Fungal Genetics and Biology 46 (2009) 232–242

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Modified oxidosqualene cyclases in the formation of bioactive secondary metabolites: Biosynthesis of the antitumor clavaric acid

Ramiro P. Godio [a], Juan F. Martín [a,b,*]

[a] Instituto de Biotecnología de León (INBIOTEC), Avda. Real, 1. 24006 León, Spain
[b] Área de Microbiología, Facultad de Ciencias Biológicas y Ambientales, Universidad de León, Campus de Vegazana, s/n. 24071 León, Spain

ARTICLE INFO

Article history:
Received 31 July 2008
Accepted 5 December 2008
Available online 24 December 2008

Keywords:
Hypholoma
Clavaric acid
Triterpenoids
Basidiomycete

ABSTRACT

Hypholoma sublateritium is a basidiomycete that produces the antitumor compound clavaric acid. We cloned a gene encoding an oxidosqualene cyclase (*occ*) that is involved in the conversion of oxidosqualene to clavaric acid. Mutants disrupted in *occ* by gene replacement were unable to synthesize clavaric acid, but did not require sterols for growth. Amplification of the *occ* gene produced a 35–67% increase in clavaric acid yield. Northern analysis of *occ* gene expression confirmed that there is a clear correlation of *occ* expression and clavaric acid biosynthesis. Analysis of the *occ*-encoded oxidosqualene cyclase revealed that it has VSDCVGE motif instead of the consensus VSDCTAE sequence of the active center. In summary, there is an oxidosqualene cyclase specific for secondary metabolite biosynthesis; this is in agreement with the finding of two squalene cyclases in the sequenced genomes of basidiomycetes.

1. Introduction

Mushrooms have been extensively used in traditional medicine because some species produce a large number of bioactive compounds (Wasser, 2002). Many of those compounds, including some promising antitumor compounds are triterpenoid secondary metabolites synthesized by the isoprenoid pathway (Erkel and Anke, 1997; Lee et al., 1998; Vilella et al., 2000; Calvo et al., 2002).

In 1998 researchers at Merck isolated from a mushroom a potent inhibitor of the RAS protein farnesyltransferase that was named clavaric acid (Lingham et al., 1998; Jayasuriya et al., 1998). The producer mushroom *Hypholoma sublateritium* is widespread in the north hemisphere.

Clavaric acid, as many other sterol-related compounds, is synthesized through the intermediate 2,3-oxidosqualene (OS) and the early steps of the pathway including the oxidation of squalene to OS are common for the lanosterol and clavaric acid pathways (Godio et al., 2007). During sterol biosynthesis (primary metabolism) OS is converted to lanosterol by the enzyme oxidosqualene lanosterol cyclase (OSLC) (E.C.5.4.99.7) that catalyzes an intramolecular cyclization of OS forming six new carbon to carbon bonds creating a molecule with seven stereoisomeric centers (Abe and Prestwich, 1999).

A family of OSLC-related enzymes is involved in the cyclization of OS to a variety of intermediates of secondary metabolites. Those enzymes named oxidosqualene cyclases (OSC) generate a variety of polycyclic triterpenoid compounds that are widely distributed in plants, fungi and bacteria (Abe et al., 1993; Abe, 2007). Analysis of the differences in the cyclization mechanism is one of the key aspects for understanding how the diversity of secondary metabolites arises from the common intermediate OS (Abe and Prestwich, 1999).

Since OSLCs and OSCs belong to the same protein family, it seems that small differences in the active center(s) of those enzymes are responsible for the final triterpenoid product of the cyclization reaction. The OSLCs and OSCs provide the OS-binding pocket and their reactive centers determine the stability of alternative possible cyclization intermediates, guiding the reaction to the formation of the different triterpenoids (Fessenden and Fessenden, 1982; Van Tamelen, 1982). Several OSLCs have been cloned from vertebrates (Roessner et al., 1993; Baker et al., 1996; Sung et al., 1995; Young et al., 1996; Abe and Prestwich, 1995a,b; Corey et al., 1996), but surprisingly very little information is available about fungal OSCs, although they give rise to a variety of triterpenoid secondary metabolites; in fungi, only the OSLCs of the model yeast *Sacharomyces cerevisiae* (Shi et al., 1994) and the ascomycete *Cephalosporium caerulens* (Abe et al., 2001) have been studied to some extent. However, no OSCs from basidiomycetes have been reported, despite their involvement in the biosynthesis of different secondary metabolites.

The primary sequence of some vertebrate OSLCs has confirmed that these enzymes have repeated 'QW' motifs, containing the aro-

* Corresponding author. Address: Área de Microbiología, Departamento de Biología Molecular, Facultad de CC. Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n. 24071, León, Spain. Fax: +34 987 291506.
E-mail address: jf.martin@unileon.es (J.F. Martín).

1087-1845/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2008.12.002

matic amino acid rich sequence ((K/R)(G/A)XX(F/Y/W)(L/I/V)XXXQXXXGXW) with a helix structure. The exact role of these motives on the active center (Dougherty, 1996) or in the stabilization of the protein structure (Wendt et al., 1997) is still an open question.

The basidiomycetes OSCs might be more similar to the cycloartenol cyclases of plants (Corey et al., 1993; Morita et al., 1997; Kushiro et al., 1998; Kawano et al., 2002; Zhang et al., 2003; Hayashi et al., 2004). More information about OSCs from basidiomycetes and other poorly studied fungi is necessary to understand the differences in the molecular mechanisms that originate the formation of bioactive secondary metabolites as compared to those of primary metabolism enzymes that form lanosterol.

We report in this article an oxidosqualene–clavarinone cyclase of *H. sublateritium* that is different from the primary metabolism enzyme of this same fungus. The gene encoding this enzyme is essential for clavaric acid biosynthesis, but not for lanosterol biosynthesis. Its overexpression leads to a clear increase in clavaric acid production; this is the first example of an OS cyclase for the biosynthesis of a bioactive secondary metabolite in basidiomycetes.

2. Materials and methods

2.1. Microbial strains and culture media

H. sublateritium HS898 (ATCC74314) (Vilella et al., 2000) and *H. sublateritium* (HSCanales, isolated in Canales, León, Spain) dikaryotic strains were routinely grown on solid MEA (20 g/l malt extract, 5 g/l peptone, 20 g/l glucose, 15 g/l agar) medium at 20 °C. To obtain arthrospores the cultures were grown on solid PDA medium (Difco, Detroit, Mich.) at 20 °C for 3 weeks and collected as described previously (Godio et al., 2007). Liquid *H. sublateritium* cultures were grown in 100 ml of MEA broth at 20 °C and 200 rpm. For clavaric acid production studies or transcriptional analysis, *H. sublateritium* wild type strain and the transformants were grown in MEA broth or minimal medium (MM) (glucose, 20 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; H_2KPO_4, 0.5 g; HK_2PO_4, 0.6 g; $CuSO_4 \cdot 5H_2O$, 0.4 mg (1.6 μM); $MnCl_2 \cdot 4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; biotin, 5 μg; distilled water up to 1 l) with different nitrogen sources (asparagine–glutamate, 15 mM each or ammonium acetate 10 mM and 30 mM). Erlenmeyer flasks (250 ml) containing 50 ml medium were inoculated with two agar plugs (each 0.25 cm^2) cut out from a colony grown on solid MEA medium. Incubation was carried out at 20 °C under static conditions, since previous studies revealed that static incubations were optimal for clavaric acid production. The growth in solid media was estimated as previously described by Panagou et al. (2005). Images were captured with a high-sensitivity Canon EOS 10D (DS6031) (Canon USA Inc., NY, USA) digital camera and processed with Image-Pro Express version 6.0 (Media Cybernetics) image analysis software. All plates were photographed every day. Each plate was corrected for background by subtracting the image of an uninoculated (blank) plate incubated together with the test plates. Images were 512 by 512 pixels. Visible growth is expressed in integrated optical density (IOD) units.

Agrobacterium tumefaciens AGL1 was used as a host strain for transformation in conjugation experiments (Godio et al., 2004).

2.2. Preparation of ergosterol and lanosterol

Ergosterol and lanosterol solutions were prepared at a stock concentration of 12 mg/ml in ethanol and Tween 80 (1:1, v/v). The solution was heated at 65 °C for 5 min before using it.

2.3. DNA procedures and plasmids

All basic DNA methods were performed as described by Sambrook and Russel (2001). Genomic DNA from both wild type and hygromycin-resistant *H. sublateritium* transformants was obtained by freezing cells from a 20-day liquid culture in liquid nitrogen and grounding the frozen pellet to a fine powder in a mortar, as described previously (Godio et al., 2004).

2.4. OS clavarinone cyclase over expression constructions

In order to construct the expression vector pHgOCCover, the intermediate plasmid pgdhA-2OCCover (Fig. 1A) was made by inserting the *occ* gene sequence obtained by PCR amplification (3.0 kb) with primers POSC1F and OSC1R, in the *Sma*I restriction site of the plasmid pgdhA-2 (Godio et al., 2007). Finally, pHgOCCover was constructed by inserting the cassette containing the *occ* gene with the original *occ* promoter from pgdhA-2OCCover (obtained by PCR amplification with primers POSC1F and TGDHR) by blunt-end ligation at the *Pvu*II-*Sma*I site in the plasmid pBGgHg (Chen et al., 2000) (Fig. 1A).

2.5. Construction pBG-SKCICLAi for occ gene disruption

In order to construct the expression vector pBG-SKCICLAi, the intermediate plasmid pGEM-OSC12i (Supplementary Fig. S1) was made by inserting the hygromycin-resistant cassette (obtained by *Eco*RV/*Xmn*I digestion of pBGgHg plasmid) in the blunt-ended *Bgl*II restriction site of the vector pGEM-OSC12. This vector was digested with *Not*I and the *Not*I fragment was subcloned in the plasmid pBluescript II SK (+), previously digested with *Not*I to obtain the intermediate plasmid pBSCICLAi. Finally, pBG-SKCICLAi was constructed by inserting the *Bam*HI/*Bst*XI disruption at the *Bam*HI/*Bst*XI sites in the plasmid pBG-SK.

2.6. Southern blot analysis

Southern blotting was performed by standard procedures (Sambrook and Russel, 2001). Digoxigenin labeling, hybridization and detection were performed with the Genius Kit (Roche Diagnostics). Hybridizations were performed at 42 °C using a 5×SSC solution containing 0.1% lauryl-sarcosine, 0.02% SDS, 40% formamide and 2% blocking reagent. The hybridization signals were visualized with a chemiluminescent substrate for alkaline phosphatase (CDP-Star), according to the manufacturer's (Roche) protocol. For Southern experiments, a 800-bp *Eco88*I DNA fragment, internal to the *occ* gene, was used as probe.

2.7. Primers used for cloning and sequencing the occ gene of H. sublateritium

Six OSLC and OSC sequences were used to design the primer pairs OSCL2 and OSCL7 according to the codon usage in filamentous fungi. PCR amplifications were performed with *Taq* DNA polymerase (Promega, Madison, WI), using genomic DNA of *H. sublateritium* as template. The resulting 3.0 and 3.5 kb DNA fragments were sequenced to confirm the presence of the gene of interest, and used as a probe to screen the *H. sublateritium* genomic library.

2.8. PCR analysis

Disruption of *occ* gene was achieved by the double recombination strategy. The presence of the *occ* disruption cassette (construction pBG-SKCICLAi) in the transformants was confirmed by PCR using primers F1C and C2 (both internal to the *occ* gene, Fig. 2a).