_{pau}$) was inactivated using a PCR-targeted gene disruption system. Construction of the $wblA_{pau}$ mutant (named *P. autotrophica* $\Delta wblA_{pau}$) was confirmed by PCR analysis. The PCR-amplified 0.3 kb band was observed in genomic DNA isolated from *P. autotrophica*, whereas a band of expected size (1.6 kb) was detected in *P. autotrophica* $\Delta wblA_{pau}$. A 1.8 kb fragment was observed in the cosmid used for the PCR-targeted $wblA_{pau}$ gene disruption, whereas no PCR-amplified fragments were detected in *P. autotrophica* wild-type or $\Delta wblA_{pau}$ strain (Figs. 3A and 3B). This result suggests that the $wblA_{pau}$ gene from *P. autotrophica* was inactivated by the apramycin resistance gene/*oriT* cassette. No notable visual phenotypic difference was observed between *P. autotrophica* and *P. autotrophica* $\Delta wblA_{pau}$. However, approximately 180% greater NPP production was observed from *P. autotrophica* $\Delta wblA_{pau}$ compared with *P. autotrophica* wild type over the same 3-day period in YEME production culture media (Fig. 3C).

Discussion

Natural products continue to play a highly significant role in drug discovery and development. Among them, polyene macrolides are the most potent broad-spectrum antifungals available for clinical use. Previously, the rare actinomycete *P. autotrophica* KCTC9441 was determined to contain genes related to polyene biosynthesis, such as Nystatin-like *Pseudonocardia* Polyene (NPP). However, the low level of NPP production implies that this metabolic pathway is tightly controlled by regulatory genes, such as the global down-regulatory gene *wblA*. Whole genome sequencing and construction of a total genomic DNA library of *P. autotrophica* enabled the successful isolation and inactivation of a *wblA* ortholog gene, $wblA_{pau}$, from *P. autotrophica*. The $wblA_{pau}$ gene from *P. autotrophica*, usually considered as a non-*Streptomyces* rare actinomycete strain, showed a much less degree of amino acid identity with *wblA* genes from various *Streptomyces* species, implying that the biological significance of $wblA_{pau}$ in *P. autotrophica* might be similar to a previously known *wblA*, a global antibiotic down-regulatory gene in *Streptomyces*. About 80% greater NPP production was observed in *P. autotrophica* $\Delta wblA_{pau}$ compared with *P. autotrophica* wild type, and approximately 12% less NPP production was identified upon expression of *wblA* (SCO3579) under the strong constitutive *ermE** promoter. These results suggest that $wblA_{pau}$ could be a negative regulatory gene for NPP production in *P. autotrophica*. Interestingly, there was no significant difference in NPP production between *P. autotrophica* with empty vector and *P. autotrophica* expressing *wblA*

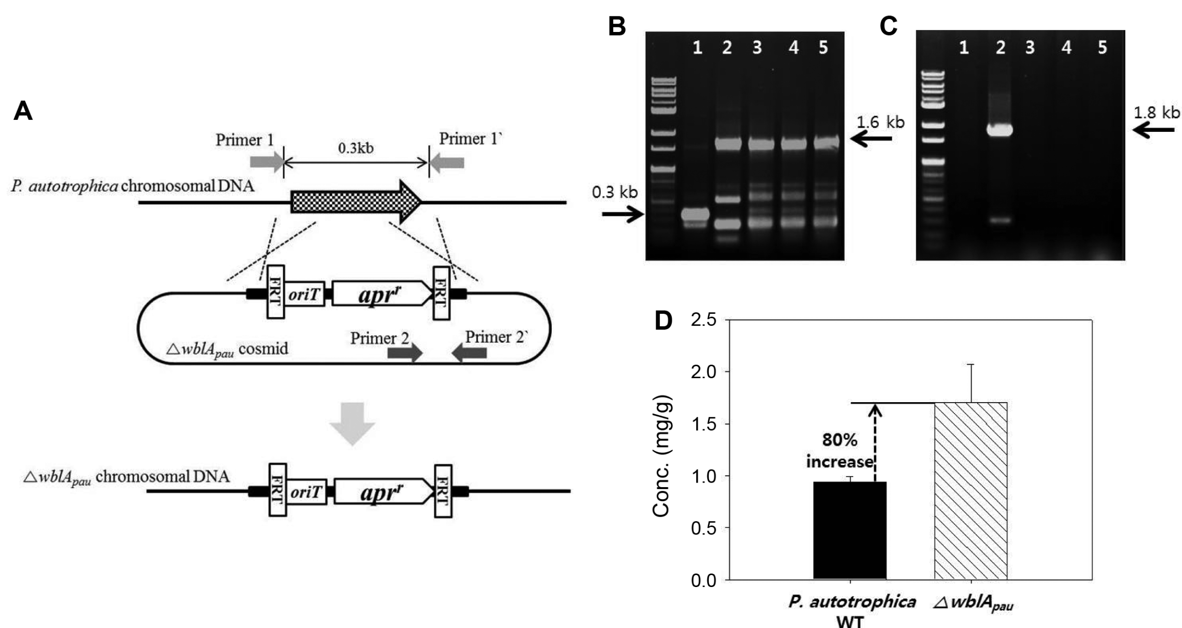

Fig. 3. *wblA*$_{pau}$ gene inactivation using a PCR-targeted gene disruption method in *P. autotrophica*.
(**A**) A schematic representation of the PCR-targeted gene replacement of *wblA*$_{pau}$. (**B**) Gene replacement was confirmed by PCR using the primer pair #1 and 1′. (**B**) The absence of cosmid in *P. autotrophica* total chromosomal DNA was confirmed by PCR using the primer pair #2 and 2′; lane 1, *P. autotrophica* genomic DNA; lane 2, cosmid containing *wblA*$_{pau}$ replaced with apramycin resistance gene/*oriT*; lanes 3~5, *P. autotrophica wblA*$_{pau}$ disruption mutant strains genomic DNA. (**D**) *P. autotrophica* wild type and *P. autotrophica wblA*$_{pau}$ cultured in YEME medium for 3 days. The averages of triplicates are shown with error bars.

(SCO3579) under the *S. coelicolor* promoter. It is not clear whether or not the *S. coelicolor* promoter operates in *P. autotrophica* or NPP biosynthesis is already tightly regulated in *P. autotrophica*. Previously, we identified six putative NPP pathway-specific regulatory genes to be present in its biosynthetic gene cluster. Although we could not check the transcriptome profile in the *wblA* over-expression strain, we expected *wblA* overexpression regulated NPP pathway-specific regulatory genes. As a result, low NPP production was revealed upon expression of *wblA* under the strong constitutive *ermE** promoter. In conclusion, the identification and inactivation of the global antibiotic down-regulatory gene *wblA* ortholog should be a critical strategy for improving secondary metabolite overproduction in not only *Streptomyces* but also in non-*Streptomyces* rare actinomycete species.

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