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Production improvement of antifungal, antitrypanosomal nucleoside sinefungin by *rpoB* mutation and optimization of resting cell system of *Streptomyces incarnatus* NRRL 8089

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Sinefungin, a nucleoside antibiotic with potent antifungal, antiviral, and anti-trypanosome activities, has been a target for production enhancement in the past decades through medium optimization and strain improvement. For the purpose of introducing a more rational approach, we induced *rpoB* mutation in the producer strain, *Streptomyces incarnatus* NRRL 8089, by optimized UV-irradiation, and a resulting rifampicin-resistant strain rif-400 increased the sinefungin production by 7-fold. The growth and melanin production were obviously accelerated in the rifampicin-resistant high-producer mutant, while the morphological differentiation such as aerial mycelia and spiked-spore formation was retained. Molecular cloning and DNA sequencing identified a single mutation A1340G in the *rpoB* gene, which encodes the β-subunit of RNA polymerase, and the resulting amino acid substitution Asp447Gly corresponded to one of mutations that reportedly allowed the transcriptional up-regulation of actinorhodin production in *S. coelicolor* A3(2). Sinefungin production was further enhanced by resting cell system using the *rpoB* mutant strain in the presence of 10 mM L-Arg. D-Arg or L-ornithine did not enhance the sinefungin production, and > 50 mM urea strongly suppressed the nucleoside antibiotic production, supporting the proposed biosynthetic mechanism by which urea is liberated from the guanidino-group-bearing intermediate that is produced by enzymatic condensation of L-Arg and ATP.

[**Key words:** *Streptomyces incarnatus*; Sinefungin; *rpoB* gene; Secondary metabolism; Rifampicin-resistance]

Sinefungin is a nucleoside antibiotic, in which a molecule of L-ornithine is linked to the 5′-end of adenosine through a C–C bond (Fig. 1). The antibiotic was independently isolated at Eli Lilly Research Laboratories from the fermentation broth of *Streptomyces griseolus* (1) and at Rhone-Poulenc Laboratories from the culture broth of *Streptomyces incarnatus* NRRL 8089 (Florent, Y., Lunel, Y., and Mancy. D.: Nouvelle substance antifonginique, sa preparation et les compositions qui la contiennet, Fr. 7611141, Apr. 15, 1976). This nucleoside antibiotic exhibits antifungal (2) and antiviral activity (3) as well as potent activity against a number of protozoal parasites including malaria and trypanosome (4–7). The structure of sinefungin is highly analogous to *S*-adenosyl-L-methionine (SAM), and sinefungin inhibits SAM-dependent methyltransferases in a competitive mode *in vitro* (8–10). Several studies suggested that the potent antiviral and antifungal activities are most likely due to the selective inhibition of pathogen-encoded mRNA cap-methylating enzymes, especially RNA (guanine-N7) methyltransferase, which adds a methyl group to Gppp-RNA to form the m7GpppRNA cap (11–13). This capping is necessary for the translation initiation and also for protecting RNA molecules from degradation by 5′-exoribonucleases (12, 14).

While the mode of action has been studied in detail, the biosynthetic mechanism of this unique nucleoside compound remained to be elucidated for over decades. Early attempts to identify a biosynthetic precursor suggested that L-ornithine is the direct precursor substrate for the biosynthesis of sinefungin in *S. griseolus* (15, 16). However, subsequent studies revealed that ^{14}C-labeled sinefungin was more efficiently produced from ^{14}C-labeled L-arginine by cell-free extract of a high-producer variant of *S. incarnatus* (17) and that of *S. griseolus* (18). Enzymes involved in sinefungin biosynthesis have not been characterized any further due to the low expression and/or instability of the biosynthetic enzymes.

The first attempt to improve the sinefungin production was carried out by Malina et al., who reported that the original sinefungin production 40 μg/ml by the wild-type strain was increased to 126 μg/ml through medium improvement, and it was further improved to the high yield of 664 μg/ml through mutagenesis and protoplast regeneration (19). This remarkable outcome, however, was based on the estimation of sinefungin production by agar diffusion paper disk method, and no chromatographic measurement was used for sinefungin assay. When the HPLC system was introduced in their following

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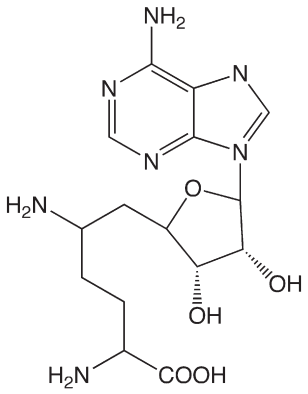

FIG. 1. Structure of sinefungin.

study, the sinefungin production by the high-producer strain was reported to be 30 μg/ml (17). Although bioassay is a convenient method to detect a small amount of antibiotic produced in the culture broth, it has an inherent risk of poor reproducibility and low specificity.

Recently, there have been an increasing number of reports, which describe that certain mutations in *rpoB* gene, encoding RNA polymerase β-subunit protein, or *rpsL* gene encoding ribosomal S12 protein, effectively enhanced secondary metabolite production in gram-positive bacteria. These phenomena have been applied to various secondary metabolites such as polyketide antibiotics in *S. coelicolor* and *S. lividans* (20–22), tripyrrole antibiotic and lipopeptide in *S. lividans* (23), and amino sugar antibiotics in *Bacillus subtilis* (24). While the antibiotic-resistance guided mutation has been applied to various class of secondary metabolism, there has been no application of *rpoB* mutation to increase any nucleoside antibiotic production; only one previous study reports the effect of the mutation of *rpsL* gene on the nucleoside antibiotics fosmycin (25).

Nucleoside antibiotics constitute an important group of secondary metabolites with high selective toxicity to eukaryotic pathogens. Given the key roles played by nucleosides and nucleotides in biochemical processes, they should have high potential in fundamental application as specific inhibitors of a high variety of biochemical reactions along with anti-viral and anti-protozoa activity. Nucleoside antibiotics are at present definitely worthwhile for evaluation for viral diseases including human immunodeficiency and trypanosomal diseases such as malaria (26). Accordingly, demands are growing for the development of efficient approaches to improve the production of nucleoside antibiotics.

In the present study, we investigated whether *rpoB* mutation could also improve the sinefungin production by the wild-type *S. incarnatus*, which produced only a small amount of sinefungin (2 μg/ml) by our HPLC analysis. Remarkably, sinefungin production was increased approximately 7-fold by a single round of mutation conferring rifampicin-resistance. DNA sequence of the *rpoB* gene identified a point mutation which corresponded to one of *rpoB* mutations previously reported for *S. lividans* 66 (23). The enhanced sinefungin production by *rpoB* mutation allowed us to study the effects of precursor supplementation using the resting cells of *S. incarnatus* rif-400. Addition of 10 mM L-Arg stimulated the sinefungin production by 5-fold, but D-Arg and L-ornithine did not increase the production, supporting the hypothesis that L-Arg but not L-ornithine is the direct precursor for the ornithine moiety of sinefungin.

MATERIALS AND METHODS

Bacterial strain and culture conditions *S. incarnatus* NRRL 8089 was obtained from the Agricultural Research Service, United States Department of Agriculture. Medium F contained 1% (w/v) glucose, 0.5% glycerol, 1% corn steep liquor (CSL), 1% soybean flour, 0.5% yeast extract, 0.5% NaCl, and 0.2% $CaCO_3$. Medium CM contained 1% glucose, 0.5% glycerol, 0.3% CSL, 0.3% meat extract, 0.3% malt extract, 0.3% yeast extract, 0.2% $CaCO_3$, and 0.001% thiamine. Medium H contained 1% glucose, 0.5% glycerol, 1% CSL, 1% soybean flour, 0.5% yeast extract, 0.5% NaCl, and 0.2% $CaCO_3$. Medium S contained 1% glucose, 0.5% glycerol, 0.4% yeast extract, and 0.2% $CaCO_3$, pH 7. Potato-Dextrose broth was from DIFCO. GYM33 medium contained 1% D-glucose, 1% yeast extract, 1% malt extract, 3% starch, and 3% soybean powder. Yeast-Nitrogen base plate for sinefungin bioassay contained 6.7% (w/v) yeast nitrogen base (DIFCO), 5% D-glucose, and 1.5% agar.

Mutation by ultraviolet light irradiation Mycelia of wild-type *S. incarnatus* grown overnight in 5 ml of tryptic soy broth (TSB) medium (Becton-Dickinson) was spread on the agar plates, and the plates were irradiated with UV light (Toshiba GL15 lamp: 3.3×10^{-4} J/cm^2/s) for 0, 30, 50, 70, and 90 s. The plates were incubated at 30 °C in the dark for 3 days. Growing colonies were transferred to a pair of agar-plates of minimum media with or without 0.1% (w/v) L-arginine. The survival rate and the L-Arg auxotroph mutants were plotted as a function of irradiation time. The rifampicin-resistant strain was screened on TSB plate medium that contained 0.4 mg/ml of rifampicin.

DNA sequencing of the *rpoB* gene The *rpoB* gene fragment (2989 bp) was amplified from the genome DNA of *S. incarnatus* and the rif-400 mutant by PCR using DNA polymerase KOD FX (TOYOBO) and oligonucleotide primers rpo-F (5′-ATCAA GTCCC AGACG GTCTT CAT-3′) and rpo-R (5′-CTCAG GGTTG TAAGT CTGTG GTCTG AAAC-3′), which were designed on the sequences of *rpoB* genes from *S. coelicolor* A3(2) (GenBank Accession No. AL160431) and *S. avermitilis* (GenBank Accession No. BA000030). The PCR products from wild-type and rif-400 strains were sequenced in the forward and reverse directions using the oligonucleotide primers designed from the sequencing results. The coding region between *rplL* and *rpoB* genes was amplified by PCR with the set of primers rpl-F (5′-AAGAA GATCC AGGTC AAGGTC-3′) and pr-15 (5′-ACACC GACCA TGTCG CGCTT-3′), which were designed by the sequence conserved in *S. coelicolor* A3(2) and *S. avermitili*. DYEnamic ET terminator cycle sequencing kit (Amersham) and the capillary DNA sequencer model 310 (Applied Biosystems, Foster City, CA) were used.

Production of sinefungin by resting-cell system The resting cell systems were prepared with the mycelia of wild-type strain grown in the S-medium for 7 days, or that of rif-400 strain grown for 1 day or 2 days in the S-medium. The mycelia were harvested and washed twice with 50 mM potassium phosphate buffer at pH 7, and resuspended in 50 ml of 50 mM potassium phosphate solution that was adjusted at pH 6. Supplemental substances such as L-Arg, L-ornithine, and urea were added to the cell suspension when the effects of supplemented compounds were to be tested. Then, the cell suspensions were incubated at 30 °C under aeration by 200 rpm using a reciprocal shaker INOVA4230 (New Brunswick Scientific, USA). The cells were completely removed by centrifugation and the supernatant solution was loaded on Dowex 50W X8 cation exchange column (Φ10×30 mm). The solution eluted by 0.1% ammonium hydroxide was concentrated and lyophilized to dryness, and the components were dissolved in 50 μl of sterile water for bioassay and analytical HPLC for the determination of sinefungin.

Determination of sinefungin by bioassay and analytical HPLC Sinefungin was conveniently determined by bioassay using *Candida albicans* as the test organism. Eluate from the cation exchange column was neutralized before adsorbed on 8Φ mm paper disk (Advantec, Tokyo). The disk was dried in the stream of air in a clean bench, and put on the Yeast-Nitrogen base agar plate, on which cells of *C. albicans* are dispersed in invisible amount. The plate is then incubated overnight at 30 °C. The diameter of inhibitory zone X (mm) designated in $\log_{10}$X was linear with respect to the amount of sinefungin in a range of 1 to 60 μg loaded on the paper disk. Sinefungin production was also determined by analytical HPLC using a CAPCELL PAK SCX column (4.6 mm I.D.×150 mm, Shiseido Fine Chemicals), TOSOH liquid chromatography pump, and a Hitachi photodiode array detector. The mobile phase was 0.5 M ammonium formate, pH4.5, at a flow rate of 0.5 ml/min. The chromatogram was also monitored by absorption at 260 nm for sinefungin determination.

Urea assay The urea formation in the culture broth was monitored by urease-glutamate dehydrogenase-coupled subtraction assay (27) with modification necessary for the photometric assay. The culture broth was centrifuged to remove the mycelia, and the supernatant solution was diluted 10-fold by 50 mM Tris–HCl buffer, pH 8. The assay mixture is made up by mixing 100 μl of 8.5 mM NADH, 100 μl of 25 mM α-ketoglutarate, 100 μl of urease solution 100 U in the Tris buffer, pH 8, 100 μl of 250 U/ml L-glutamate dehydrogenase (GLDH), and 2.5 ml of 50 mM Tris–HCl buffer, pH 8. The decrease of absorption at 340 nm was linear with respect to time between 100 and 200 s upon the initiation of the coupled enzyme reaction, and the slope was calibrated with the standard urea solution. Since the culture broth contains background ammonia, a control experiment without urease was carried out to subtract the value from that of the complete assay solution. This urease-GLDH-coupled subtraction assay allowed urea determination in the range from 0.125 to 4.0 mM. Urea was assayed by the average of duplicate determination.

RESULTS

Medium selection for sinefungin production A wild-type strain, *S. incarnatus* NRRL 8089, was grown for 7 days at 30 °C in