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Characterization of the ferrichrome A biosynthetic gene cluster in the homobasidiomycete *Omphalotus olearius*

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Abstract

Under iron deprivation *Omphalotus olearius* was found to produce the hydroxamate siderophore ferrichrome A. A gene cluster consisting of three genes: *fso1*, a nonribosomal peptide synthetase whose expression is enhanced in the absence of iron; *omo1*, a L-ornithine-N^5-monooxygenase; and *ato1*, an acyltransferase probably involved in the transfer of the methylglutaconyl residue to N^5-hydroxyorinithine was identified. The *fso1* sequence is interrupted by 48 introns and its derived protein sequence has a similar structure to the homologous genes of *Ustilago maydis* and *Aspergillus nidulans*. This is the first report of a nonribosomal peptide synthetase gene and a biosynthetic gene cluster in homobasidiomycetes.

Keywords: *Omphalotus*; Nonribosomal peptide synthetase; Siderophore; Ferrichrome A; Gene cluster

1. Introduction

Iron is an essential element for the growth and proliferation of nearly all organisms. Under iron starvation, most microorganisms excrete siderophores, low-molecular-mass compounds with a very high affinity for iron. Their function is to mediate iron uptake by microbial cells. Most fungal siderophores are of the hydroxamate group [1], divided into three structural families: fusarinines, coprogens and ferrichromes [2]. All share the basic structural unit N^5-acyl-N^5-hydroxyornithine. Ferrichrome A (Fig. 1) consists of a hexapeptide ring made up of one glycine, two serine, and three N^5-hydroxyornithine amino acid residues, the latter acylated by *trans*-(α-methyl)-glutaconic acid residues [3]. Ferrichrome A was first isolated from the smut fungus *Ustilago sphaerogena* [4].

Hydroxamate siderophore biosynthesis takes place in at least three steps. The first is the hydroxylation of L-ornithine catalysed by a L-ornithine-N^5-monooxygenase. The second is the acylation of N^5-Hydroxy-L-ornithine to form N^5-acyl-N^5-hydroxy-L-ornithine, catalysed by an acyl-CoA:N^5-hydroxy-L-ornithine *N*-acyl transferase. The third step comprises the linking of the hydroxamic acids and, in the case of ferrichromes, the incorporation of the other amino acids by a modular nonribosomal peptide synthetase (NRPS) [5]. Three domains are necessary for a basic NRPS module: an adenylation domain (A) that selects the amino acid and activates it as amino acyl adenylate, a peptidyl carrier protein domain (PCP or T domain) that binds the co-factor 4′-phosphopantetheine and to which the amino acid is attached, and a

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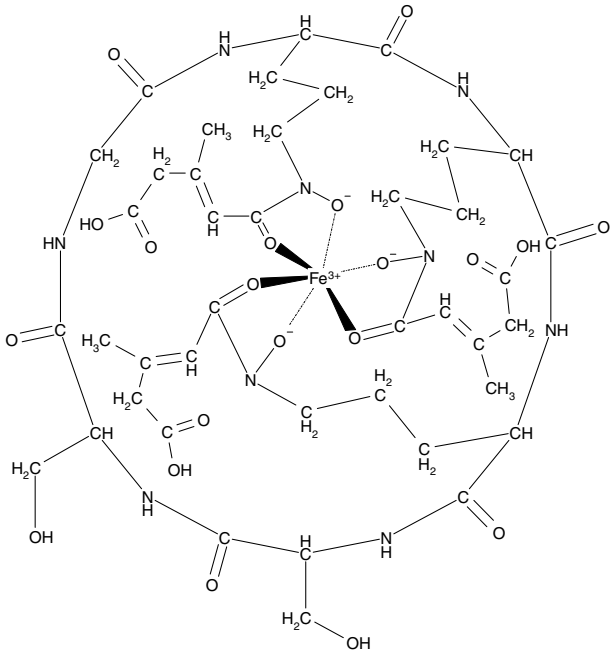

Fig. 1. Ferrichrome A structure.

condensation domain (C) that catalyses the peptide bond formation [6,7].

Production of siderophore biosynthetic enzymes was shown to be repressed by iron in cell-free experiments for rhodotorulic acid [8], fusigen [9] and ferrichrome [10]. In *Ustilago maydis* (heterobasidiomycete) and *Aspergillus Emericella nidulans* (ascomycete), the expression of siderophore biosynthetic genes is enhanced in iron depleted medium, and is under the control of the GATA family transcription factors Urbs1 [11] and SREA [12], respectively.

We describe here the first NRPS gene as well as the first iron regulated siderophore biosynthetic gene cluster in a higher basidiomycete.

2. Materials and methods

2.1. Organisms, media, and cultivation conditions

Omphalotus olearius TA90170 was isolated from spore prints of fruiting bodies. The culture is deposited at the IBWF. The strain was cultivated and maintained in YMG medium (glucose 4 g/l, yeast extract 4 g/l, malt extract 10 g/l; pH 5.5). For cultures on solid media, 1.5% of agar were added. Fermentation up to 2 l were carried out in Erlenmeyer flasks with agitation (120 rpm) at 28 °C. Larger fermentations were carried out in a Braun Biostat A-20 fermenter containing 20 l of YMG medium with aeration (3 l air/min) and agitation (120 rpm) at 28 °C. For iron depletion studies mycelia of *O. olearius* were washed four times with Sundström minimal medium [13] (20 g/l glucose, 1.4 g/l L-asparagine, 0.35 g/l KH_2PO_4, 0.15 g/l $K_2HPO_4 \cdot 3H_2O$, 0.5 g/l $Na_2S0_4 \cdot 10H_2O$, 0.1 mg/l thiamine, 5 mg/l $CaCl_2$, 20 mg/l $MgCl_2 \cdot 6H_2O$, 0.27 g/l sodium citrate, 0.26 g/l citric acid, 0.22 g/l $MnSO_4 \cdot 4H_2O$, 0.20 g/l $ZnSO_4 \cdot 7H_2O$), transferred to 0.25–2 l flasks with Sundström minimal medium and incubated with agitation (120 rpm) at 28 °C.

The *Escherichia coli* strain DH5α (Gibco BRL, Rockville, MD, USA) was used for cloning and plasmid propagation. *E. coli* XL1 b [P2] was used as host for bacteriophage lambda FIX II (Stratagene) and was grown in NZY medium. DNA from lambda clones was purified by standard techniques and cloned into pUC18 [14].

2.2. DNA and RNA isolation

For genomic DNA isolation, lyophilized mycelium of *O. olearius* was ground to a fine powder using a mortar. Extraction buffer (1 M Tris–HCl, pH 8.0, 0.1 mM EDTA, 1% SDS, 200 μg/ml proteinase K and 100 μg/ml DNase-free RNase) was added and the mixture incubated for 50 min at 56 °C. The mixture was extracted once with phenol and three times with phenol/chloroform/isoamylalcohol (25:24:1 v/v/v) followed by a final extraction with chloroform/isoamylalcohol (24:1 v/v). To remove proteins, 0.1 vol of 5 M potassium acetate was added to the supernatant, the mixture incubated 1 h on ice and centrifuged. DNA was precipitated with 2 vol of ethanol and washed with 70% ethanol.

RNA was isolated by grinding lyophilized mycelium under liquid nitrogen. The fine powder was extracted with the Total RNA Isolation System (Promega) according to the manufacturer's instructions. DNase treatment was performed by dissolving the RNA pellets in 200 μl buffer (0.1 M sodium acetate, 5 mM $MgSO_4$, pH 5.0) containing 2 units DNase and incubated for 20 min at room temperature, followed by a final phenol/chloroform extraction. RNA was precipitated with 2 vol ethanol and dissolved in 500 μl of RNase-free water. mRNA was isolated from total RNA using the "PolyATract mRNA Isolation System" (Promega).

2.3. Isolation and characterization of ferrichrome A

Mycelia and culture broth were separated by filtration from 6 to 8 week old culture of *O. olearius*, grown in iron depleted medium. $FeCl_3$ was added to the culture filtrate which was then loaded onto a XAD-16 column and washed with three column volumes of water. Ferrichrome A was eluted with methanol. After evaporation of the solvent, the extract was dissolved in methanol and subjected to gel filtration on Sephadex LH20 in methanol. The fractions containing ferrichrome A were pooled, concentrated and purified by preparative HPLC (RP-18 250 × 4 mm, elution with 20% acetonitrile, 80%