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Ligninolytic peroxidase gene expression by *Pleurotus ostreatus*: Differential regulation in lignocellulose medium and effect of temperature and pH

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ABSTRACT

Pleurotus ostreatus is an important edible mushroom and a model lignin degrading organism, whose genome contains nine genes of ligninolytic peroxidases, characteristic of white-rot fungi. These genes encode six manganese peroxidase (MnP) and three versatile peroxidase (VP) isoenzymes. Using liquid chromatography coupled to tandem mass spectrometry, secretion of four of these peroxidase isoenzymes (VP1, VP2, MnP2 and MnP6) was confirmed when *P. ostreatus* grows in a lignocellulose medium at 25 °C (three more isoenzymes were identified by only one unique peptide). Then, the effect of environmental parameters on the expression of the above nine genes was studied by reverse transcription-quantitative PCR by changing the incubation temperature and medium pH of *P. ostreatus* cultures pre-grown under the above conditions (using specific primers and two reference genes for result normalization). The cultures maintained at 25 °C (without pH adjustment) provided the highest levels of peroxidase transcripts and the highest total activity on Mn^{2+} (a substrate of both MnP and VP) and Reactive Black 5 (a VP specific substrate). The global analysis of the expression patterns divides peroxidase genes into three main groups according to the level of expression at optimal conditions (*vp1/mnp3* > *vp2/vp3/mnp1/mnp2/mnp6* > *mnp4/mnp5*). Decreasing or increasing the incubation temperature (to 10 °C or 37 °C) and adjusting the culture pH to acidic or alkaline conditions (pH 3 and 8) generally led to downregulation of most of the peroxidase genes (and decrease of the enzymatic activity), as shown when the transcription levels were referred to those found in the cultures maintained at the initial conditions. Temperature modification produced less dramatic effects than pH modification, with most genes being downregulated during the whole 10 °C treatment, while many of them were alternatively upregulated (often 6 h after the thermal shock) and downregulated (12 h) at 37 °C. Interestingly, *mnp4* and *mnp5* were the only peroxidase genes upregulated under alkaline pH conditions. The differences in the transcription levels of the peroxidase genes when the culture temperature and pH parameters were changed suggest an adaptive expression according to environmental conditions. Finally, the intracellular proteome was analyzed, under the same conditions used in the secretomic analysis, and the protein product of the highly-transcribed gene *mnp3* was detected. Therefore, it was concluded that the absence of MnP3 from the secretome of the *P. ostreatus* lignocellulose cultures was related to impaired secretion.

1. Introduction

Pleurotus ostreatus, the oyster mushroom, is the second in importance edible fungus worldwide (Sánchez, 2010). From an ecophysiological point of view, *Pleurotus* species belong to the group of fungi causing the so-called white rot of wood and other lignocellulosic materials, due to their ability to degrade the recalcitrant lignin polymer that protects polysaccharides in vascular plants (Ruiz-Dueñas and Martínez, 2009). Among these fungi, *Pleurotus* species are of biotechnological interest because some of them degrade lignin selectively (i.e., with a limited attack on cellulose) when growing on cereal straw and related materials (Martínez et al., 1994). Biological delignification with lignin-degrading fungi saves energy and chemicals in the manufacture of cellulose pulp from woody (Young and Akhtar, 1998) and non-woody (Camarero

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et al., 1998) plant feedstocks, and can be also of interest in the production of second generation bioethanol (Salvachúa et al., 2011).

Since the sequencing of white-rot *Phanerochaete chrysosporium* genome in 2004 (Martinez et al., 2004), many other basidiomycete genomes have been sequenced, up to a total of 90 by the JGI (http://www.jgi.doe.gov), as those of *Postia placenta* (first brown-rot fungal genome) (Martinez et al., 2009), *Ceriporiopsis subvermispora* (first selective white-rot fungal genome) (Fernández-Fueyo et al., 2012) and *P. ostreatus*. According to the genomic data, ligninolytic peroxidases – including lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) – are exclusive of lignin-degrading white-rot basidiomycetes, being absent from polysaccharide-degrading brown-rot basidiomycetes (Floudas et al., 2012). The above distribution of ligninolytic peroxidase genes in basidiomycete genomes confirms its central role in lignin biodegradation. *In vitro* degradation of lignin was first reported for LiP (Tien and Kirk, 1983; Hammel et al., 1993) and recently for VP (Fernández-Fueyo et al., 2014), being ligninolytic enzymes largely investigated because of their biotechnological interest (Martínez et al., 2009).

The existence of multiple isoforms (isoenzymes) is a well-known phenomenon among degradative enzymes secreted by fungi, already described years ago for ligninolytic peroxidases (Farrell et al., 1989; Glumoff et al., 1990). However, the biochemical and operational differences between isoenzymes remain largely unknown. The availability of genomes provides evidence on the large and widespread duplication of peroxidase genes in white-rot basidiomycetes (Martinez et al., 2004; Floudas et al., 2012; Fernández-Fueyo et al., 2012; Ruiz-Dueñas et al., 2013). Our preliminary *in silico* analysis of the *P. ostreatus* genome (Ruiz-Dueñas et al., 2011) indicated the presence of nine genes encoding five MnP and four VP isoenzymes, and the absence of genes encoding LiP and generic peroxidases (a fourth non-ligninolytic peroxidase family at the class II of the superfamily of plant-fungal-prokaryotic peroxidases) (Ruiz-Dueñas and Martínez, 2010). The heterologous expression of these nine genes showed the existence of three VPs and six MnPs in *P. ostreatus* (including an unusual MnP that had been *in silico* classified as a VP) with significant differences in their pH and temperature stabilities suggesting environmental regulation (Fernández-Fueyo et al., 2014).

Media composition and fungal growth conditions strongly affect the production of ligninolytic enzymes and the extent of lignin degradation, e.g. no ligninolytic peroxidase activity is produced by *Pleurotus* species in the synthetic medium used to produce these enzymes in *P. chrysosporium*, while activity was found in peptone and lignocellulose cultures (Martínez et al., 1996). Transcriptional regulation of *vp* and *mnp* genes has been investigated in *Pleurotus* species (Ruiz-Dueñas et al., 1999; Cohen et al., 2001). Differential expression is also characteristic among the members of a gene family, including ligninolytic peroxidase isoenzymes (Salame et al., 2010; MacDonald et al., 2011; Wymelenberg et al., 2011). Recently, the effect of natural (lignocellulosic) vs. simple (glucose) C sources has been addressed in different transcriptomic studies on sequenced wood-rotting basidiomycetes (Martinez et al., 2009; Sato et al., 2009; Wymelenberg et al., 2011; Fernández-Fueyo et al., 2012). Reverse transcription followed by quantitative PCR (RT-qPCR) represents the most powerful technology to quantitatively amplify trace amounts of mRNA (Heid et al., 1996; Pfaffl, 2004). Moreover, RT-qPCR is considered the gold standard for measuring gene expression (Qin et al., 2006) because of its high sensitivity and specificity, robust reproducibility, and wide dynamic range (Pfaffl and Hageleit, 2001). However, this technique requires the careful selection and validation of reference genes (internal standards), which are processed in parallel with the target gene (Ling and Salvaterra, 2011). Moreover, it is critical to determine the amplification efficiency (Pfaffl, 2001; Ramakers et al., 2003), which is used in mathematical models for the accurate estimation of the expression levels.

In the present study, we analyze the ligninolytic peroxidases secreted by *P. ostreatus* when grown in a culture medium with lignocellulose as the sole carbon (and nitrogen) source, by activity estimation and isoenzyme identification by nanoflow liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS) of a whole secretome hydrolyzate. Then, we designed specific primers for each of the corresponding transcripts, and used RT-qPCR to quantify the differential expression of the nine peroxidase genes when the cultures were transferred to extreme pH (3 and 8) and temperature (10 °C and 37 °C), compared with those maintained at optimal conditions (25 °C and pH 5.5), using two reference genes that were validated for the present qPCR experiments. Finally, an intracellular proteomic study was performed to explain some differences between the results from the previous transcriptomic and secretomic analyses.

2. Material and methods

2.1. P. ostreatus strain and genome

Monokaryotic *P. ostreatus* PC9 (CECT-20311) was used in this study. This strain is a protoclone obtained by dedikaryotization from the commercial dikaryon N001 (CECT-20600) (Larraya et al., 1999). The fungus was maintained in 2% malt extract agar.

The genomic sequence of *P. ostreatus* PC9 is available at the JGI website (http://genome.jgi-psf.org/PleosPC9_1), together with that of the second monokaryon PC15, after sequencing in a project coordinated by A.G. Pisabarro. The manual annotation of nine ligninolytic peroxidase gene models in the PC9 genome, including intron positions and N/C termini, has been already described (Ruiz-Dueñas et al., 2011).

In addition, 1 kb promoter fragment from each of the nine *P. ostreatus* (PC9) ligninolytic peroxidase genes was manually analyzed looking for the presence of conserved regulatory and other promoter elements (Janusz et al., 2013) (see Supplemental Information Section S1, for the specific sequences searched).

2.2. Fungal growth in lignocellulose medium

Lignocellulose cultures were carried out at 25 °C on 5 g of a mixture of milled wheat straw and small poplar chips (particle size <4 mm, ratio 1:1) soaked with 35 mL of distilled water (unadjusted pH 5.5) in glass flasks at a surface to volume ratio of 1 cm^{-1} (hereinafter referred as standard conditions). Inocula consisted of 15 mL of homogenized actively growing mycelium from liquid cultures grown at 200 rpm in the dark in M7GY medium, comprising (per liter) 2 g ammonium tartrate, 0.5 g $MgSO_4 \cdot 7\ H_20$, 1 g KH_2PO_4, 0.5 g KCl, 10 g glucose, and 1 ml element's trace solution (0.1 g $Na_2B_4O_7 \cdot H_2O$, 0.07 g $ZnSO_4$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, 0.01 g $MnSO_4 \cdot 4\ H_2O$, 0.05 g $FeSO_4 \cdot 7\ H_2O$, 0.01 g $(NH_4)_6Mo_7O_2 \cdot 4\ H_2O$ per liter) (Castanera et al., 2012).

To study the expression of the nine ligninolytic peroxidase genes when temperature turned to be nearly limiting for fungal growth, twenty-four 7-day-old flask cultures were transferred to 10 °C or 37 °C (twelve flasks per condition) and incubated for additional 24 h to study the effect of temperature change on the transcript levels along the time (twelve additional flasks were maintained at 25 °C and used as reference). In a similar way, other twenty-four 7-day-old cultures were adjusted to pH 3 or 8 (twelve flasks per condition) by adding 0.2 M Brighton&Robin buffer pH 2 or 9, respectively, and incubated for 24 h to study the effect of extreme pH on the transcription levels (the twelve flasks

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