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ORIGINAL PAPER

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# Identification and functional characterisation of *ctr1*, a *Pleurotus ostreatus* gene coding for a copper transporter

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**Abstract** Copper homeostasis is crucial for the maintenance of life. In lignin-degrading fungi, copper is essential for the phenol oxidase enzymes that provide this activity. In this paper we report the characterization of a gene (*ctr1*) coding for a copper transporter in the white rot fungus *Pleurotus ostreatus*. The gene was identified in a cDNA library constructed from 4-day-old vegetative mycelium grown in liquid culture. The results presented here demonstrate that: (1) *ctr1* functionally complements the respiratory deficiency of a yeast mutant defective in copper transport, supporting the idea that the Ctr1 protein is itself a copper transporter; (2) transcription of *ctr1* is detectable in *P. ostreatus* at all developmental stages and in all tissues (with the exception of lamellae), and is negatively regulated by the presence of copper in the culture medium; (3) *ctr1* is a single-copy gene that maps to *P. ostreatus* linkage group III; and (4) the regulatory sequence elements found in the promoter of *ctr1* are similar to those found in other copper-related genes described in other systems. These results provide the first description of a copper transporter in this white rot fungus and should be useful for further studies on copper metabolism in higher basidiomycetes.

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## Introduction

Copper is an indispensable cation for the maintenance of living cells because of its particular redox properties. It acts as a cofactor in proteins involved in respiration and photosynthesis, the elimination of free radicals, iron homeostasis, normal cell growth and development (Koch et al. 1997). If copper accumulates to high levels in cells, cell death can occur due to changes in redox processes and the formation of destructive hydroxyl free radicals. Aerobic organisms have developed complex homeostatic mechanisms to combat copper toxicity (Puig and Thiele 2002).

In eukaryotes, two copper uptake activities with different affinities and capacities have been reported. In *Saccharomyces cerevisiae*, the model organism where copper transporter mechanisms have been most extensively studied (Labbe and Thiele 1999), one low-affinity (CTR2) and two redundant high-affinity [CTR1 (Dancis et al. 1994b) and CTR3 (Knight et al. 1996)] copper transporters have been identified. Complementation of yeast mutants that are defective in copper uptake with animal, plant, and fungal cDNAs has resulted in the identification of new proteins of this family. All copper transporters share structurally conserved domains such as three transmembrane regions, a variable number of methionines arranged as MxxM and MxM motifs at the N-terminal end, and a number of functionally important charged amino acids at the C-terminal end. In contrast to the case in ascomycetous fungi, such as *S. cerevisiae*, *Candida albicans* and *Podospora anserina*, copper homeostasis in basidiomycetes remains largely unexplored, and only two genes for P-type ATPases [ *tahA* (Uldschmid et al. 2002) and *ctaA* (Uldschmid et al. 2003)] involved in copper trafficking in the white rot fungus *Trametes versicolor* have been described.

The edible basidiomycete *Pleurotus ostreatus* (the oyster mushroom) is able to secrete a number of ligninolytic enzymes that can be used in the bioconversion of agricultural wastes and biodegradation of organopollutants, xenobiotics and industrial contaminants (Cohen et al. 2001). *Pleurotus* carries out lignin degradation by secreting enzymes belonging, among others, to the family of laccases (Giardina et al. 1996). These enzymes are members of the blue copper oxidase family which contain four copper atoms per molecule. The biological roles of fungal laccases involve ligninolytic activities, fruit body formation, pigmentation and pathogenicity. Moreover, the enzymatic properties of copper-dependent laccases have led to investigations of their use in selected bioremediation strategies and biotechnological applications (Gianfreda et al. 1999; Larsson et al. 2001). Studies of the expression of laccase izoenzymes in *Pleurotus* have demonstrated that copper regulates transcription of the corresponding structural genes (Palmieri et al. 2000).

By analysing gene expression during vegetative submerged growth of *P. ostreatus*, we have isolated a gene (*ctr1*) that codes for a copper transporter and is able to complement the respiratory deficiency of a *ctr1* null–mutant of *S. cerevisiae*. We describe its exon–intron organisation, its genomic environment, its chromosomal location and its conservation in other *Pleurotus* species. This is the first report of a protein that facilitates copper transport in the genus *Pleurotus* and provides a basis for further investigations of the regulation of copper acquisition in higher fungi.

## Materials and methods

### Strains and culture conditions

*Pleurotus ostreatus* N001 was the dikaryotic strain used in this work. Its characteristics, as well as those of its monokaryotic protoclones, have been described elsewhere (Larraya et al. 1999b, 2000). *Pleurotus* and yeast cultures were grown in liquid SMY broth [1% (w/v) sucrose, 1% (w/v) malt extract, 0.45% (w/v) yeast extract, pH 5.6], Malt Extract Medium, or MM (minimal medium) [2 mM $MgSO_4$, 0.4 mM $KH_2PO_4$, 5.7 mM $K_2HPO_4$, 1 mM $(NH_4)_2HPO_4$], and incubated at 24°C in the dark. Fruiting was induced by a cold shock (4°C, overnight) and subsequent incubation under a regime of 12 h light/12 h darkness.

*Saceharomyces cerevisiae* strain 83 (*ctr1-3*), a mutant that is deficient in copper uptake and lacks all three *CTR* genes, is unable to respire due to lack of cytochrome oxidase activity (Dancis et al. 1994a). This strain was used for functional complementation analysis. A related wild-type *S. cerevisiae* strain (CECT 1328) was used as the positive control. For growth under non-selective conditions, YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] was used as the culture medium. The ability to grow on a non-fermentable carbon source was assayed by replacing the glucose in YPD with 3% (v/v) glycerol or ethanol (YPG or YPE, respectively). Iron starvation was achieved by addition of the iron chelator 3-(2-pyrydyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (BPS, 750 μM) to YPD medium. To ensure starvation for copper, the copper chelator bathocuproinedisulfonic acid (BCS, 33 μM) was added to the medium together with 1 mM ascorbic acid. Copper-rich medium was made by adding $CuSO_4$ (100 μM) to YPD after autoclaving. $Ura^+$ yeast transformants were selected on SC-ura (glucose synthetic media lacking uracil) medium [2% (w/v) glucose, 0.7% (w/v) yeast nitrogen base without amino acids, 0.09% (w/v) synthetic complete drop out medium mix without uracil]. Yeast transformation was performed using the lithium acetate method according to Gietz and Woods (1994).

*Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used for cloning, maintenance, and propagation of plasmids. *E. coli* K803 [ *supF met, hsdS*($r^-$ , $m^-$) *supF*] was used for bacteriophage propagation. Bacterial cultures were grown on LB (Scharlab, Barcelona, Spain) and NZ media [1% (w/v) peptone of casein acid hydrosylate, 85.5 mM NaCl, 0.1% (w/v) casamino acids, 0.16 M $MgSO_4$, pH 7.5]. λEMBL4 phage was used for construction of the genomic library. General molecular manipulations and PCR were performed as described by Sambrook et al. (1989) and Dieffenbach and Dveksler (1993), respectively.

### Construction of cDNA and genomic libraries of *P. ostreatus* N001

High-quality mRNA was purified from *P. ostreatus* N001 grown for 4 days on SMY using a messenger RNA isolation kit (Stratagene), and cloned directionally into the pBluescript II SK(+) vector (pBluescript II XR cDNA Library Construction Kit; Stratagene). The library was then transformed into *E. coli* XL1-Blue cells and amplified.

Genomic DNA was purified from vegetative *P. ostreatus* N001 mycelium as described elsewhere (Larraya et al. 1999a). The purified DNA was sheared by passing it through a 0.7×31 mm needle five times, and the ends of the fragmented DNA were repaired by ligation to *Bam*HI-adapters using T4 DNA polymerase. The mixture was then size-separated by centrifugation on a 10–40% (w/v) sucrose gradient, and pooled fragments ranging in size from 12 to 22 kb were ligated to λEMBL4 arms (cut with *Bam*HI) and packaged using Stratagene packaging extracts.

### Plasmid construction

Clones were picked from the cDNA library at random and sequenced. Sequencing of plasmid pMN4-0012 revealed a cDNA insert of 790 bp containing the ORF for a putative copper transporter. To obtain the corre-

sponding genomic sequence, PCR was carried out with the two primers BamHI-5′ (5′-cgggatccATGTCTCACGGTGAC-3′; the sequence in upper case corresponds to positions 1–15 of the cDNA sense strand, the sequence in lower case includes the *BamH*I site introduced by the primer) and EcoRI-3′ (5′-cggaattcCTAGTGAAAGCCATGG-3′; upper case corresponds to the stretch from the stop codon to position 628 of the cDNA antisense strand, the sequence in lower case includes the *Eco*RI site introduced by the primer). PCR products were purified, cleaved with *Bam*HI and *Eco*RI, and cloned into the yeast vector p426GPD (Mumberg et al. 1995) cut with *Bam*HI and *Eco*RI. The resulting plasmid p426GPD-MN4-0012 was transformed into *S. cerevisiae* strain 83. Yeast transformants containing the p426GPD plasmid were selected by plating on SC-ura medium. Functional complementation by the *ctr1* gene from *Pleurotus* was tested by screening for growth of the $Ura^+$ clones on YGP and YPE plates. Plasmids containing the *ctr1* gene were rescued from the yeast transformants using a glass-bead lysis procedure (Hoffman and Winston 1987) and transformed into *E. coli*. Inserts were isolated from purified plasmids and subcloned into the pBluescript vector. The presence of *ctr1* in recombinant plasmids was confirmed by sequencing of miniprep-grade DNA purified from bacteria.

PCRs were performed in a final volume of 25 µl containing 200 ng of genomic DNA as template, 2 mM $MgCl_2$, 67 mM TRIS-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.1 g/l Tween-20, each dNTP at 200 µM, 1 U of Taq polymerase (Eurobiotaq, Ecogen, Barcelona, Spain) and 50 pmol of the specific primer oligonucleotides. PCRs were incubated for 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1.5 min at 60°C and 1.5 min at 72°C) and a final 5-min incubation at 72°C. One-tenth of each reaction volume was assayed by electrophoresis on a 0.8% (w/v) agarose gel run in IX-TAE (Sambrook et al. 1989).

### Genetic linkage analysis

The gene *ctr1* was mapped to the genetic linkage map of *P. ostreatus* var. *florida* using the procedure described by Larraya et al. (2000). For RFLP analysis, hybridisations were performed at 65°C as described by Church and Gilbert (1984) under high-stringency conditions. The linkage analysis was performed using the MAPRF program (Lebrun et al. 2001).

### Expression analysis

Total RNA from *P. ostreatus* strain N001 and from the transformed yeast strain was extracted using the procedure described by Wessels et al. (1987). RNA was quantified by UV spectrophotometry and its integrity was visually assessed on ethidium bromide-stained agarose gels after electrophoresis. Northern blots were performed according to Sambrook et al. (1989). Total RNA (1–5 µg) isolated from the indicated tissues was used as the template for cDNA synthesis by reverse transcription (5′/3′ RACE kit; Roche Diagnostics, Mannheim, Germany) using the primer EcoRI-3′ (see above). The cDNA was used as the template for PCR with the specific oligonucleotides designed for amplification of the *ctr1* transcript.

### DNA sequence analysis

The sequence of both strands of all constructs was determined by automated sequencing. Multiple alignments were carried out automatically, using the program CLUSTAL W (Thompson et al. 1994). The programs BLASTX and BLASTN (Altschul et al. 1997) were used to search the databases. Predictions of membrane-spanning domains were obtained with HMMTOP, an automatic server for predicting transmembrane helices and topology of proteins (Tusnády and Simon 2001).

### Sequence accession number

The annotated genomic sequence of *P. ostreatus ctr1* has been deposited in the EMBL and GenBank nucleotide sequence databases under the Accession No. AJ705045.

## Results

### Identification and characterisation of *P. ostreatus ctr1*

A cDNA library was constructed using RNA isolated from a static culture of the dikaryotic *P. ostreatus* strain N001 grown for 4 days in liquid SMY cultures, with the aim of identifying new *P. ostreatus* genes expressed at this developmental stage. Among the clones isolated from this library was one that contained an approximately 770-bp insert with a continuous ORF coding for a protein of 189 amino acid residues. When this sequence was used as the query in a BLASTX search for similar proteins several known or hypothetical proteins from different organisms (*Neurospora crassa*, *Caenorhabditis elegans*, *S. cerevisiae*, *P. anserina*, and *Homo sapiens*) were identified. The putative protein coded for by the *P. ostreatus* cDNA showed a significant level of similarity to PaCtr2 (20% identity), a low-affinity copper transporter from *P. anserina* (Borghouts et al. 2002) and to CTR1 (20% identity), a high-affinity copper transporter from *S. cerevisiae* (Dancis et al. 1994b). These similarities suggested that the *Pleurotus* cDNA coded for a putative copper transporter, which we called Ctr1. Furthermore, the alignment of the *P. ostreatus* Ctr1 sequence with those of other copper transporters revealed great similarity in size (Fig. 1) and the presence of a conserved sequence motif ($MLX_2M$), present in all these copper transporters at their C-terminal ends.

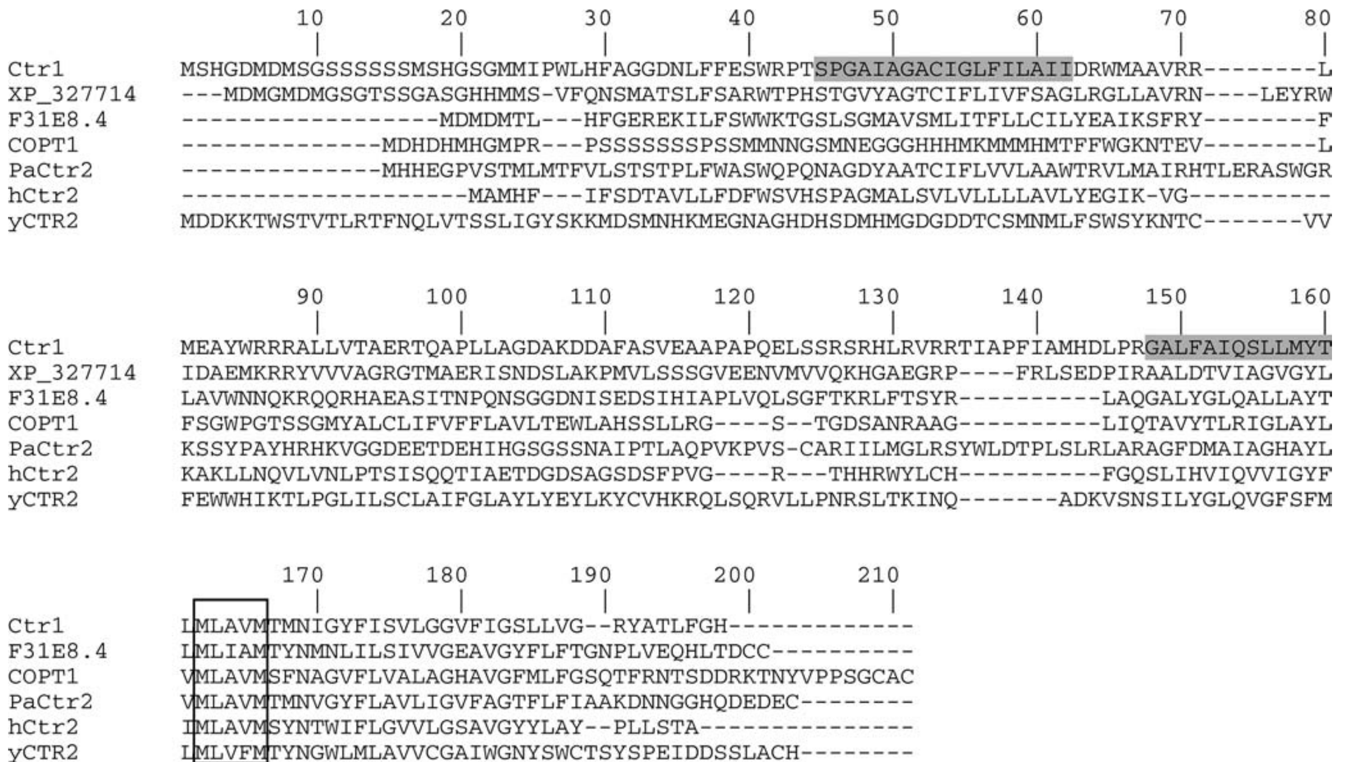

**Fig. 1** Alignment of the predicted amino acid sequence of the *P. ostreatus* Ctr1 protein with *N. crassa* predicted protein XP_327714 (Galagan et al. 2003), hypothetical protein F31E8.4 from *Caenorhabditis elegans*, *Arabidopsis thaliana* COPT1 (Kampfenkel et al. 1995), *Podospora anserina* PaCtr2 (Borghouts et al. 2002), human hCtr2 (Zhou and Gitschier 1997), and *Saccharomyces cerevisiae* yCTR2 (Kampfenkel et al. 1995). Gaps have been introduced to improve the homologies between the proteins. The *box* (positions 162–166) indicates the conserved $MLX_2M$ motif, which lies within the third predicted transmembrane domain (162–179) in Ctr1. The other two putative transmembrane domains in Ctr1 (45–62, 140–167) are *highlighted*

Restriction fragment length polymorphism (RFLP) analysis, performed using the *ctr1* cDNA as probe on Southern blots of *P. ostreatus* DNA, revealed that it corresponded to a single-copy gene (data not shown). The genomic region containing *ctr1* was selected from a *P. ostreatus* N001 genomic λ library using *ctr1* cDNA as probe, and a 4410-bp *Eco*RI fragment was sequenced. Comparison of the genomic and cDNA sequences allowed us to deduce the complete mRNA sequence coding for *ctr1*, which consists of a 570-bp ORF flanked by a 65-bp 5′UTR, and a 130-bp 3′UTR followed by the polyA tail. A putative polyadenylation signal (ATATAAA) was present 77 nucleotides upstream of the −3 relative to the putative start codon, in accordance with the 'first-AUG-rule' (Kozak 1984). The gene is made up of three exons of 239, 234 and 97 bp, respectively, interrupted by two introns of 67 and 55 bp long; the splice junctions conform to the GT...AG rule proposed for filamentous fungal genes (Gurr et al. 1988). The GC content of the complete *ctr1* gene is 50.5% (exons 54.9%; introns 42.6%).

A BLASTX search using the 4,410 bp sequence as the query suggested that a gene for a hypothetical protein belonging to the family of rhodanases was located 511 bp upstream from the *ctr1* start codon and oriented divergently to *ctr1*. A BLASTN search of EST databases revealed that a region starting at position +1737 from the *ctr1* start codon (385 bp downstream of *ctr1* stop codon) showed marked homology to a *P. ostreatus* EST isolated from liquid-cultured mycelia (Lee et al. 2002); this putative gene was in the same orientation that *ctr1*.

The primers 5′-BamHI and 3′-EcoRI (see Materials and methods) were used to amplify *ctr1* sequences from different strains of *P. ostreatus*, and from isolates of *P. pulmonarius*, *P. colombinus*, *P. cornucopiae*, *P. eryngii*, *P. sapidus* and *P. sajor-caju* with the aim of studying the conservation of this gene in this genus. A PCR product of similar size to the genomic sequence of *ctr1* was detected in all the strains analysed (data not shown).

The *ctr1* gene was mapped on the genetic linkage map of *P. ostreatus* var. *florida* (Larraya et al. 2000) by analysing the segregation of *ctr1* RFLP alleles in a set of 80 monokaryons derived from the dikaryotic strain N001. The linkage analysis mapped *ctr1* to linkage group III, cosegregating with marker $P17_{1425}$ and lying 6 cM from the marker $R8_{575}$ and 8 cM from *matA*.

## Functional analysis of *ctr1*

To investigate the role of Ctr1 in copper transport, a strain of *S. cerevisiae* which is defective in copper uptake, called strain 83 (*ctr1 ctr2 ctr3*), was transformed with the *ctr1* cDNA from *P. ostreatus ctr1* to test for

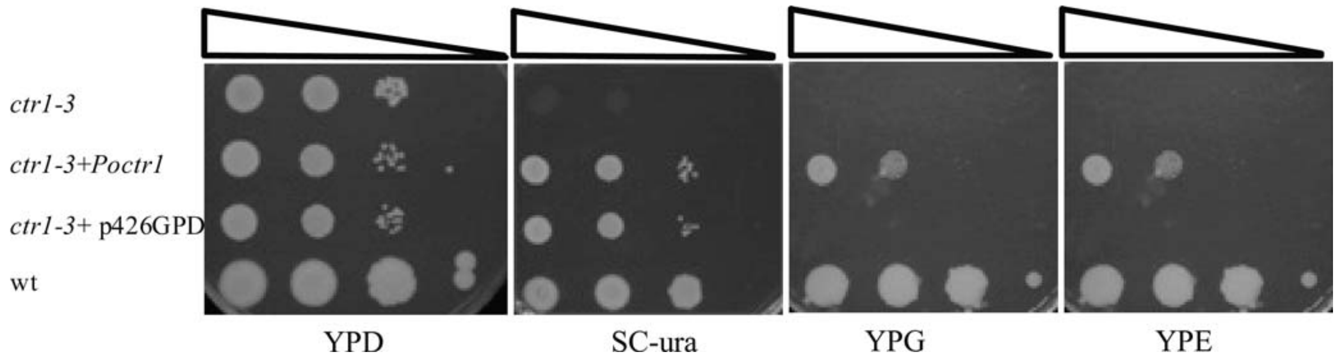


**Fig. 2** Functional analysis of the *ctr1* gene: complementation of strain 83 of *S. cerevisiae* (*ctr1-3*). Aliquots (5 μl) of $10^{-2}$ dilutions of a yeast culture were spotted on selective agar plates and incubated for 7 days at 30°C. *ctr1-3*, *S. cerevisiae* strain 83, defective in copper uptake; *ctr1-3 Poctr1*, strain 83 transformed with the plasmid p426GPD carrying the *P. ostreatus ctr1*cDNA; *ctr1-3* + p426GPD, strain 83 transformed with a control plasmid without the *ctr1 insert*; *wt*, wild-type control (yeast strain CECT 1328)

functional complementation. The cDNA was cloned into the yeast vector p426GPD and expressed in yeast under the control of the glycerol-3-phosphate dehydrogenase (GPD) gene promoter (Mumberg et al. 1995). Control transformants (*ctr1-3* + p426GPD; Fig. 2) were able to grow on SC-ura, demonstrating the presence of the expression vector, but unable to grow on YPG or YPE medium supplemented with galactose (0.02% w/v) which induces a ten-fold increase in expression of the p426GPD promoter. However, growth of the *S. cerevisiae* mutant on YPG or YPE was restored when the construct carrying the *P. ostreatus ctr1* cDNA (p426GPD-MN4-0012) was transformed into the strain (*ctr1–3* + *Poctr1*; Fig. 2). Expression of the *P. ostreatus ctr1* gene in yeast transformants growing on YPG was verified by Northern hybridisation (data not shown) and confirmed by RT-PCR analysis (Fig. 3).

### Expression of the *P. ostreatus ctr1* gene

The expression profile of *ctr1* gene in *P. ostreatus* was studied by Northern analysis using dikaryon N001 grown in submerged culture in media containing a range of copper concentrations (Fig. 4). The dikaryon used in these analyses had previously been cultivated on minimal medium to standardise the mycelial growth. *ctr1* was expressed at similar levels in the control and under copper or iron limitation (Fig. 4, lanes 1–3). A lower expression level, however, was detected in mycelia grown on YPD supplemented with copper. These results were confirmed by RT-PCR (data not shown).

The temporal and spatial expression pattern of *ctr1* in *P. ostreatus* was studied in dikaryon N001 and in its protoclones PC9 and PC15 by RT-PCR. The experiment was performed with DNAse I-treated RNA samples obtained from mycelia grown on SMY for various times, and with RNA samples obtained from complete mature fruit-bodies and gills (Fig. 5). Expression of *ctr1* was detected in the vegetative mycelium during all stages of culture, in both monokaryons (PC9 and PC15) and in the dikaryon N001. However, although *ctr1* expression was detected in fruit bodies, no transcript could be found in the lamellae.

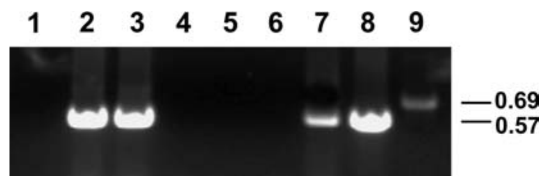


**Fig. 3** Expression of the *P. ostreatus ctr1* gene in yeast strains detected by RT-PCR. RNAs from two yeast colonies carrying p426GPD-MN4-0012 (*lanes 2* and *3*) or p426GPD (*lane 4*), the yeast mutant *ctr1-3* (*lane 5*) and wild type *S. cerevisiae* cells (*lane 6*) were used as templates. RNA from the N001 dikaryon cultivated on YPD (*lane 7*), the *ctr1* cDNA cloned in the pBluescript vector (*lane 8*), and dikaryotic genomic DNA (*lane 9*) were used as positive controls

## Discussion

In this work, we have isolated a clone, from a *P. ostreatus* cDNA library constructed out of early dikaryotic

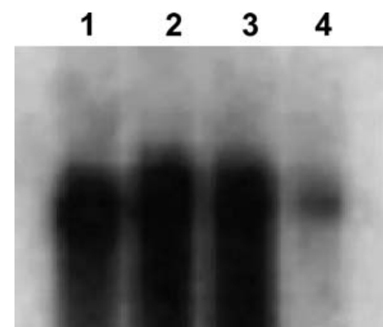


**Fig. 4** Expression analysis of *ctr1* in *P. ostreatus*. RNA was purified from N001 grown on YPD (*lane 1*), YPD supplemented with the copper chelator BCS (*lane 2*), YPD supplemented with the ferrous iron chelator ferrozine BPS (*lane 3*), and YPD supplemented with $CuSO_4$ (*lane 4*). The blot was probed with *ctr1* cDNA and the film was overexposed to show the low expression level detected in lane 4. The RNA was stained with ethidium bromide as a loading control

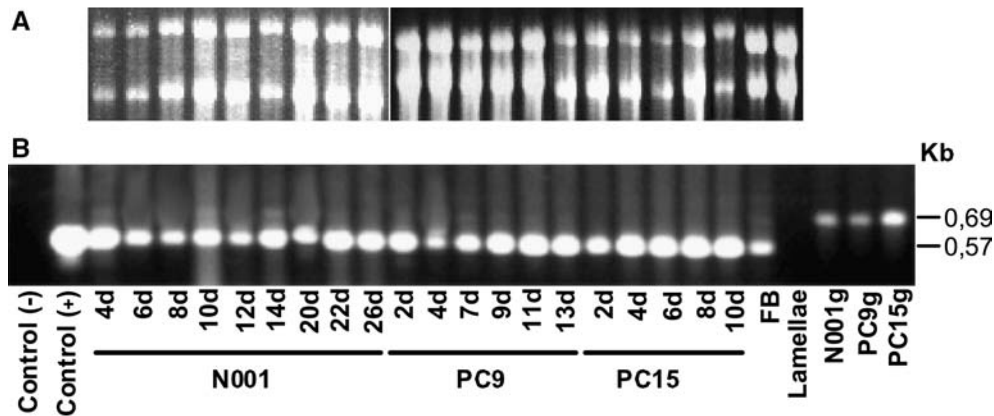


**Fig. 5** Time course of *ctr1* expression in *P. ostreatus*, measured by RT-PCR. **a** Total RNAs were purified from the dikaryon N001 and the monokaryotic strains PC9 and PC15 grown in SMY medium the number of days indicated, and stained with ethidium bromide as a loading control. **b** *ctr1* transcripts were amplified using the specific primers described in Materials and methods together with RNA purified at the times or developmental stages indicated as template. *ctr1* cDNA (control +) and genomic DNA (N001g, PC9g and PC15g) were used as positive controls

vegetative mycelium, that codes for a protein which is very similar to copper transporters previously described in yeasts, filamentous fungi and animals. Conceptual translation of this cDNA indicates that it encodes a 189-amino acid protein with a theoretical molecular mass of 20.6 KDa and a pI of 8.06. The protein is similar in size to a number of copper transporter proteins such as yCtr2 from *S. cerevisiae* (Kampfenkel et al. 1995), PaCtr2 from *P. anserina* (Borghouts et al. 2002) and the predicted protein XP_327714 from *N. crassa* (Galagan et al. 2003) (which comprise 189, 188 and 185 residues, respectively). Hydrophobicity analysis (data not shown) predicted three hydrophobic regions with three 18-amino acid transmembrane domains (TMDs) at positions 45–62, 140–157 and 162–179, suggesting that Ctr1 is an integral membrane protein. The cysteine residues within the TMDs described in CTR3 (Peña et al. 2000), and the methionine and histidine-rich regions at the N-terminal domain described in most of the copper transporters (Dancis et al. 1994a) have been proposed as candidate domains for copper binding. No cysteine-rich regions are present in the predicted TMDs of the Ctr1 protein from *P. ostreatus*, but examination of its N-terminal region reveals several methionine and serine residues, suggesting a possible role for this segment in copper binding. An abundance of serine residues in the N-terminal region is also a feature of the copper transporter COPT1 from *A. thaliana* (Sancenón et al. 2003) and substitution of the histidine residues by another polar amino acid such as serine could be the result of a conservative change that maintains the ability of the protein to interact with copper. A serine residue at position 15 in the *P. ostreatus* protein (that hypothetically corresponds to the outer N-terminal part of the protein) was predicted as a potential glycosylation site by the NetOGlyc server (Gupta et al. 1999). Both the potential glycosylation site and the typical $MLX_2M$ motif conserved along copper transporters are found in the second transmembrane domain of the *P. ostreatus* Ctr1 protein.

Given the lack of copper-deficient mutants in *P. ostreatus*, we expressed the *P. ostreatus crt1* gene in a well characterised *S. cerevisiae crt1-3* null mutant that is defective in copper transport, in order to test whether Ctr1 functions as a copper transporter. The cDNA was able to rescue the respiration-deficient phenotype of the *S. cerevisiae* strain. Northern analysis performed on yeast transformants grown in media with non-fermentable carbon sources show strong *ctr1* expression, providing further support for its role in copper transport.

The expression of *ctr1* was also studied at various times during the growth of vegetative *P. ostreatus* mycelium, in mature fruit-bodies and in lamellae. Expression of the gene was detected in all of the samples examined, with the exception of the lamellae. Therefore, we assume that Ctr1 plays an important role in copper uptake during the whole developmental program of *P. ostreatus*.

To investigate the regulation of *ctr1* expression under different culture conditions, we monitored its transcription in submerged cultures of the dikaryon grown under control conditions, under either copper or iron starvation, and in the presence of an excess of copper. The gene was expressed under all the conditions assayed, although lower transcription levels were detected when the mycelium was cultivated in the presence of excess copper. This result suggests the possibility that *ctr1* expression is regulated by the presence of the metal. Regulation by copper has also been seen in the case of *tahA* gene of *T. versicolor* that codes for a copper chaperone protein (Uldschmid et al. 2002). The expression of this gene is induced under elevated copper concentrations and repressed under copper starvation. The different transcription regulation by copper found in *P. ostreatus ctr1* and *T. versicolor tahA* genes suggests that they play different functions in the context of cellular copper transport. Negative regulation by copper has been described for the Mac1 protein of *S. cerevisiae*, a transcriptional activator of copper uptake. Mac1p binds via its N-terminal domain to GCTC elements in the promoters of the yeast genes *CTR1* and *FRE1*, which

code for a copper transporter and a metal reductase, respectively (Jamison McDaniels et al. 1999; Serpe et al. 1999). Interestingly, we have found that the core sequence for Mac1p binding is repeated five times in the sequence upstream of *ctr1* gene, supporting a role for copper in the regulation of *ctr1* expression. Furthermore, the Cu-signalling element (CuSE) with the consensus sequence 5′-DWDDHGCTGD-3′ (D = A, G, or T; H = A, C, or T, and W = T or A) defined in *Schizosaccharomyces pombe* as a *cis*-acting element found in the promoter of genes regulated by copper, occurs at position −233 in the promoter of *P. ostreatus ctr1*. In *S. pombe*, CuSE is the binding site for the transcription factor encoded by *cuf1* (Beaudoin et al. 2003). Although the homologous *cuf1* gene has not been functionally identified in *P. ostreatus*, a sequence with significant homology to *S. pombe cuf1* has been detected (our unpublished results).

Taken together, our results indicate that the *P. ostreatus* protein Ctr1 is a membrane-bound copper transporter, which is conserved in other *P. ostreatus* strains and in species belonging to the same genus. Comparison of the Ctr1 sequence with other copper transporters, and the change in expression level observed under copper deprivation indicates that Ctr1 may act as a low-affinity copper transporter. Detailed studies will be needed to clarify the substrate specificity and the kinetic characteristics of this novel transport protein. Moreover, the possibility that this transporter may be a capable of transporting iron and other metals must be kept in mind. The heterologous complementation test described in this work to prove the putative role of *P. ostreatus* Ctr1 will be a valuable strategy with which to study the function of other genes from *P. ostreatus*.

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