J. Microbiol. Biotechnol. (2009), **19**(9), 869–872
doi: 10.4014/jmb.0811.629
First published online 4 March 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Combination Strategy to Increase Cyclosporin A Productivity by *Tolypocladium niveum* Using Random Mutagenesis and Protoplast Transformation

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Received: November 9, 2008 / Accepted: January 27, 2009

The cyclic undecapeptide cyclosporin A (CyA), one of the most valuable immunosuppressive drugs, is produced nonribosomally by a multifunctional cyclosporin synthetase enzyme complex by the filamentous fungus *Tolypocladium niveum*. To increase CyA productivity by wild-type *T. niveum* (ATCC 34921), random mutagenesis was first performed using an antifungal agar-plug colony assay (APCA) selection approach. This generated a mutant strain producing more than 9-fold greater CyA than the wild-type strain. Additionally, a foreign bacterial gene, *Vitreoscilla* hemoglobin gene (VHb), was transformed *via* protoplast regeneration and its transcription was confirmed by RT–PCR in the UV-irradiated mutant cell. This led to an additional 33.5% increase of CyA production. Although most protoplast-regenerated *T. niveum* transformants tend to lose CyA productivity, the optimized combination of random mutagenesis and protoplast transformation described here should be an efficient strategy to generate a commercially valuable, yet metabolite low-producing, fungal species, such as CyA-producing *T. niveum*.

Keywords: *Tolypocladium niveum*, cyclosporin A, mutagenesis, protoplast transformation

Tolypocladium niveum, also known as *Trichoderma polysporum* [16], *T. inflatum* [18], or *Beauveria nivea* [24], is a slow-growing, white-colony-forming fungal species that belongs to the family of conidiophore-generating Ascomycota [10]. Cyclosporin A (CyA), a major secondary metabolite of *T. niveum*, is a cyclic undecapeptide with some unusual amino acids, including α-aminobutyric acid and (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (MeBmt). CyA is generated *via* a non ribosomal biosynthetic pathway by a multi functional cyclosporin synthetase enzyme complex [8]. Although CyA was initially developed as an antifungal in the early 1970s [4], it is currently one of the most important immunosuppressive drugs for organ transplantation, as well as for patients with autoimmune diseases, owing to its superior T-cell-specific calcineurin inhibition *via* TGF-β stimulation and low-level myelotoxicity [20].

Microbial strain improvement to maximize metabolite productivity is important to the microbial fermentation industry. Although recently developed rational approaches based on Omics-driven genetic engineering have been applied to various commercially valuable microbial systems [13, 19, 7, 6], traditional random mutagenesis [1, 26] is still routinely used, especially for strains with little available genetic information or that are recalcitrant to genetic manipulation [17, 23]. Thus, both traditional and rational strain improvement approaches are equally important and should be well-balanced to achieve maximum metabolite productivity. Here, we briefly describe a combination strategy for *T. niveum* strain improvement using random mutagenesis with an antifungal agar-plug colony assay (APCA) selection method and a foreign bacterial gene transformation *via* protoplast regeneration.

It is taken as a given that the amount of antifungal compound in an agar-plug colony assay (APCA) is proportional to the size of the inhibition zone on the plate [21]. To test the feasibility of the APCA method for CyA-producing *T. niveum*, several paper discs containing various amounts of CyA were first applied to a lawn of *Aspergillus niger* cultured on a ME medium (malt extract 20 g, glucose 20 g, peptone 1 g, agar 20 g, in 1 l of distilled water) for 2 days. As shown in Fig. 1A, the sizes of the inhibition zones were reasonably well-correlated with the amounts of CyA. Thus, the CyA productivity of *T. niveum* from an agar-plug colony could be directly measured based on the size of an inhibition zone against *A. niger*.

To find an optimum condition for *T. niveum* (ATCC 34921) mutagenesis, a short wavelength (254 nm) UV lamp (Vilber lourmat VL-4LC, France) was applied at a

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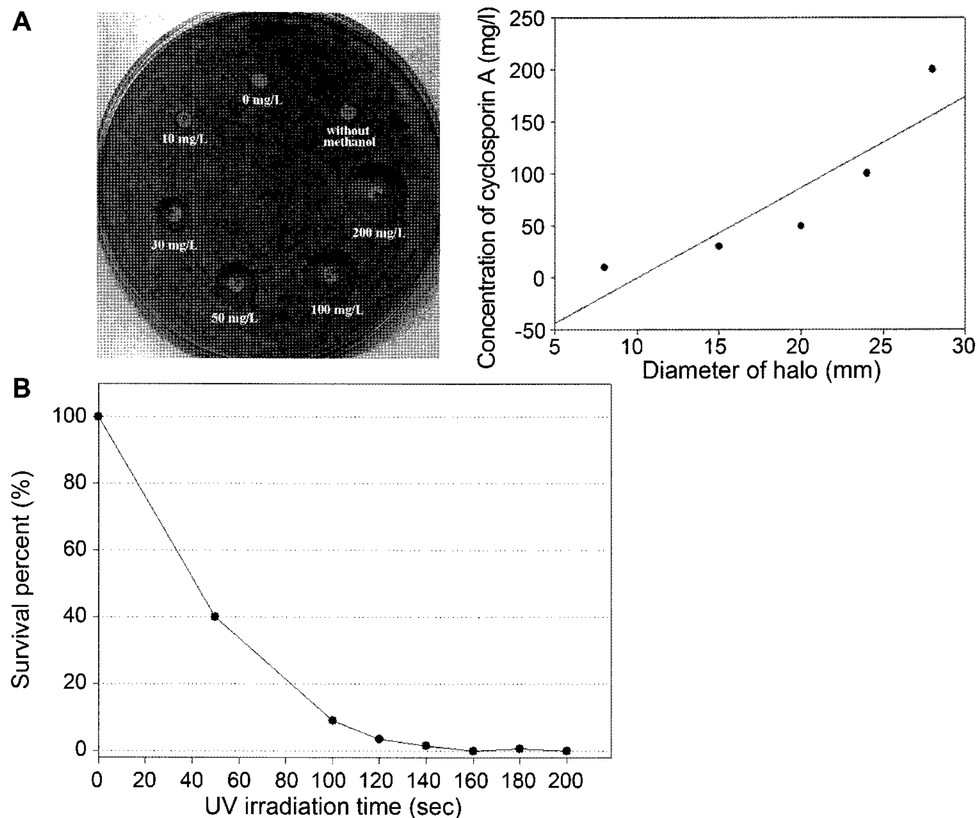

Fig. 1. A. Photograph of CyA-containing paper disc antifungal assay against *A. niger* (left) and correlation between CyA concentration and size of inhibition zone with CyA-containing paper discs (right). **B**. Survival (%) of *T. niveum* versus UV irradiation time.

distance of 15 cm to approximately 6.8×10^5 spores of 7-day-old *T. niveum* cultured in SSMA medium [9]. We followed spore spreading using various time-point samplings during a total 200-s UV exposure. As shown in Fig. 1B, a 160-s UV exposure was ideal for *T. niveum* mutagenesis with a 0.01% survival rate. Approximately 750 *T. niveum* mutant colonies were randomly selected and used for the APCA method against *A. niger*. Each colony was grown on SM agar medium for 7 days, and then *T. niveum* plugs were applied to ME medium containing *A. niger*. After culture in 28°C for 2 days, the inhibition zones were confirmed.

Based on visual measurements, 13 colonies showing larger inhibition zones than the wild-type control were selected and placed into liquid culture for CyA quantification [9]. Three additional colonies showing inhibition zones similar in size to the wild type were also selected as negative controls. Although the size of the inhibition zone does not exactly correlate with the volumetric concentration of CyA, colonies showing larger inhibition zones tend to produce more CyA in liquid culture (Fig. 2A). In particular, the mutant designated M196, which produced approximately 27 mg/l of CyA in culture, exhibited 9-fold greater CyA volumetric production than the typical wild-type strain that produced approximately 3 mg/l (Fig. 2B). This result implies that the traditional method of UV mutagenesis with APCA selection is very efficient and cost-effective for *T. niveum* strain improvement to increase CyA productivity.

To test the feasibility of applying a genetic engineering method to the CyA-overproducing *T. niveum* mutant, a protoplast transformation method was performed in the M196 strain using a *Vitreoscilla* sp. gene, VHb, as a model system. The VHb gene is a hemoglobin-like gene present in the chromosome of the aerobic Gram-negative bacterium, *Vitreoscilla* sp., whose protein plays an important role in oxygen transfer [11, 17, 22, 25]. Heterologous expressions of the VHb gene in several microorganisms, including *E. coli*, stimulates ATP generation by promoting efficient oxygen transfer in an oxygen-limited environment, leading to enhanced cell growth and protein synthesis [2, 5, 14, 15, 27]. A VHb gene cloned in a fungal expression vector, pBARGPE1, containing a selection marker of an Ignite/basta-resistance (*bar*) gene [12] was kindly provided by Prof. G. T. Chun of Kangwon University, Korea. To introduce the VHb gene into the *T. niveum* M196 mutant strain, spores were harvested from an SSMA plate in 5 ml of 20% glycerol solution and inoculated into 50 ml of minimal medium [9], followed by a 2-day incubation at 28°C with constant shaking at 200 rpm. After this 2-day preculture, the cells were harvested by centrifuging at 7,000 rpm for 10 min

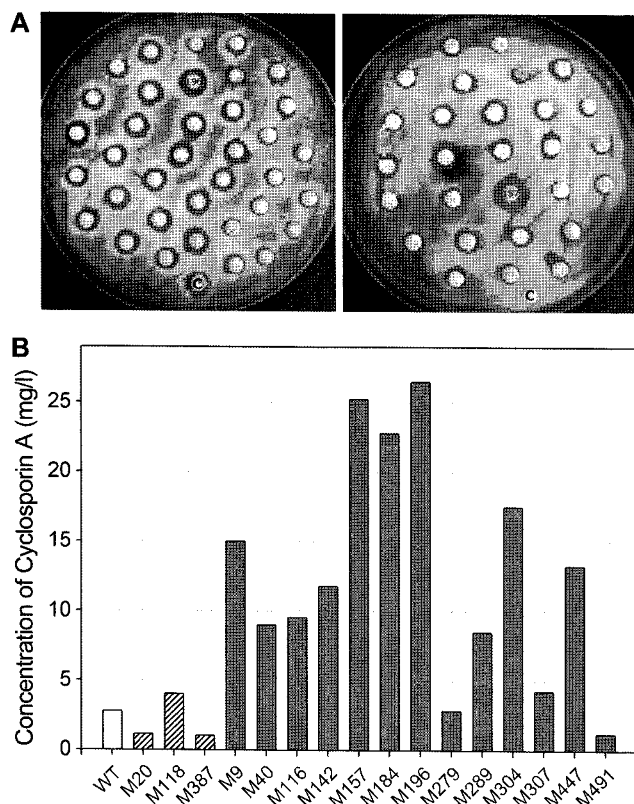

Fig. 2. A. Photographs of agar-plug colony assay (APCA) against *A. niger.* **B.** CyA productivities of selected UV-irradiated *T. niveum* mutants.

Mutants showing small inhibition zones are marked with striped lines, and black for mutants having high antifungal activity.

and washed with 0.6 M KCl. Treatment with 2% lysozyme solution in 5 ml of OM-A buffer (1.2 M $MgSO_4$, 10 mM Phosphate buffer, pH 5.0) with gentle shaking at 28°C at 80 rpm for about 2 h generated relatively well-isolated protoplasts (Fig. 3A).

The harvested protoplasts were washed 4 times using trapping buffer (1.2 M D-sorbitol, 0.1 M Tris–Cl, pH 7.5), and then resuspended in 100 µl of STC buffer (1.2 M D-sorbitol, 10 mM $CaCl_2$, 10 mM Tris–Cl, pH 7.5). The mixture containing 100 µl of a protoplast suspension with 5.7 ng of plasmid DNA was incubated for 30 min on ice, followed by 20 min incubation at room temperature in the presence of 900 µl of PEG (MW 4000; Fluka) solution. The mixture was gently mixed with 5 ml of soft minimal medium agar containing 1.2 M D-sorbitol, and then applied on top of a minimal medium agar plate. Phosphinothricin overlay after 16 – 18 h inoculation and 4 – 10 day additional incubation at 30°C generated several phosphinothricin-resistant transformed colonies. Eight randomly isolated transformants, which were all confirmed by bar gene-specific PCR amplification (data not shown), were selected along with the protoplast-regenerated M196 control strain. CyA production in liquid culture was determined. As shown in Fig. 3B, 2 strains

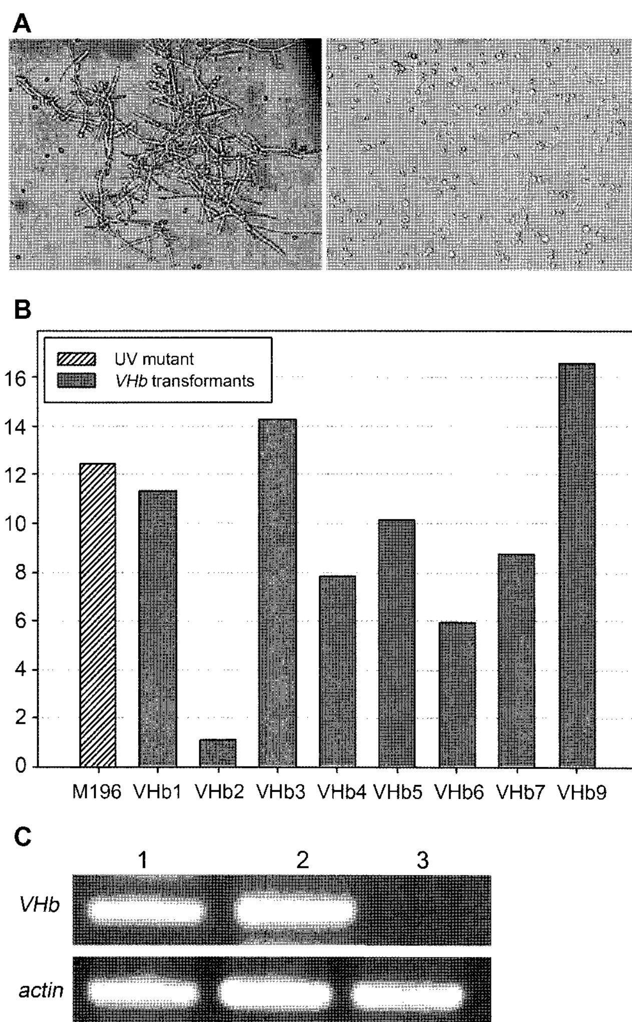

Fig. 3. A. Mycelia of *T. niveum* before (left) and after (right) lysozyme treatment. **B.** CyA productivities of VHb-containing transformants. **C.** RT–PCR analysis of RNA samples isolated from VHb2 (lane 1), VHb9 (lane 2), and M196 (lane 3) strains for VHb-specific (upper) and actin-specific (lower).

The RT–PCR primer pairs (5'-3') were as follows: *VHb* (tattgaaggagcatggc gttacc, tgagcgtacaaatctgcttccac), *act* (ctgggaygayatggaraagat, gytcrgccagga tcttcat).

designated VHb3 and VHb9 showed approximately 14.9% and 33.5% higher CyA productivities compared with the protoplast-regenerated M196 control strain, respectively. Unfortunately, most of the transformants, including the M196 control strain, showed decreased CyA production, probably due to either the protoplast regeneration procedure or the presence of the selection antibiotics in the culture. To determine if variable CyA production among the transformants was due to the VHb gene expression, 2 strains showing the lowest (VHb2) and the highest (VHb9) productivities were chosen for RT–PCR analysis. RT–PCR was performed with slight modification of a previously reported method [3]. As shown in Fig. 3C, the VHb genes were properly transcribed in both transformants, suggesting that a foreign bacterial

gene could be successfully transformed into a UV-mutagenized *T. niveum via* protoplast regeneration.

In conclusion, a *T. niveum* VHb9 strain showing improved CyA productivity could be selected using both random mutagenesis and protoplast transformation. Although it remains to be determined if the CyA-overproducing VHb9 strain was obtained by bacterial foreign VHb gene expression or by random selection during protoplast regeneration, the optimized combination of random mutagenesis and protoplast regeneration should be an efficient strategy to generate a commercially valuable, yet metabolite low-producing, fungal species, such as CyA-producing *T. niveum*.

Acknowledgments

The authors are very grateful for the technical support provided by the ERC of Inha University. This work was financially supported a 21C Frontier R&D program MG3 Grant from the Korean Ministry of Education, Science and Technology Policy Directions for the 21st Century.

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