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Culture-based and sequence-based insights into biosynthesis of secondary metabolites by *Aspergillus terreus* ATCC 20542

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ABSTRACT

Aspergillus terreus ATCC 20542 was cultivated in various culture media in order to activate its genome-encoded biosynthetic pathways and explore the secondary metabolic repertoire. In addition to mevinolinic acid (lovastatin) and its precursor monacolin L, a number of other secondary metabolites were found in the analyzed cultures, namely terreic acid, citrinin, (+)-geodin, terrein, and dehydrocurvularin. In contrast to previously described gene clusters responsible for production of lovastatin, monacolin L, (+)-geodin and dehydrocurvularin, the gene clusters of *A. terreus* associated with the formation of terreic acid, citrinin and terrein still await identification. Putative gene clusters potentially related to citrinin and terreic acid biosynthesis were suggested in the publicly available genome of *A. terreus* NIH 2624. The functions of putative genes in the previously identified cluster of (+)-geodin biosynthesis were predicted by confronting the annotation results with the proposed biosynthetic pathway and published biochemical studies on the underlying enzymes. Since there were no available data regarding genetic aspects of terrein biosynthesis, the candidate gene cluster potentially responsible for the production of terrein was not suggested.

1. Introduction

Secondary metabolism of filamentous fungi harbors a plethora of bioactive low-molecular-weight compounds, including polyketides and non-ribosomal peptides (Keller et al., 2005). Biosynthesis of polyketides involves the action of polyketide synthases (PKSs), large multidomain enzymes responsible for providing chemical scaffolds that are subsequently modified by a set of tailoring enzymes (Chiang et al., 2010a; Cox, 2007). The production of non-ribosomal peptides is based on the catalytic capabilities of non-ribosomal peptide synthetases (NRPSs) (Strieker et al., 2010). The genes responsible for assembling a particular metabolite are usually clustered in fungal genomes, with the core synthase gene accompanied by the genes of dedicated regulatory proteins, transporters and post-synthase tailoring enzymes (Brakhage, 2013). The enzymes encoded by a single cluster can be compared to a biosynthetic factory responsible for generating a final product.

The availability of genome sequences opens the door for genome mining efforts focused on discovery of novel fungal metabolites. Importantly, the true metabolic potential of fungi is still far from being fully discovered and most of the putative biosynthetic gene clusters are currently neither characterized nor experimentally associated with the corresponding metabolic products (Sanchez et al., 2012; Winter et al., 2011). In order to elucidate the biosynthetic repertoire of a fungus it is essential to awake the silent gene clusters and thus activate the largest possible part of the secondary metabolic network (Brakhage and Schroeckh, 2011). An approach known as OSMAC (One Strain-Many Compounds) is based on the idea to manipulate media composition and process parameters in order to trigger biosynthesis of as many secondary metabolites as possible by a single strain (Bode et al., 2002). If the cultivation conditions and environmental stimuli resemble the ones that activate the cluster in the natural habitat of the examined strain, the fungus responds by producing the corresponding molecule.

Sequence similarity search is often performed in order to predict the functions of cluster-encoded proteins and suggest the link between the biosynthetic gene cluster and the corresponding metabolite. However, the area of fungal secondary metabolism is still poorly explored and the number of experimentally validated reference sequences deposited in databases remains relatively low. In the absence of closely related entries sharing high sequence identity, bioinformatic analyses of secondary metabolism necessarily involve the alignments with more distant proteins and the pairwise identity values between 30% and 60% are often taken into

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consideration when analyzing fungal biosynthetic gene clusters (Chiang et al., 2009, 2010b; Davison et al., 2012; Keller et al., 2005; Zabala et al., 2012). While being insufficient for the confident and precise function assignment, the computational comparison does provide a valuable basis for hypotheses and further experimental characterization of genes involved in the secondary metabolism of fungi.

Aspergillus terreus has gathered much attention due to its long catalog of documented secondary metabolites. The website devoted to *Aspergillus* species and aspergillosis (https://www.aspergillus.org.uk) provides the lists of metabolic products discovered in *Aspergillus* spp. with *A. terreus* being a species associated with a remarkably large number of molecules. This fungus has made a successful industrial career as a producer of lovastatin, a cholesterol-lowering polyketide compound (Kennedy et al., 1999) and itaconic acid, an important bulk chemical employed in polymer industry (Li et al., 2011). While some of the *A. terreus* metabolites were discovered in the first half of the 20th century, much of the current effort is focused on the identification and functional characterization of gene clusters. Since the genome sequence of *A. terreus* became available, several biosynthetic clusters have been identified and characterized, including the ones responsible for the biosynthesis of isoflavipucine (Gressler et al., 2011), terretonin (Guo et al., 2012), dehydrocurvularin (Xu et al., 2013) and acetylaranotin (Guo et al., 2013). Importantly, the majority of the putative biosynthetic loci in the genome of *A. terreus* are still not functionally linked to any metabolic products (Sanchez et al., 2012).

The aim of this work was to explore the biosynthetic repertoire of *A. terreus* ATCC 20542 (the lovastatin-producing strain) by subjecting the fungus to a set of diverse culture media. Various combinations of carbon and nitrogen sources were applied to trigger cellular processes related to the production of secondary metabolites. Experimental work was followed by an attempt to identify the candidate gene clusters responsible for the biosynthesis of the detected molecules in *A. terreus*. The genomic sequence of NIH 2624 strain was used, since it is the only *A. terreus* strain with the publicly available genome sequence. Nevertheless, the organization of respective gene clusters is likely to be shared among the metabolite-producing strains due to a relatively small evolutionary distance. Finally, the findings of the in silico analysis were confronted with the metabolic pathways in order to discuss the potential biosynthetic role of each cluster-encoded protein.

2. Materials and methods

2.1. Strain and growth media

A. terreus ATCC 20542 was the strain used in the course of the experimental works.

The culture media were based on several combinations of nutrients and their compositions were as follows:

- *GLY + LAC + YE* (glycerol: 15 g l^{-1} (0 in the preculture); lactose: 10 g l^{-1}; yeast extract: 4 g l^{-1} (8 g l^{-1} in the preculture); KH_2PO_4: 1.51 g l^{-1});
- *GLY + YE* (glycerol: 25 g l^{-1} (0 in the preculture); lactose (used only in the preculture): 10 g l^{-1}; yeast extract: 4 g l^{-1}(8 g l^{-1} in the preculture); KH_2PO_4: 1.51 g l^{-1});
- *LAC + YE* (lactose: 25 g l^{-1} (10 g l^{-1} in the preculture); yeast extract: 4 g l^{-1}(8 g l^{-1} in the preculture); KH_2PO_4: 1.51 g l^{-1});
- *GLU + YE* (glucose 45 g l^{-1} (10 g l^{-1} in the preculture); yeast extract: 12.5 g l^{-1}; KH_2PO_4: 5 g l^{-1}; K_2HPO_4: 5 g l^{-1});
- *GLU + PEPT* (glucose 45 g l^{-1} (10 g l^{-1} in the preculture); casein peptone: 12.5 g l^{-1}; KH_2PO_4: 5 g l^{-1}; K_2HPO_4: 5 g l^{-1});
- *GLU + MSG* (glucose 45 g l^{-1} (10 g l^{-1} in the preculture); monosodium glutamate: 12.5 g l^{-1}; KH_2PO_4: 5 g l^{-1}; K_2HPO_4: 5 g l^{-1});
- *FRU + YE* (fructose: 45 g l^{-1} (10 g l^{-1} in the preculture); yeast extract: 12.5 g l^{-1} (8 g l^{-1} in the preculture); KH_2PO_4: 1.51 g l^{-1});
- *STRCH + YE* (starch: 10 g l^{-1} (0 in the preculture); glucose (used only in the preculture): 10 g l^{-1}; yeast extract: 4 g l^{-1} (8 g l^{-1} in the preculture); KH_2PO_4: 1.51 g l^{-1}).

In addition, all the eight media described above contained the following components: $MgSO_4{\cdot}7H_2O$: 0.52 g l^{-1}, NaCl: 0.4 g l^{-1}, $ZnSO_4{\cdot}7H_2O$: 1 mg l^{-1}, $Fe(NO)_3{\cdot}9H_2O$: 2 mg l^{-1}, biotin: 0.04 mg l^{-1} and 1 ml of trace element solution per 1 l of medium (the solution of trace elements contained: $Na_2B_4O_7{\cdot}10H_2O$: 100 mg l^{-1}, $MnCl_2$: 50 mg l^{-1} $Na_2MoO_4{\cdot}2H_2O$: 50 mg l^{-1}, $CuSO_4{\cdot}5H_2O$: 250 mg l^{-1}).

The remaining three culture media were composed as follows:

- *GLU + NITR* (glucose: 50 g l^{-1} (10 g l^{-1} in the preculture); $NaNO_3$: 2 g l^{-1}; K_2HPO_4: 1 g l^{-1}; KCl: 0.5 g l^{-1}; $MgSO_4{\cdot}7H_2O$: 0.5 g l^{-1}; $FeSO_4{\cdot}7H_2O$: 0.01 g l^{-1}; $ZnSO_4{\cdot}7H_2O$: 0.01 g l^{-1}; $CuSO_4{\cdot}5H_2O$: 0.005 g l^{-1}; malt extract: 0.5 g l^{-1}; yeast extract: 0.2 g l^{-1});
- *SUCR + YE* (sucrose: 150 g l^{-1} (20 g l^{-1} in the preculture); yeast extract: 20 g l^{-1}; $MgSO_4{\cdot}7H_2O$: 0.5 g l^{-1}; $ZnSO_4{\cdot}7H_2O$: 0.01 g l^{-1}; $CuSO_4{\cdot}5H_2O$: 0.005 g l^{-1});
- *PDB* – potato dextrose broth (glucose: 20 g l^{-1} (10 g l^{-1} in the preculture); $ZnSO_4{\cdot}7H_2O$: 0.01 g l^{-1}; $CuSO_4{\cdot}5H_2O$: 0.005 g l^{-1}; all the components were dissolved in the liquid obtained after a 25-min boiling of 200 g potatoes in 1 l of water).

The pH level value of all growth media was adjusted to 6.5. The media were sterilized by autoclaving for 20 min at 121 °C.

2.2. Cultivation method

A. terreus ATCC 20542 was grown on malt agar slants for 10 days in order to induce sporulation. The preculture was prepared by washing the spores with the preculture medium in order to achieve approximately 10^9 spores per l. The 24-h preculture was used for the inoculation. The volume of inocula was chosen so as to achieve the initial biomass concentration between 0.1 and 0.15 g l^{-1}.

The fungus was cultivated in 500 ml shake flasks (working volume was equal to 150 ml). The temperature of the cultivation and the speed of the rotary shaker were kept constant at the level of 30 °C and 110 rpm, respectively. Sampling was made in 96 and 120 h of the experiment. After filtration of biomass the samples were tested for the presence of secondary metabolites secreted by the fungus.

2.3. Analytical method

Ultra performance liquid chromatography (UPLC® Acquity)-mass spectrometry (SYNAPT G2) system was employed for metabolite detection. A Waters Acquity UPLC® BEH Shield RP18 column (2.1 mm × 100 mm × 1.7 μm) was used for separation of molecules (eluent flow rate: 0.2 ml min^{-1}, column temperature: 40 °C). The gradient of acetonitrile:water (both acidified with formic acid at the final concentration of 0.1%) ranging from 0:100 (v/v) to 60:40 (v/v) was applied (Bizukojc et al., 2012). Prior to injection, the samples were pretreated by filtering the liquid through 0.2 μm syringe filters. The molecular mass values of metabolites and the corresponding fragmentation ions were determined by using quadrupole-TOF mass spectrometer SYNAPT G2 (Waters, USA). The following values of instrument parameters were kept throughout the analysis: temperature of the source: 120 °C, desolvation temperature: 200 °C, desolvation gas flow: 500 l h^{-1},