Cloning and characterization of the genes encoding the high-affinity iron-uptake protein complex Fet3/Ftr1 in the basidiomycete *Phanerochaete chrysosporium*

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MCO1, a multicopper oxidase from *Phanerochaete chrysosporium* exhibiting strong ferroxidase activity, has recently been described. This enzyme shows biochemical and structural similarities with the yeast Fet3p, a type I membrane glycoprotein that efficiently oxidizes Fe(II) to Fe(III) for its subsequent transport to the intracellular compartment by the iron permease Ftr1p. The genome database of *P. chrysosporium* was searched to verify whether it includes a canonical *fet3* in addition to *mco1*, and single copies of *fet3* and *ftr1* orthologues were found, separated by a divergent promoter. *Pc-fet3* encodes a 628 aa protein that exhibits overall identities of about 40 % with other reported Fet3 proteins. In addition to a secretion signal, it has a C-terminal transmembrane domain, characteristic of these cell-surface-attached ferroxidases. Structural modelling of Pc-Fet3 revealed that the active site has all the residues known to be essential for ferroxidase activity. *Pc-ftr1* encodes a 393 aa protein that shows about 38 % identity with several Ftr1 proteins from ascomycetes. Northern hybridization studies showed that the mRNA levels of both genes are reduced upon supplementation of the growth medium with iron, supporting the functional coupling of Fet3 and Ftr1 proteins *in vivo*.

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INTRODUCTION

Although iron is an essential element for life, it becomes toxic to organisms when it exceeds the physiological requirements. Thus, a regulated uptake mechanism is fundamental to ensure adequate levels of iron inside the cells. The molecular bases underlying intracellular iron homeostasis have been only recently characterized, and the work conducted in yeast has been fundamental to unveiling its details. In *Saccharomyces cerevisiae*, both high- and

low-affinity iron-uptake systems have been described (Kosman, 2003). The high-affinity iron-uptake system (K_m 0.15 μ M) requires the reduction of Fe(III) to Fe(II) by metalloreductases such as Fre1/Fre2, which are located on the cell surface. The Fe(II) is subsequently translocated through the plasma membrane by the concerted action of Fet3p and Ftr1p (Radisky & Kaplan, 1999). Fet3p oxidizes Fe(II) to Fe(III), which then enters the cell via the iron permease Ftr1p (Stearman *et al.*, 1996).

Fet3p is a blue copper protein belonging to the family of multicopper oxidases (MCO), which also includes laccases, ascorbate oxidases and ceruloplasmin, among other proteins (Solomon *et al.*, 1996). It possesses four structural copper atoms distributed in three different centres that have distinct spectroscopic and functional properties. The type 1 copper (T1, blue copper) is responsible for the strong absorption of these proteins at 600 nm, and it is the primary electron acceptor from the substrate. The type 2 copper (T2) and the binuclear type 3 copper (T3) centres are arranged in a trinuclear cluster that is responsible for the binding and reduction of molecular oxygen (Solomon *et al.*, 1996).

Several differences can be observed among the various members of the MCO family. Ceruloplasmin is the most

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Abbreviations: CDH, cellobiose dehydrogenase; MCO, multicopper oxidase; TM, transmembrane.

The GenBank/EMBL/DDBJ accession numbers for the cDNA sequences of *Pc-fet3* and *Pc-ftr1* are DQ464016 and DQ464017, respectively.

Two supplementary multiple-sequence alignments are available with the online version of this paper.

complex member of this group, with a six-domain structure possessing three T1 copper centres plus one T2/T3 cluster (Zaitsev *et al.*, 1999). Ascorbate oxidase is a dimer composed of two laccase-like monomers (Messerschmidt *et al.*, 1992), while laccase is the simplest member of the family, containing only one T1 and one T2/T3 centre (Piontek *et al.*, 2002). In this regard, ferroxidases are similar to laccases, since they also have only one T1 and one T2/T3 copper centre.

Fet3p from *Sac. cerevisiae* has been extensively characterized and its three-dimensional structure has been recently solved (Taylor *et al.*, 2005). It exhibits spectroscopic properties that are typical of blue copper oxidases, which can be predicted from the primary sequence (Hassett *et al.*, 1998). Its substrate specificity differs considerably from that of other MCOs: it is able to oxidize aromatic amines and iron, but not phenolic compounds, which are characteristic substrates of laccases (de Silva *et al.*, 1997; Baldrian, 2006). The crystal structure of the *Sac. cerevisiae* Fet3p and its subsequent superposition with laccase from *Trametes versicolor* (Piontek *et al.*, 2002) showed several differences in the surroundings of the aromatic-substrate-binding pocket observed in laccases (Taylor *et al.*, 2005). It has been generally stated that Fet3p and ceruloplasmin are the only members of the MCO family that show ferroxidase activity. We have recently described MCO1, a new member of this family from the ligninolytic fungus *Phanerochaete chrysosporium*. In contrast to most white-rot fungi, *P. chrysosporium* does not produce a conventional laccase (Larrondo *et al.*, 2003). MCO1 can efficiently oxidize iron and aromatic amines but not phenolic compounds (Larrondo *et al.*, 2003), as reported for Fet3p. The analysis of the primary structure of MCO1 and, in particular, the lack of the C-terminal transmembrane domain distinctive of Fet3 proteins, support the contention that MCO1 represents an additional clade of the MCO family, different from laccases and Fet3 proteins (Larrondo *et al.*, 2003). Moreover, a recent and detailed phylogenetic analysis of more than 350 MCOs, including the four MCOs from *P. chrysosporium* (Larrondo *et al.*, 2004), supports the latter assertion (Hoegger *et al.*, 2006).

We decided to explore the publicly available genome database of *P. chrysosporium* to find out whether this fungus possesses an actively transcribed gene encoding Fet3, in addition to the gene encoding the MCO1 ferroxidase. This study led not only to the finding of *Pc-fet3*, whose identity was confirmed by cDNA cloning and sequencing, but also to the uncovering of *Pc-ftr1*, separated from the former gene by a divergent promoter. Here we describe the characterization of this genomic cluster and the analysis of the *Pc-Fet3* active

site through comparative structure modelling. In addition, we present preliminary expression studies that support the role of *Pc-fet3* and *Pc-ftr1* in iron uptake.

METHODS

Search for *Pc-fet3* and *Pc-ftr1*. The *P. chrysosporium* database (<http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>) was searched for the presence of gene(s) encoding a Fet3 protein using the *Sac. cerevisiae* Fet3p sequence (GenBank accession no. CAA89768) for a tBLASTN query ($E 10^{-5}$). Only one *fet3* gene model was identified. The manual examination of the neighbouring regions of the putative *fet3* revealed a sequence with a high similarity to the *ftr1* gene from *Sac. cerevisiae* (Fig. 1). Both gene models were manually corrected and the new predictions were confirmed by cDNA cloning and sequencing (see below). No other *fet3* or *ftr1* sequences were identified.

Strain and culture conditions. *P. chrysosporium* homokaryotic strain RP-78 was obtained from the Center for Mycology Research, Forest Products Laboratory, Madison, WI, USA. Spores (10^7 per flask) were inoculated in 100 ml defined medium containing wood-derived crystalline cellulose (Avicel PH-101, Fluka Chemika) as the sole carbon source (Wymelenberg *et al.*, 2002). Cultures were incubated at 37 °C for 6 days with constant agitation (300 r.p.m.). When indicated, cultures were supplemented with FeCl₃ to a final concentration of 0.25 mM.

RNA extraction. After 6 days of growth, the mycelium was separated from the culture fluid by filtration through Miracloth (Calbiochem) and immediately frozen in liquid nitrogen. The frozen mycelium was ground to a powder in a mortar containing liquid nitrogen, and total RNA was extracted as described by Manubens *et al.* (2003).

cDNA cloning and analysis. Poly(A) mRNA was obtained from 100 µg total RNA using the mRNA DIRECT micro kit (Dynal) according to the manufacturer's instructions. *Pc-fet3* and *Pc-ftr1* cDNAs were obtained by reverse transcription using the Moloney murine leukaemia virus reverse transcriptase (Invitrogen) for 45 min at 42 °C. RT-PCR was conducted as described by Larrondo *et al.* (2003) using high-fidelity DNA polymerase (Pfu, Stratagene). The RT-PCR amplification of *Pc-fet3* cDNA was primed using direct (5'-CTCCTCACACAGAGCCCTCTA-3') and reverse (5'-ACAGTACA-TCCTGTCCACCA-3') oligonucleotides, located at 23 and 38 nt from the predicted start and stop codons, respectively. The corresponding direct and reverse oligonucleotides employed for amplification of *Pc-ftr1* cDNA were 5'-AATGGGCAAGAACGTCTTCT-3' and 5'-TT-AGTGCTTCTCATCCGTCT-3', respectively. Nucleotide sequences were determined with the ABI Prism Big Dye terminator cycle sequencing kit on ABI automated sequencers (Applied Biosystems). Sequence editing and analysis were conducted with DNASTar software. The cDNA sequences of *Pc-fet3* and *Pc-ftr1* have been deposited in the GenBank database under accession nos. DQ464016 and DQ464017, respectively. The deduced protein sequence of both *Pc-fet3* and *Pc-ftr1* was further analysed by using the SignalP and TMHMM servers (www.cbs.dtu.dk/services) for the detection of putative secretion signals and transmembrane (TM) domains, respectively (Bendtsen *et al.*, 2004; Krogh *et al.*, 2001).

Fig. 1. Schematic representation of the *fet3/ftr1* locus. Both are single-copy genes located in Scaffold 5. The arrows indicate the transcriptional orientation of the genes.

Three-dimensional modelling of Pc-Fet3. A comparative model of the Pc-Fet3 protein sequence was built with MODELLER software (Sali & Blundell, 1993) using as a template the recently reported X-ray structure of Fet3p (PDB code 1zpu) from *Sac. cerevisiae* (Taylor *et al.*, 2005). The template structure shares 38 % sequence identity with Pc-Fet3. The sequence-structure alignment used to build the model was generated through two independent but consecutive steps. First, a multiple sequence alignment among several known fungal ferroxidases was built using the MALIGN command and the default similarity matrix (as1.sim.mat) available in MODELLER. The sequences included were: *P. chrysosporium* Fet3 (ABE60664), *Arxula adeninivorans* Fet3 (CAB90817), *Candida albicans* Fet3 (CAA70509), *Candida glabrata* Fet3 (BAB62813), *Neurospora crassa* Fet3 (CAD21075), *Pichia pastoris* Fet3 (CAC33177), *Sac. cerevisiae* Fet3p (CAA89768) and *Schizosaccharomyces pombe* Fet3 (NP_594494). These sequences share between 42 and 73 % sequence identity. In this step, and based on the high overall sequence similarity shared among the sequences, stringent gap penalties were used to ensure a proper alignment of the core regions. Gap opening and extension penalties of –1000 and –500 were used. Then, an alignment between the Pc-Fet3 sequence and the previous multiple sequence alignment was calculated. In this case, the gap opening and extension penalties were relaxed to default values: –500 for gap opening and –100 for gap extension. This final alignment was used to build the comparative model of Pc-Fet3, based on the known structure of Sc-Fet3p. The three-dimensional modelling was carried out using the MODEL routine from the MODELLER software. The final model for Pc-Fet3 protein includes the coordinates for those residues ranging between positions 18 and 561 (i.e. the model was built only for the central region that could be aligned with the template structure, lacking 17 residues from the N terminus and 71 residues from the C terminus). The numbering of the residues in the model corresponds to the sequential numbers in the complete Pc-Fet3 sequence.

Northern-blot hybridization. For Northern-blot hybridization studies, 10 µg total RNA was fractionated by electrophoresis in a formaldehyde-agarose gel (1.2 %, w/v) and analysed for the presence of mRNAs encoding *Pc-fet3* and *Pc-ftr1* as described by Manubens *et al.* (2003). The cDNA probe for *Pc-fet3* was prepared with the oligonucleotide primers 5'-GAACATGGCGAATGCAGA-3' (direct) and 5'-ATGCGGTCGGCACTCGCCC-3' (reverse), whereas the probe for *Pc-ftr1* was obtained with the same primers as used for the isolation of its complete cDNA. As a control, levels of mRNA from the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene were also monitored using a cDNA probe primed with the direct (5'-CCTGCACCACCAACTGCCT-3') and reverse (5'-TCGTCTCGGTGTAGCCCGA-3') oligonucleotides. All cDNA probes were prepared by PCR using [α - 32 P]dCTP as described by Mertz & Rashtchian (1994).

Multiple-sequence analysis. Multiple-sequence alignments were constructed with the CLUSTALW method using the MegAlign software

(DNASTar). Default gap opening and extension penalties (15 and 0.20, respectively; Gonnet matrix) were used to construct the alignments.

RESULTS

Identification and characterization of *Pc-fet3*

The *P. chrysosporium* database was searched for the presence of a gene encoding a Fet3 protein, as described in Methods. A single gene was identified, located in Scaffold 5 between coordinates 1 329 663 and 1 331 733 (Fig. 1). The identity of this gene was confirmed by isolation and characterization of the cDNA from mycelium grown in Avicel medium as described in Methods. Comparison of the cDNA with the genomic sequence confirmed the presence of five introns (Fig. 2), which contrasts with the 19 introns present in the *Pc-mco1* gene (Larrondo *et al.*, 2004). The deduced protein has 628 aa, with a predicted peptide signal of 16 aa and a putative TM domain located in the C terminus, between residues 572 and 594.

A multiple alignment was conducted with all the Fet3 sequences present in the National Center for Biotechnology Information (NCBI) database, as well as with the MCOs from *P. chrysosporium* (Larrondo *et al.*, 2004) using CLUSTALW (see Fig. S1, available as supplementary data with the online version of this paper). The *P. chrysosporium* *fet3* gene (*Pc-fet3*) encodes a putative MCO with high homology to other fungal ferroxidases. Among the well-characterized Fet3 proteins, the highest similarities were to *N. crassa* (39.2 %) and *Sch. pombe* (37.1 %). The alignment and the manual examination of the Pc-Fet3 coding sequence revealed that the catalytic histidines participating in the copper centres (Piontek *et al.*, 2002) are encoded in exons II, III, V and VI. In contrast, in Pc-MCO1, these histidines are encoded in exons III, V, VI, VII, XVI, XIX and XX. Interestingly, the His codons located in exons V to VII are split by introns, exons VI and VII being only 5 and 3 nt long, respectively. Regarding the Glu residue involved in iron oxidation (see below), it is present in a modular exon in Pc-MCO1 and in a much larger exon in Pc-Fet3. For comparative purposes, these amino acids, with some of their neighbouring residues, are shown for both *Pc-fet3* and *Pc-mco1* in Fig. 2. On the other hand, as reported for other Fet3 enzymes, as well as for Pc-MCO1, the axial type 1

Fig. 2. Intron-exon composition of *Pc-fet3* and *Pc-mco1*. Catalytic histidines (H, bold) involved in copper binding, and the glutamic acid (E, underlined) believed to be involved in iron oxidation, are indicated in their corresponding sequence contexts.

canonical ligand corresponds to a Leu residue (L506) instead of the Met or Phe residues observed in ascorbate oxidases and some laccases, respectively (Xu *et al.*, 1998). Further analysis of this alignment can be found in the Discussion.

Analysis of the active site in the Pc-Fet3 model

A three-dimensional model of the Pc-Fet3 protein was built using as a template the recently solved structure of Sc-Fet3p (Taylor *et al.*, 2005). Table 1 shows the structural mapping of important residues from the active site of Sc-Fet3p into the Pc-Fet3 model. The active site of the Pc-Fet3 model is illustrated in Fig. 3. All the copper ligands of the T1 and T2/T3 centres in Sc-Fet3p are conserved in the Pc-Fet3 model, which include ten histidines and one cysteine. The acidic residues E185, D283 and D409 from Sc-Fet3p that are critical for iron binding are also present in the Pc-Fet3 model (E182, D304 and D426). The acidic residues involved in oxygen binding and turnover, D94 and D458 in Sc-Fet3p, are also conserved in Pc-Fet3 (D89 and D470). Therefore, all the residues that have been described as critical for ferroxidase activity in Sc-Fet3p are present in structurally equivalent positions in the three-dimensional model of Pc-Fet3. The only observed difference between Sc-Fet3p and Pc-Fet3 active sites was the variation of M345 in Sc-Fet3p to F364 in Pc-Fet3. Interestingly, this latter residue is also a Phe in all the basidiomycete Fet3-like sequences available in NCBI to date and in the *Sch. pombe* Fet3 protein analysed in this work (see consensus sequence at position 481 of the alignment in Fig. S1, available as supplementary data with the online version of this paper). This residue, along with another methionine that is conserved in the Pc-Fet3 model (M302), has been described as important for cuprous oxidase activity (Taylor *et al.*, 2005), but it has no impact on ferroxidase activity.

Identification and characterization of *Pc-ftr1*

Inspection of the flanking regions of *Pc-fet3* revealed a sequence with 43 % identity to *Sac. cerevisiae ftr1* (data not

shown). As previously mentioned, Ftr1 corresponds to the permease component of the Fet3/Ftr1 high-affinity iron-uptake complex (Severance *et al.*, 2004). *Pc-ftr1* is located in Scaffold 5 between coordinates 1 332 556 and 1 334 016, just 0.8 kb away from *Pc-fet3*. The two genes are in opposite transcriptional orientations and therefore separated by a divergent promoter (Fig. 1). Primers were designed to amplify the entire predicted coding region. Comparison of the *Pc-ftr1* gene with its cDNA showed the presence of three introns (data not shown). The cDNA encodes a 393 aa protein with a deduced molecular mass of 43.0 kDa. To confirm whether Pc-Ftr1 possesses the TM domains observed in other Ftr1 proteins (Severance *et al.*, 2004), an *in silico* analysis was conducted using the TMHMM software. This approach led to the identification of seven putative TM domains in positions that are equivalent to those observed in other Ftr1 sequences. These TM domains are highlighted in the multiple alignment of several Ftr1 ascomycete sequences built using CLUSTALW (see Fig. S2, available as supplementary data with the online version of this paper).

Expression of *Pc-fet3* and *Pc-ftr1*

Expression of the *fet3/ftr1* locus was analysed by Northern-blot hybridization as indicated in Methods. As shown in Fig. 4, both *Pc-fet3* and *Pc-ftr1* transcripts decreased dramatically upon addition of FeCl₃ to a final concentration of 250 µM. Negligible levels of these transcripts were observed 6 h after the addition of this salt to the culture medium. These results are in agreement with those obtained with other fungi (Kosman, 2003).

DISCUSSION

We have previously described the *Pc-mco1* gene from the basidiomycete *P. chrysosporium* (Larrondo *et al.*, 2003). This gene, a component of a cluster containing four MCO sequences (Larrondo *et al.*, 2004), encodes a novel MCO with strong ferroxidase activity. This constitutes an

Table 1. Structural mapping of key residues from the active site of the Sc-Fet3p X-ray structure into the Pc-Fet3 comparative model

This table shows the key residues for ferroxidase activity (copper ligands, iron binding, and oxygen binding and turnover) and also the putative residues involved in cuprous oxidase activity (Taylor *et al.*, 2005). Variations of identity between equivalent residues in the two structures are shown in bold type.

Sc-Fet3 X-ray structure	Pc-Fet3 model	Function
H413, C484, H489	H430, C496, H501	Cu1 ligands (T1 centre)
H128, H418, H483	H125, H435, H495	Cu2 ligands (T3 centre)
H83, H126, H485	H78, H123, H497	Cu3 ligands (T3 centre)
H81, H416	H76, H433	Cu4 ligands (T2 centre)
E185, D283, D409	E182, D304, D426	Fe(II) binding
D94, D458	D89, D470	O ₂ binding and turnover
M281, M345	M302, F364	Cu(I) binding

Fig. 3. Stereo views of the T1 and the T2/T3 Cu centres in the three-dimensional model of Pc-Fet3. Both sites are shown with the features that define ferroxidase activity. Identity and numbering of structurally equivalent residues from Sc-Fet3p are shown in parentheses. (A) In the T1 Cu site, two histidines (H430, H501) and a cysteine (C496) act as Cu1 ligands, whereas an aspartic acid (D426) and a glutamic acid (E182) participate in the electron transfer from the Fe(II) substrate to the Cu1 atom. These two residues, along with another aspartic acid (D304), are also involved in iron binding. (B) In the T2/T3 Cu site, two histidines (H76 and H433) act as Cu4 ligands. Two aspartic acids that have been suggested to participate in oxygen binding and turnover (Taylor *et al.*, 2005) are also shown.

unusual property in fungal MCOs distinct from Fet3 (Larrondo *et al.*, 2003). In this work, we describe *Pc-fet3*, which encodes a canonical Fet3-like ferroxidase in *P. chrysosporium*. There appears to be a single copy of this gene, as in *Pichia pastoris* (Paronetto *et al.*, 2001), *Sac.*

cerevisiae (Askwith *et al.*, 1994) and *C. albicans* (Eck *et al.*, 1999). In addition, we identified *Pc-fts1*, a gene encoding an iron permease that has been described as the functional partner of Fet3 (Stearman *et al.*, 1996). The adjacent genes lie in opposite transcriptional orientation and are separated by a divergent promoter. Based on the genome sequence, their location is distant from the MCO cluster (Larrondo *et al.*, 2004).

Comparative analysis of Pc-Fet3 with additional Fet3 enzymes from other fungi, as well as with Pc-MCO1, may help to further clarify the structural features underlying ferroxidase activity. In the last 10 years, a considerable amount of structural/functional information has been obtained for the *Sac. cerevisiae* Fet3p. In addition, preliminary characterization of the *Sch. pombe* (Askwith & Kaplan, 1997), *C. albicans* (Eck *et al.*, 1999), *Pichia pastoris* (Bonaccorsi di Patti *et al.*, 1999; Paronetto *et al.*, 2001) and *A. adenivorans* (Wartmann *et al.*, 2002) orthologues has contributed to a more detailed understanding of their properties and regulation. Nevertheless, no information regarding *fet3* genes from basidiomycetes is available. The massive release of fungal genomes has revealed the existence of sequences predicting putative Fet3 homologues in almost all sequenced organisms, with the exception of *Coccidioides immitis*, *Aspergillus nidulans* and *Coprinus cinereus* (Hoegger *et al.*, 2006). This increase in

Fig. 4. Effect of Fe(III) on transcript levels from *Pc-fet3* and *Pc-fts1*. *P. chrysosporium* was grown for 6 days in Avicel medium, after which it was harvested (C, control) or exposed to FeCl₃ to a final concentration of 250 µM for the indicated times. After the addition of this salt, cultures were harvested and total RNA was purified for Northern hybridization experiments.

the number of sequences populating the ferroxidase branch of the MCO family has been accompanied by a concomitant increase in members of the separate clade where Pc-MCO1 is located. As we had previously anticipated (Larrondo *et al.*, 2003), Pc-MCO1 has defined a new growing branch of the MCO family distinct from fungal laccases and ferroxidases. Hoegger *et al.* (2006) have given this new branch the name 'ferroxidases/laccases'.

Alignment of Pc-Fet3 with all the characterized and putative Fet3 sequences available in NCBI (see supplementary Fig. S1), as well as with the MCOs from *P. chrysosporium*, shows that Pc-Fet3 possesses a 24 aa insertion between residues 196 and 219, which is coincident with the presence of seven extra residues in the same area of Pc-MCO1. This insertion is not present in any of the ascomycete entries. Notably, insertions of variable size are consistently present in this area in the basidiomycete sequences so far deposited at NCBI (see consensus sequence between positions 299 and 324 of the alignment shown in Fig. S1). Three different Fet3 models from *Cryptococcus neoformans*, and one from *Auricularia polytricha*, *Cryptococcus bacillisporus* and *Ustilago maydis*, suggest that basidiomycete Fet3-like sequences might indeed have a distinctive insertion in this rather conserved domain. This observation could be of interest considering that one of the residues involved in iron oxidation (E182 in Pc-Fet3) is located nearby.

Site-directed mutagenesis in *fet3* from *Sac. cerevisiae* has revealed the involvement of residues E185 and Y354 in catalysis. Replacement of the former by Ala leads to a 95 % reduction in enzymic activity (Bonaccorsi di Patti *et al.*, 2000). This E185, which corresponds to E182 in Pc-Fet3 and to E214 in Pc-MCO1 (Larrondo *et al.*, 2004), is not present in ascorbate oxidases and laccases. With respect to Y354, its replacement by Phe in Sc-Fet3p reduces ferroxidase activity by only 50 %, indicating that it is not as critical for catalysis as E185 (Bonaccorsi di Patti *et al.*, 2000). This residue, conserved in all ascomycete Fet3 proteins analysed in this work, was also mapped into equivalent structural positions of the Sc-Fet3p X-ray structure and in the Pc-Fet3 model (Fig. 3, Table 1). All the basidiomycete ferroxidases (including Pc-MCO1) have an Arg residue in the equivalent position, with the sole exception of the *U. maydis* Fet3-like sequence, which also possesses a Tyr. Recent studies have also highlighted the importance of D283 and D409 from Sc-Fet3p in catalysis. These residues, according to the crystal structure of Sc-Fet3p, are located near the T1 copper centre (Taylor *et al.*, 2005). The former amino acid is conserved in all the ferroxidases analysed in this study with the exception of the MCOs from *P. chrysosporium* (e.g. MCO1 has a Thr in the equivalent position). The D409, which corresponds to D426 in Pc-Fet3, is conserved in all the proteins analysed, including Pc-MCO1 (see consensus sequence at positions 411 and 554 of the alignment in Fig. S1). These two residues also mapped into equivalent positions at the active site of the Pc-Fet3 model and the Sc-Fet3p X-ray structure

(Fig. 3, Table 1). On the other hand, the three residues described to be involved in iron binding, as well as the two residues involved in oxygen binding and turnover, are also conserved in Pc-Fet3. Their corresponding structural positions in the Pc-Fet3 model map into equivalent structural positions in the known X-ray structure of Sc-Fet3p (Fig. 3, Table 1). Therefore, comparative analysis of the active sites suggests that *Pc-fet3* encodes a canonical and active ferroxidase.

Pc-ftr1 encodes a 393 aa protein that shows about 38 % identity with several well-characterized Ftr1 sequences from ascomycetes, as based on CLUSTALW. Site-directed mutagenesis experiments in *Sac. cerevisiae ftr1* have indicated the importance of two REXLE (Arg-Glu-Xaa-Leu-Glu) motifs in TM domains 1 and 4 (Severance *et al.*, 2004). As expected, Pc-Ftr1 contains these two key conserved motifs (see Fig. S2). The predicted TM 1 (aa 10–32) in Pc-Ftr1 contains a REXLE motif (RETLE) and the TM 4 (aa 180–202) contains a REXZE motif (REGME), where X and Z are most commonly Gly and Leu, respectively. Substitutions of Arg or Glu by Ala in these motifs inactivate iron uptake (Stearman *et al.*, 1996; Severance *et al.*, 2004). We also found an EELWE motif associated with TM 3 and a DAXE motif located in the extracellular loop 6 that are highly conserved among Ftr1 proteins (data not shown) (Severance *et al.*, 2004). Interestingly, the Glu residue present in the DAXE motif (DASE in *Sac. cerevisiae* Ftr1p) is conserved in all Ftr1 proteins analysed, with the sole exception of Pc-Ftr1, where it is an Ala (see consensus sequence at position 362 of the alignment in Fig. S2). Site-directed mutagenesis experiments in the DASE motif of *Sac. cerevisiae* Ftr1p (Glu to Ala substitution, the same amino acid present in Pc-Ftr1) showed an enhanced capacity for iron uptake (Severance *et al.*, 2004). The relevance of these observations to Pc-Ftr1 activity remains uncertain, but an efficient Fet3/Ftr1 system in *P. chrysosporium* could have important implications for its lignocellulose degradation capabilities (see below). Additional differences between Pc-Ftr1 and Ftr1 sequences from other organisms include a 38 aa insertion near the N terminus (aa 42–79) that is also present in *N. crassa* Ftr1 and a DVD insertion (aa 277–279) in front of the aforementioned DAXE motif that is only present in Pc-Ftr1 (see Fig. S2).

The regulation of the expression of *Pc-fet3* and *Pc-ftr1* is similar to that of the corresponding orthologues in *Sac. cerevisiae*, where mRNA levels from both genes decrease upon iron supplementation (Askwith *et al.*, 1994; Stearman *et al.*, 1996). The coordinate expression of both genes is not only consistent with the presence of a divergent promoter, but also with the functional coupling of both proteins at the plasma membrane.

Iron has been implicated as an important component in the degradation of lignocellulose by wood-rotting fungi. Fe(II) can react with H₂O₂ (a metabolite that can reach millimolar levels in *P. chrysosporium* cultures), producing

hydroxyl radicals through the Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{Fe}^{3+} + \cdot\text{OH}$). This highly reactive oxygen species acts as a diffusible oxidant attacking nonspecifically all wood polymers. It is probably the main agent causing rapid cellulose depolymerization by brown rot fungi (Xu & Goodell, 2001; Cohen *et al.*, 2002, 2004; Hammel *et al.*, 2002), and to a lesser extent, by white rot fungi as well. Wood contains enough iron to make the generation of hydroxyl radicals through the Fenton reaction feasible (Koenigs, 1974), which contrasts with other biological systems, where iron is normally sequestered in redox-inactive complexes. Although the participation of hydroxyl radicals was long ago postulated in *P. chrysosporium* (Forney *et al.*, 1982; Kutsuki & Gold, 1982; Bes *et al.*, 1983; Kirk & Nakatsubo, 1983; Faison & Kirk, 1983; Evans *et al.*, 1984), subsequent studies have shown that the attack of lignin model compounds by Fenton chemistry leads to products different from those detected in ligninolytic cultures or by isolated peroxidases (Kirk *et al.*, 1985). Nevertheless, there is evidence that supports a role for Fenton chemistry in the degradation of lignocellulose by *P. chrysosporium* (Kremer & Wood, 1992a, b; Backa *et al.*, 1993; Wood, 1994; Henriksson *et al.*, 1995; Tanaka *et al.*, 1999). It has been shown that cellobiose dehydrogenase (CDH), an oxidative enzyme secreted by both brown- and white-rot fungi, is capable of generating hydroxyl radicals by reducing Fe(III) and producing H_2O_2 (Kremer & Wood, 1992a, b; Henriksson *et al.*, 1995). Although the real impact of these hydroxyl radicals in lignin mineralization remains controversial, increasing evidence supports their active role in accelerating the depolymerization of cellulose by disrupting its crystalline structure and facilitating the subsequent attack by hydrolytic enzymes (reviewed by Mason *et al.*, 2003). Another important fungal pathway for the generation of hydroxyl radical involves an extracellular hydroquinone-quinone redox cycle. Thus, the brown rotters *Gloeophyllum trabeum* and *Postia placenta* generate a hydroquinone-driven Fenton system which seems to be largely responsible for their ability to attack wood (Kerem *et al.*, 1999; Jensen *et al.*, 2001; Cohen *et al.*, 2002; reviewed by Hammel *et al.*, 2002). Whether some of these iron-siderophore complexes produced by wood rotters also serve a role in iron uptake remains to be determined (Fekete *et al.*, 1989; Hernandez-Macedo *et al.*, 2002; Assmann *et al.*, 2003).

On the other hand, iron is a critical structural element for the striking set of over 150 cytochrome P450s that this micro-organism possesses (Martinez *et al.*, 2004; Doddapaneni & Yadav, 2005). Moreover, it is also present in the active sites of ligninolytic enzymes such as LiPs (lignin peroxidases), MnPs (manganese peroxidases) and CDH. However, in spite of the importance of iron in the process of wood decay by fungi, little is still known about its uptake and homeostasis and the impact of these processes in wood-rotting. In this regard, we would like to suggest that the presence of two types of ferroxidases in this fungus might contribute to the spatio-temporal

coordination of ligninolytic and cellulolytic activities by controlling the levels of ferrous iron available for Fenton chemistry. As previously proposed (Larrondo *et al.*, 2003), the iron oxidase activity of the extracellular Pc-MCO1 may modulate iron-based reactions further away from the hyphae, having a potential impact on CDH-iron reactions as well as on iron-siderophore chemistry. On the other hand, the Fet3/Ftr1 protein complex may accomplish a similar role at the plasma membrane by controlling iron uptake and transport inside the cell while ensuring that its intracellular concentrations are in accordance with the physiological needs.

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REFERENCES

- Askwith, C. & Kaplan, J. (1997). An oxidase-permease-based iron transport system in *Schizosaccharomyces pombe* and its expression in *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 401–405.
- Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M. & Kaplan, J. (1994). The *FET3* gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76**, 403–410.
- Assmann, E. M., Ottoboni, L. M., Ferraz, A., Rodriguez, J. & De Mello, M. P. (2003). Iron-responsive genes of *Phanerochaete chrysosporium* isolated by differential display reverse transcription polymerase chain reaction. *Environ Microbiol* **5**, 777–786.
- Backa, S., Gierer, J., Reitberger, T. & Nilsson, T. (1993). Hydroxyl radical activity associated with the growth of white-rot fungi. *Holzforschung* **47**, 181–187.
- Baldrian, P. (2006). Fungal laccases – occurrence and properties. *FEMS Microbiol Rev* **30**, 215–242.
- Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**, 783–795.
- Bes, B., Ranjeva, R. & Bondet, A. M. (1983). Evidence for the involvement of activated oxygen in fungal degradation of lignocellulose. *Biochimie* **65**, 283–289.
- Bonaccorsi di Patti, M. C., Bellenchi, G. C., Bielli, P. & Calabrese, L. (1999). Release of highly active Fet3 from membranes of the yeast *Pichia pastoris* by limited proteolysis. *Arch Biochem Biophys* **372**, 295–299.
- Bonaccorsi di Patti, M. C., Felice, M. R., Camuti, A. P., Lania, A. & Musci, G. (2000). The essential role of Glu-185 and Tyr-354 residues in the ferroxidase activity of *Saccharomyces cerevisiae* Fet3. *FEBS Lett* **472**, 283–286.
- Cohen, R., Jensen, K. A., Houtmanm, C. J. & Hammel, K. E. (2002). Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose. *FEBS Lett* **531**, 483–488.
- Cohen, R., Suzuki, M. R. & Hammel, K. E. (2004). Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl Environ Microbiol* **70**, 324–331.

- de Silva, D., Davis-Kaplan, S., Fergestad, J. & Kaplan, J. (1997). Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. *J Biol Chem* **272**, 14208–14213.
- Doddapaneni, H. & Yadav, J. S. (2005). Microarray-based global differential expression profiling of P450 monooxygenases and regulatory proteins for signal transduction pathways in the white rot fungus *Phanerochaete chrysosporium*. *Mol Genet Genomics* **274**, 454–466.
- Eck, R., Hundt, S., Hartl, A., Roemer, E. & Kunkel, W. (1999). A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption. *Microbiology* **145**, 2415–2422.
- Evans, C., Farmer, J. Y. & Palmer, J. M. (1984). An extracellular heme protein from *Coriolus versicolor*. *Phytochemistry* **23**, 1247–1250.
- Faison, B. D. & Kirk, T. K. (1983). Relationship between lignin degradation and production of reduced oxygen species by *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **46**, 1140–1145.
- Fekete, F. A., Chandhoke, V. & Jellison, J. (1989). Iron-binding compounds produced by wood-decaying basidiomycetes. *Appl Environ Microbiol* **55**, 2720–2722.
- Forney, L. J., Reddy, C. A., Tien, M. & Aust, S. D. (1982). The involvement of hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white rot fungus *Phanerochaete chrysosporium*. *J Biol Chem* **257**, 11455–11462.
- Hammel, K. E., Kapich, A. N., Jensen, K. E., Jr & Ryan, Z. C. (2002). Reactive oxygen species as agents of wood decay by fungi. *Enzyme Microb Technol* **30**, 445–453.
- Hassett, R. F., Yuan, D. S. & Kosman, D. J. (1998). Spectral and kinetic properties of the Fet3 protein from *Saccharomyces cerevisiae*, a multinuclear copper ferroxidase enzyme. *J Biol Chem* **273**, 23274–23282.
- Henriksson, G., Ander, P., Pettersson, B. & Pettersson, G. (1995). Cellobiose dehydrogenase (cellobiose oxidase) from *Phanerochaete chrysosporium* as a wood-degrading enzyme. Studies on cellulose, xylan, and synthetic lignin. *Appl Microbiol Biotechnol* **42**, 790–796.
- Hernandez-Macedo, M. L., Ferraz, A., Rodríguez, J., Ottoboni, L. M. & De Mello, M. P. (2002). Iron-regulated proteins in *Phanerochaete chrysosporium* and *Lentinula edodes*: differential analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis profiles. *Electrophoresis* **23**, 655–661.
- Hoegger, P. J., Kilaru, S., James, T. Y., Thacker, J. R. & Kües, U. (2006). Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS J* **273**, 2308–2326.
- Jensen, K. A., Houtman, C. J., Ryan, Z. C. & Hammel, K. E. (2001). Pathways for extracellular Fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl Environ Microbiol* **67**, 2705–2711.
- Kerem, Z., Jensen, K. A. & Hammel, K. E. (1999). Biodegradative mechanism of the brown rot basidiomycete *Gloeophyllum trabeum*: evidence for an extracellular hydroquinone-driven fenton reaction. *FEBS Lett* **446**, 49–54.
- Kirk, T. K. & Nakatsubo, F. (1983). Chemical mechanism of an important cleavage reaction in the fungal degradation of lignin. *Biochim Biophys Acta* **756**, 376–384.
- Kirk, T. K., Mozuch, M. D. & Tien, M. (1985). Free hydroxyl radical is not involved in an important reaction of lignin degradation by *Phanerochaete chrysosporium* Burds. *Biochem J* **226**, 455–460.
- Koenigs, J. W. (1974). Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown rot basidiomycetes. *Wood Fiber Sci* **6**, 66–80.
- Kosman, D. J. (2003). Molecular mechanisms of iron uptake in fungi. *Mol Microbiol* **47**, 1185–1197.
- Kremer, S. M. & Wood, P. M. (1992a). Production of Fenton's reagent by cellobiose oxidase from cellulolytic cultures of *Phanerochaete chrysosporium*. *Eur J Biochem* **208**, 807–814.
- Kremer, S. M. & Wood, P. M. (1992b). Evidence that cellobiose oxidase from *Phanerochaete chrysosporium* is primarily an Fe(III) reductase. *Eur J Biochem* **205**, 133–138.
- Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**, 567–580.
- Kutsuki, H. & Gold, M. H. (1982). Generation of hydroxyl radical and its involvement in lignin degradation by *Phanerochaete chrysosporium*. *Biochem Biophys Res Commun* **109**, 320–327.
- Larrondo, L. F., Salas, L., Melo, F., Vicuña, R. & Cullen, D. (2003). A novel extracellular multicopper oxidase with ferroxidase activity in *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **69**, 6257–6263.
- Larrondo, L. F., González, B., Cullen, D. & Vicuña, R. (2004). Characterization of a multicopper oxidase gene cluster in *Phanerochaete chrysosporium* and evidence of altered splicing of the *mco* transcripts. *Microbiology* **150**, 2775–2783.
- Manubens, A., Avila, M., Canessa, P. & Vicuña, R. (2003). Differential regulation of genes encoding manganese peroxidase (MnP) in the basidiomycete *Ceriporiopsis subvermispora*. *Curr Genet* **43**, 433–438.
- Martinez, D., Larrondo, L. F., Putnam, N., Gelpke, M. D., Huang, K., Chapman, J., Helfenbein, K. G., Ramaiya, P., Detter, J. C. & other authors (2004). Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat Biotechnol* **22**, 695–700.
- Mason, M. G., Nicholls, P. & Wilson, M. T. (2003). Rotting by radicals – the role of cellobiose oxidoreductase? *Biochem Soc Trans* **31**, 1335–1336.
- Mertz, L. M. & Rashtchian, A. (1994). Nucleotide imbalance and polymerase chain reaction: effects on DNA amplification and synthesis of high specific activity radiolabeled DNA probes. *Anal Biochem* **221**, 160–165.
- Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A. & Finazzi-Agro, A. (1992). A refined crystal structure of ascorbate oxidase at 1.9 Å resolution. *J Mol Biol* **224**, 179–205.
- Paronetto, M. P., Miele, R., Maugliani, A., Borro, M. & Bonaccorsi di Patti, M. C. (2001). Cloning of *Pichia pastoris* Fet3: insights into the high affinity iron uptake system. *Arch Biochem Biophys* **392**, 162–167.
- Piontek, K., Antorini, M. & Choinowski, T. (2002). Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *J Biol Chem* **277**, 37663–37669.
- Radisky, D. & Kaplan, J. (1999). Regulation of transition metal transport across the yeast plasma membrane. *J Biol Chem* **274**, 4481–4484.
- Sali, A. & Blundell, T. L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* **234**, 779–815.
- Severance, S., Chakraborty, S. & Kosman, D. J. (2004). The Ftr1p iron permease in the yeast plasma membrane: orientation, topology and structure–function relationships. *Biochem J* **380**, 487–496.
- Solomon, E. I., Sundaram, U. M. & Machonkin, T. E. (1996). Multicopper oxidases and oxygenases. *Chem Rev* **96**, 2563–2606.
- Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D. & Dancis, A. (1996). A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science* **271**, 1552–1557.

Tanaka, H., Itakura, S. & Enoki, A. (1999). Hydroxyl radical generation by an extracellular low-molecular weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. *Holzforschung* **53**, 21–28.

Taylor, A. B., Stoj, C. S., Ziegler, L., Kosman, D. J. & Hart, P. J. (2005). The copper-iron connection in biology: structure of the metallo-oxidase Fet3p. *Proc Natl Acad Sci U S A* **102**, 15459–15464.

Wartmann, T., Stephan, U. W., Bube, I., Boer, E., Melzer, M., Manteuffel, R., Stoltenburg, R., Guengerich, L., Gellissen, G. & Kunze, G. (2002). Post-translational modifications of the *AFET3* gene product: a component of the iron transport system in budding cells and mycelia of the yeast *Arxula adeninivorans*. *Yeast* **19**, 849–862.

Wood, P. M. (1994). Pathways for production of Fenton's reagent by wood-rotting fungi. *FEMS Microbiol Rev* **13**, 313–320.

Wymelenberg, A. V., Denman, S., Dietrich, D., Bassett, J., Yu, X., Atalla, R., Predki, P., Rudsander, U., Teeri, T. T. & Cullen, D. (2002).

Transcript analysis of genes encoding a family 61 endoglucanase and a putative membrane-anchored family 9 glycosyl hydrolase from *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **68**, 5765–5768.

Xu, F., Berka, R. M., Wahleithner, J. A., Nelson, B. A., Shuster, J. R., Brown, S. H., Palmer, A. E. & Solomon, E. I. (1998). Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. *Biochem J* **334**, 63–70.

Xu, G. & Goodell, B. (2001). Mechanisms of wood degradation by brown-rot fungi: chelator-mediated cellulose degradation binding of iron by cellulose. *J Biotechnol* **87**, 43–57.

Zaitsev, V. N., Zaitseva, I., Papiz, M. & Lindley, P. F. (1999). An X-ray crystallographic study of the binding sites of the azide inhibitor and organic substrates to ceruloplasmin, a multi-copper oxidase in the plasma. *J Biol Inorg Chem* **4**, 579–587.

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