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Improvement of Sinefungin-Producing Strain of *Streptomyces incarnatus* by Conferring Rifampicin-Resistance through Ultraviolet Light Irradiation and Protoplast Regeneration

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Secondary metabolite production by gram-positive bacteria is strictly regulated at the transcription of the biosynthetic genes to mRNA in response to certain stringent conditions. Therefore, some mutational disruption of regulatory domains of the bacterial RNA polymerase might increase the production of the antibiotics. In this study, we have attempted to improve the sinefungin-producing strain of *Streptomyces incarnatus* NRRL 8057 by irradiating ultraviolet light on the protoplast, and selecting mutants that acquired the resistance to rifampicin, the antibiotic which specifically binds to the β-subunit of bacterial RNA polymerase. After three rounds of mutation, 10 strains were obtained with varied resistance to rifampicin. A mutant which showed the highest resistance was found to have the highest sinefungin production, which was 2.4 times higher (0.45 ± 0.11 μg/ml) than the wild type strain (0.19 ± 0.07 μg/ml). The breeding approach by rifampicin-resistance may be advantageous over the classical random screening since it requires much smaller number of candidates to be examined.

Key words : Sinefungin, Protoplast regeneration, Rifampicin, RNA polymerase, *Streptomyces incarnatus*

Introduction

Sinefungin is a nucleoside antibiotic, in which a molecule of ornithine is linked to the 5'-end of adenosine through a C-C bond (Fig. 1). The nucleoside antibiotic was isolated at Rhone-Poulenc Laboratories from the culture broth of *Streptomyces incarnatus* NRRL 8089[1] and at Eli Lilly Research Laboratories from the fermentation broth of *Streptomyces griseolus*[2]. The antibiotic has a strong inhibitory effect on various fungi[3] and parasites[4–7], but is considered as nephrotoxic *in vivo*. It has been reported that the cell-free extract prepared from a high producing variant of *S. incarnatus* produced sinefungin from L-arginine and ATP in the presence of pyridoxal-5'-phosphate, dithiothreitol and magnesium ion[8]. The enzymes that are involved in sinefungin production have not been characterized due to the instability and low expression of the enzymes. Thus, the improvement in the yield of the microbial production of sinefungin would be necessary and should allow us to purify and characterize the intriguing enzymes, which condense ATP and L-arginine. It has been reported recently that certain mutations conferring resistance to streptomycin, gantamicin, and rifampicin result in increased antibiotics production by *Streptomyces*

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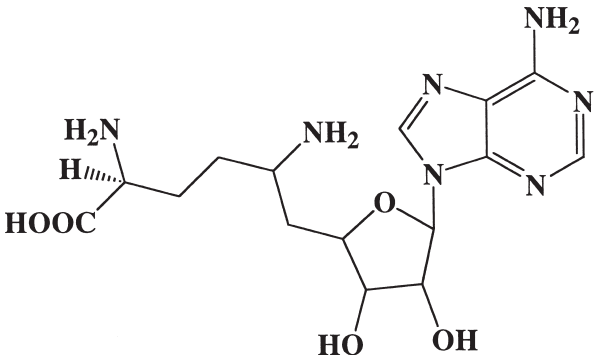

Fig. 1 Sinefungin.

coelicolor[9] and other Bacilli[10]. This paper describes the effect of the introduction of rifampicin-resistance to the sinefugin-producer strain of *S. incarnatus* NRRL 8057.

Materials and Methods

Organisms and Medium

The sinefungin producer *Streptomyces incarnatus* NRRL 8057 was obtained from the Agricultural Research Service, United States Department of Agriculture. The R6 regeneration medium contained 200 g of sucrose, 10 g of dextrin, 1 g of casamino acid, 50 mg of $MgSO_4$ $7H_2O$, 11 g of monosodium L-glutamate, 100 mg of K_2SO_4, 7 g of $CaCl_2$ $2H_2O$, 100 mL of 100 mM sodium 3-(*N*-Morphorino)propanesulfonate buffer (pH 7.2), 20 g agar and distilled water to 1000 ml. TSB agar medium contained 3 g of tryptic soy broth (DIFCO) and 1.5 g of agar in 100 mL of deionized water.

Ultraviolet Light Irradiation on Protoplast

Conditions for cultivation and preparation of protoplasts were as described previously[11]. Protoplasts were sedimented by centrifugation at 500 to 600 × g for 10 min, and spread on R6 regeneration medium. The plate was irradiated with ultraviolet light (Toshiba GL15 lamp) in a clean bench (3.31×10^{-4} J/cm²/sec), quickly contained in a dark place, and incubated at 30°C for 2 days until colonies grew visible. The plates were then covered with 3 ml of soft agar containing 60 μg/ml rifampicin, and further incubated at 30°C for 3 days.

Sample Preparation

The regenerated progeny was subcultured on 1.0 ml of TSB-agar medium in a 24-hole assay plate, and was allowed to grow for 5 days at 30°C. The culture medium was cut in halves with a toothpick, and one half was dipped in 1.0 ml of 3.0 M sodium acetate buffer, pH 5.3 and boiled for 20 min with 0.1 g of activated charcoal. Then, charcoal powder was separated from the supernatant fluid after centrifugation at 10,000 rpm for 10 min, and washed twice with 1 ml of water. Components absorbed on charcoal were eluted by soaking the charcoal powder in 1 ml of 50% aqueous acetone overnight, then ca. 20 mg of magnesium phosphate was added to the mixture and centrifuged at 10,000 rpm for 10 min. Clear supernatant solution was transferred to a new sample tube and concentrated to dryness on speed-vac concentrator. The residue was dissolved in 50 μl of sterile water.

EcoRI Methylase Inhibitor assay

Aliquot of 10 μl of the above sample was mixed with 1.2 μg of lambda DNA (TaKaRa), 3.2nmol *S*-adenosyl-L-methionine, 2 μl of *Eco*RI methylase buffer (× 10), and 10 units of *Eco*RI methylase (total volume 20 μl) and incubated at 37°C for 1 h. Two microliter of 3 M sodium acetate, pH 5.3, and 20 μl of isopropanol were added to the solution. After the solution was stand at room temperature for 10 min, DNA was precipitated by centrifugation at 14,000 rpm for 20 min. The pellet was washed with cold 70% ethanol, and dried under the reduced pressure. The residue was dissolved in 8 μl of sterile, deionized water and mixed with 1 μl of (× 10) buffer for *Eco*RI restriction enzyme. *Eco*RI restriction enzyme (10 U) was added and incubated at 37°C for 2 h. The solution was mixed with 1 μl of sample loading buffer (× 10) and run on 0.7%-agarose gel. When sinefungin was contained in the sample solution, lambda DNA was digested by *Eco*RI restriction enzyme, and the characteristic ladder was observed on the

gel. When the strain did not produce sinefungin the agarose-gel electrophoresis would show the intact lambda DNA.

Determination of Sinefungin Production by HPLC

Sinefungin production was detected by the *Eco*RI methylase inhibition assay as described above, and the amount of sinefungin produced in the medium was determined by HPLC system implemented with a cation-exchange column. A strain of *S. incarnatus* was subcultured on three TSB agar media (1 μl) contained in a 24-hole assay plate, and grown at 30°C for 5 days. Sinefungin was extracted from the culture medium as described above using activated charcoal, and determined by analytical HPLC using a CAPCELL PAK SCX column (4.6 mm I.D. × 150 mm, Shiseido Fine Chemicals), Shimadzu LC-6A liquid chromatography pump, and a Shimadzu SPD-6A uv spectrophotometric detector. The column was developed with 0.5 M ammonium formate (pH 4.0) at a flow rate of 0.5 μl/min. The chromatogram was monitored by absorption at 260 nm. The sinefungin content in each sample was estimated from the peak area of sinefungin eluted at 13.8 min. The productivity of each strain was designated as the mean and deviation calculated from the three separate cultures.

Results and Discussion

Members of the genera *Streptomyces, Bacillus,* and *Pseudomonas* are soil bacteria that produce a high proportion of agriculturally and medically important antibiotics. The development of rational approaches to improve the production of antibiotics from these organisms is therefore of considerable industrial and economic importance. It has been reported that *rpsL* mutation is likely to enhance the antibiotic production of streptomycetes by inducing the mutations on RNA binding site of ribosomal protein[12]. We first tried to obtain streptomycin resistant strains of *S. incarnatus*, but we could not obtain those mutants under various mutagenesis conditions including varied length of ultraviolet light irradiation and varied streptomycin concentrations.

Because the sinefungin producer, *S. incarnatus* NRRL 8089, did not form spores on several media tested, we attempted to separate each cell from the mycelia by digesting the cell wall and preparing protoplast. Conditions suitable for preparation and regeneration of streptomycete protoplasts have been developed by Okanishi et al[12], and the procedure has been modified and improved by Shirahata et al[13] and Baltz and Matsushima[14]. The protoplasts of *S. incarnatus* regenerated the mycelia form most efficiently on R6 medium. Ultraviolet light has been irradiated on the regeneration medium shortly after the protoplasts were spread on the plate. The irradiation time has been optimized to be 4 min, where the survival rate was in the range of 0.1 to 0.5%. Our results suggest that the protoplast regeneration can be an alternative method for using copious haploid spores, which is usually recommended to minimize the risk of the revertant appearance and unstable phenotype.

The sinefungin production by the wild type strain varied in the range of 0.13 to 0.30 μg/μl, and the mean productivity of the six wild type strains was 0.19 ± 0.07 μg/μl. The wild strain was resistant to rifampicin up to the concentration of 16 μg/ml when grown on the TSB-agar medium. The sinefungin production by wild type strains and mutants are summarized in Fig. 2. The first round of mutagenesis has yielded 3 strains, which increased the rifampicin resistance up to 64 μg/ml. One of the mutants decreased the sinefungin production to 0.16 ± 0.13 μg/ml while the other two retained the productivity at 0.23 ± 0.11 μg/ml and 0.24 ± 0.08 μg/ml. Thus, the first round of mutation did not increased the sinefungin productivity notably although the antibiotic resistance has increased almost 4-times. The second round of mutation has yielded only one mutant strain, which was resistant to 250 μg/ml rifampicin, but

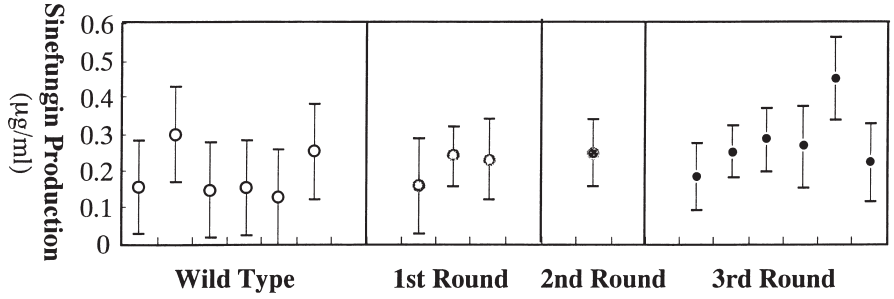

Fig. 2 Sinefungin production by wild type and mutant strains of *S. incarnatus*.
Three culture samples were prepared for the each strain grown on 1ml-TSB agar medium. The productivity (μg/ml) was the mean and deviation of the three independent cultures.

the sinefungin productivity was $0.25 \pm 0.09\,\mu$g/ml. Again, the secondary metabolite production did not increase as the increased rifampicin-resistance, which was almost 16-fold of the wild type strain. A strain that increased the sinefungin-production was found among mutants obtained by the third round of mutation. Among the 6 mutants, which survived when the rifampicin was added in the concentration of $500\,\mu$g/ml, one strain produced sinefungin at the concentration of $0.45 \pm 0.11\,\mu$g/ml. The other five mutant strains produced sinefungin in the range of 0.18 to $0.28\,\mu$g/ml.

Our principal finding in this study indicates that the introduction of rifampicin-resistance resulted in approximately 2- to 3-fold increase of the sinefungin-production by *S. incarnatus*, but this is normally the case found in classical mutagenesis and random screening. Although the sinefungin production did not increase so remarkably, the three rounds of mutation finally increased the productivity by 2.4-fold of the production by the wild type strain. It should be noted that the total number of mutants we assayed the productivity by HPLC was only 10 through the three round of mutation. Searching for high-producer strains after ultraviolet light-irradiation normally takes the trouble of assaying hundreds of strains since such the random mutation by physical or even by chemical mutagenesis normally results in 2- to 3-fold of productivity enhancement only at the small chances of 0.1-0.5%. In this point of view, the screening for antibiotic-resistance may be an effective method to reduce the trouble of examining hundreds of strains. Further mutation conferring the resistance to ribosome-binding antibiotics such as gentamicin[9] to the rifampicin-resistant mutant may further increase the sinefungin production of *S. incarnatus*.

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References

1) Florent, Y., Y. Lunel and D. Mancy. : Nouvelle substance antifonginique, sa preparation et les compositions qui la contiennet. Fr. 7611141, Apr. **15** (1976)
2) Hamill, R. L. and Hoehn, M. M. : A 9145, a new adenine-containing antifungal antibiotic. I. Discovery and isolation. J. Antibiotics., **26**, 463-465 (1973)
3) Gordee, R. S. and T. F. Butler : A 9145, a new adenine-containing antifungal antibiotic. II. Biological activity. J. Antibiotics., **26**, 466-470 (1973)
4) Bachrach, U., L. F. Schnur, J. El-On, C. L. Greenblatt, E. Pearlman, M. Robert-Gero and E. Lederer : Inhibitory activity of sinefungin and SIBA (5'-deoxy-5'-*S*-isobutylthioadenosine) on the growth of promastigoes and amastigotes of different species of *Leishmania*.

FEBS Lett., **121**, 287-291 (1980)
5) Trager, W., M. Terschakovec, P. K. Chiang and G. L. cantoni : Plasmodium falciparum : Antimalarial activity in culture of sinefungin and other methylation inhibitors. Exp. Parasitol., **50**, 83-89 (1980)
6) Dube, D. K., G. Mpimbaza, A. G. Allison, E. Lederer and L. Rovis : Antitrypanosomal activity of sinefungin. Am. J. Chem. Trop. Med. Hyg. **32**, 31-33 (1984)
7) Ferrante, A., I. Ljungstorm, G. Huldt and E. Lederer : Antitrypanosomal activity of the antifungal antibiotic sinefungin against *Entamoeba histolytica*. Trans. R. Soc. Trop. Med. Hyg., **78**, 837-838 (1984)
8) Malina, H., C. Tempete and M. Robert-Gero : Enhanced sinefungin production by meidum improvement, mutagenesis, and protoplast regeneration of *Streptomyces incarnatus* NRRL 8089. J. Antibiotics **38**, 1204-1210 (1985)
9) Hu, H. and K. Ochi : Novel Approach for Improving the Productivity of Antibiotic-Producing Strains by Inducing Combined Resistant Mutations. Appl. Environ. Microbiol. **67**, 1885-1892 (2001)
10) Hosoya, Y., O. Susumu, H. Kuramatsu and K Ochi : Acquisition of certain streptomycin-resistant (*str*) mutations enhances antibiotic production in bacteria. Antimirob. Agents. Chemother. **42**, 2041-2047 (1998)
11) Hopwood, D. A., M. J. Bibb, K. F.Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward and H. Schrempf : *In* Genetic manipulation of Streptomyces, a laboratory manual., The John Innes Foundation, Norwich England pp. 12-14, (1985)
12) Shima, J., A. Hesketh, S. Okamoto, S. Kawamoto and K. Ochi : Induction of actinorhodin production by *rpsL* (encoding ribosomal protein S12) mutations that confer streptomycin resistance in *Streptomyces coelicolor* A3(2). J. Bacteriology, **178**, 7276-7284 (1996)
13) Okanishi, M., K. Suzuki and H. Umezawa : Formation and reversion of streptomycete protoplast : cultural conditions and morphological study. J. Gen. Mirobiol. **80**, 389-400 (1974)
14) Shirahama, T., T. Furumai and M. Okanishi : A modified regeneration method for streptomycete protoplasts. Agric. Biol. Chem. **45**, 1271-1273 (1981)

UV 照射とプロトプラスト再生法による *Streptomyces incarnatus* のリファンピシン耐性とシネフンギン生産性の向上

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グラム陽性菌の二次代謝は，貧栄養条件などの緊縮応答として，その生合成遺伝子の転写段階が厳密な制御を受けている．そこで，これらのバクテリアの RNA ポリメラーゼの制御部位を突然変異導入により破壊すれば，二次代謝産物の生産性向上が期待できる．本研究ではシネフンギンを生産する *Streptomyces incarnatus* NRRL 8057のプロトプラストに紫外線を照射して，RNA ポリメラーゼ阻害剤であるリファンピシンに耐性を獲得した変異株を取得した．突然変異操作を三回行なった結果，異なるリファンピシン耐性を持つ突然変異株10株を得た．この中で最も高い耐性を示した突然変異株が最も高いシネフンギン生産能を示し，その生産性（0.45±0.11 μg/mℓ）は野生株（0.19±0.07 μg/mℓ）の約2.4倍あった．リファンピシン耐性を指標とする菌株の改良は，ランダムなスクリーニングを行なうよりも効率よく選抜を行うことができるという点で有効であるといえる．