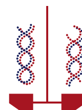

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Further enhancement of FR901469 productivity by co-overexpression of *cpcA*, a cross-pathway control gene, and *frbF* in fungal sp. No. 11243

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FR901469 is a secondary metabolite with antifungal activity, produced by fungal sp. No. 11243. In our previous study, we constructed the *frbF* overexpression mutant (TFH2-2) from the wild-type strain. FR901469 productivity of TFH2-2 was 3.4 times higher than that of the wild-type strain. To further enhance FR901469 productivity in TFH2-2, we attempted to find genes from the genome that limited the productivity as bottlenecks in this study. Based on both correlation analysis of gene expression level against FR901469 productivity and genome annotation information, the cross-pathway control gene A (*cpcA*) was most predicted as the bottleneck. The *cpcA* and *frbF* co-overexpression mutant named TFCH3 was then constructed from TFH2-2. As a result, FR901469 productivity of TFCH3 was enhanced at 1.8 times higher than that of TFH2-2. Transcriptome analysis revealed that many genes involved in amino acid biosynthesis and encoding tRNA ligases were significantly upregulated in TFCH3, which implied increase of amino acids as the substrates of FR901469 would be a reason of further productivity enhancement.

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[**Key words:** Fungal sp. No. 11243; FR901469; Secondary metabolite; Transcriptome; *cpcA*]

It is known that microorganisms produce various chemical compounds called secondary metabolites, and some of them are used as medicines or the precursors. However, productivities of secondary metabolites are mostly very low, and thus enhancing the productivities is important for medicinal uses. Recently, because of the development of genome analysis technologies and genome engineering techniques, it became easy to construct genetically modified microorganisms. Previous studies reported that secondary metabolite productivity was improved using genetic engineering such as cephalosporin C in *Acremonium chrysogenum* (1,2) and penicillin in *Aspergillus nidulans* (3). Application of genetic engineering to breeding, called molecular breeding, is expected to become the leading method for enhancing secondary metabolite productivity.

Fungal species No. 11243 (No. 11243 hereafter) was found to produce a novel antifungal antibiotic, FR901469, which is an inhibitor of β-1,3-glucan synthase and inhibits the growth of *Candida albicans* and *Aspergillus fumigatus* (4,5). FR901469 is a secondary metabolite having a macrocyclic lipopeptidolactone structure (6, Fig. 1A). In our industrial process development, a series of FR901469 high-production mutant strains has been obtained from the No. 11243 wild-type strain by random mutagenesis (Fig. 1B, C). We previously performed genome analyses of the wild-type strain (7) and these mutant strains (8) to determine mutations related to FR901469 productivity. The study suggested that both increasing expression of the putative FR901469 biosynthesis gene cluster and mutations of genes in the KOG categories ("Replication, Recombination, and Repair", "Signal Transduction Mechanisms", and "Transcription") would enhance FR901469 productivity (8). Therefore, we constructed the FR901469 high-production mutant by overexpressing a transcription factor gene, *frbF*, which probably regulates expressions of putative FR901469 biosynthesis cluster genes (9). As expected, the constructed strain successfully showed 3.4 times higher FR901469 productivity than the wild-type strain.

To further enhance the productivity, we attempted to find new target genes that are bottlenecks in the FR901469 production. Because FR901469 has 12 amino acid residues in its molecule, it is expected that amino acids are used to form FR901469 as substrates. Thus, we supposed that the productivity of FR901469 might be enhanced by increasing the intracellular amino acids. In fact, our previous examinations of culture condition showed that addition of some kinds of amino acids (e.g., proline, isoleucine, tyrosine) improved FR901469 productivity (data not shown). In this study, we identified a gene regulating amino acid biosynthesis and performed genetic engineering for further enhancing the FR901469 productivity.

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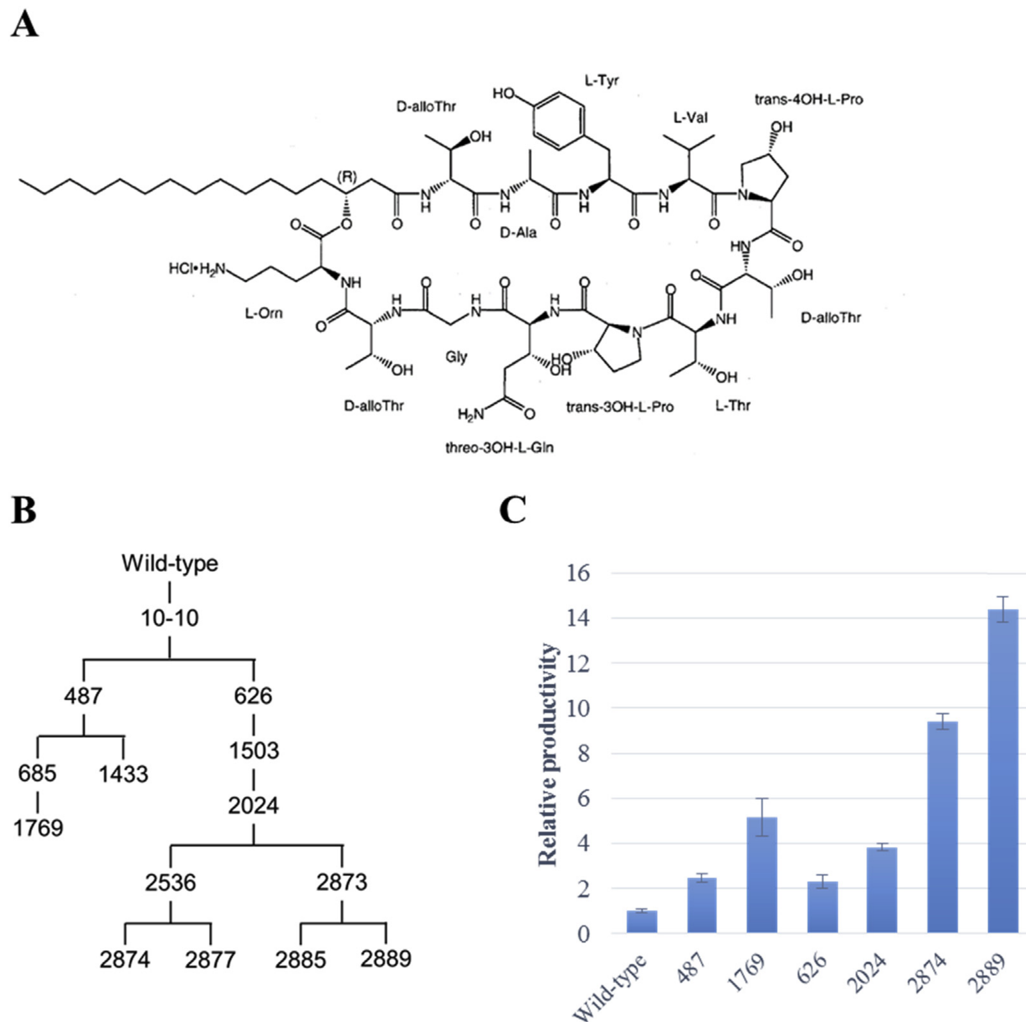

FIG. 1. Antifungal antibiotic FR901469 producing fungus No. 11243 and its mutant strains. (A) Structure of FR901469. (B) Genealogy of No. 11243 mutant strains. (C) The relative FR901469 productivity of No. 11243 wild-type and mutant strains. The value of FR901469 productivity of the wild-type strain at the 8-d culture period was set to 1.0. Three replicate experiments were performed, and the values of the means and standard deviations are represented.

MATERIALS AND METHODS

Strains and genome sequencing The gene-annotated genome sequence of No. 11243, registered to DDBJ with DF938580-DF938599 (7), was used to design primers and perform RNA-seq analysis. FR901469 high-production mutant by overexpressing *frbF*, named TFH2-2, was used for the construction of transformants (9). Putative FR901469 biosynthesis cluster genes were defined in our previous paper (9). Amino acid biosynthesis genes and tRNA ligase genes were extracted by using BlastKOALA (10). These extracted amino acid biosynthesis genes were named similarly to the *Saccharomyces cerevisiae* genes that had the same KEGG IDs. If there were more than one gene which had the same KEGG ID, they were distinguished by adding a different alphabet at the end of the gene name (e.g., *aro4a*, *aro4b*).

Plasmid construction Overexpression plasmids were constructed by fusing three DNA fragments (*adh5* gene [ANO11243_065790] promoter region, *cpcA* gene coding and terminator region, and hygromycin resistance gene [*hph*]) and the pUC19 vector using the In-Fusion HD Cloning Kit (Clontech, CA, USA). PCR was performed using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). Primers used are listed in Table S1.

Transformation For fungal transformation, a 5 mm cube excised from the plate culture of TFH2-2 was transferred to 20 mL seed medium in a 100-mL Erlenmeyer flask. The seed medium contains 4% soluble starch (Nacalai Tesque, Kyoto, Japan), 2% cottonseed flour (Sigma-Aldrich, MO, USA), 1% soybean flour (Sigma-Aldrich), 1% KH_2PO_4, and 0.2% $CaCO_3$. TFH2-2 was precultured at 25°C at 180 rpm for 5 d. The resultant seed culture (400 μL) was transferred to 20 mL transformation medium in a 100-mL Erlenmeyer flask. The transformation medium contains 1.0% soluble starch, 0.5% glucose, 2.0% Brain Heart Infusion (Becton, Dickinson and Company, NJ, USA), and 0.5% KH_2PO_4. TFH2-2 was grown at 25°C at 220 rpm for 3 d. The mycelia were collected by sterilized Miracloth (Merck Millipore, MA, USA), and washed with 10 mL of sterilized TF solution A containing 1 M KCl, 10 mM $MgCl_2$, and 25 mM $CaCl_2$. The washed mycelia were suspended in 10 mL of TF solution A supplemented with 10 mM dithiothreitol (DTT) (pH 8.0) and incubated at 30°C for 30 min. After washed with 10 mL of DTT-free TF solution A, mycelia were resuspended in 5 mL of a TF solution A containing 0.5% Yatalase (Takara, Shiga, Japan) and 0.5% Zymolyase (Nacalai Tesque). The suspension was incubated at 30°C at 80 rpm for 3 h. Cell wall debris was removed by filtration using Miracloth, and the filtrated protoplasts were collected by centrifugation at 3000 rpm at 4°C for 5 min. The protoplasts were resuspended at approximately 10^8 protoplasts/mL in sterilized TF solution B containing 1.2 M sorbitol, 50 mM $CaCl_2$, and 10 mM Tris–HCl (pH 7.5). Approximately 5 μg plasmid DNA was mixed with a 200-μL aliquot of the protoplast suspension and incubated on ice for 30 min. Then, 1 mL of sterilized TF solution C containing 40% polyethylene glycol 3350, 50 mM $CaCl_2$, and 10 mM Tris–HCl (pH 7.5) was added. After gentle mixture and incubation at 25°C for 1 h, protoplasts were overlaid on the surface of regeneration agar medium containing 0.6 M sucrose, 1% yeast extract (Becton, Dickinson and Company), 5 mM $CaCl_2$, 0.1% $ZnSO_4$, 0.1% $MnSO_4$, and 0.75% agar. In the selection of transformants, drug-resistant transformants were selected by culturing in regeneration agar medium containing 1000 g/mL hygromycin B (Wako, Osaka, Japan).

Cultivation for analysis Seed medium (20 mL) containing 4% soluble starch, 2% cottonseed flour, 1% soybean flour, 1% KH_2PO_4, and 0.2% $CaCO_3$ was poured into 100-mL Erlenmeyer flasks and sterilized at 121°C for 20 min. A 5 mm cube excised from the plate culture was transferred to each seed medium. Seed culture was done at 220 rpm at 25°C for 5 d.

Production medium (100 mL) consisting of 7% soluble starch, 1% glucose, 4% cottonseed flour, 6% corn steep liquor (Sigma-Aldrich), 1% $(NH_4)_2SO_4$, 0.5% Bacto Peptone (Becton, Dickinson and Company), 0.44% K_2HPO_4, 1.0% KH_2PO_4, 0.1%

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