Oxalate decarboxylase of the white-rot fungus *Dichomitus squalens* demonstrates a novel enzyme primary structure and non-induced expression on wood and in liquid cultures

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Oxalate decarboxylase (ODC) catalyses the conversion of oxalic acid to formic acid and CO₂ in bacteria and fungi. In wood-decaying fungi the enzyme has been linked to the regulation of intra- and extracellular quantities of oxalic acid, which is one of the key components in biological decomposition of wood. ODC enzymes are biotechnologically interesting for their potential in diagnostics, agriculture and environmental applications, e.g. removal of oxalic acid from industrial wastewaters. We identified a novel ODC in mycelial extracts of two wild-type isolates of *Dichomitus squalens*, and cloned the corresponding *Ds-odc* gene. The primary structure of the Ds-ODC protein contains two conserved Mn-binding cupin motifs, but at the N-terminus, a unique, approximately 60 aa alanine-serine-rich region is found. Real-time quantitative RT-PCR analysis confirmed gene expression when the fungus was cultivated on wood and in liquid medium. However, addition of oxalic acid in liquid cultures caused no increase in transcript amounts, thereby indicating a constitutive rather than inducible expression of *Ds-odc*. The detected stimulation of ODC activity by oxalic acid is more likely due to enzyme activation than to transcriptional upregulation of the *Ds-odc* gene. Our results support involvement of ODC in primary rather than secondary metabolism in fungi.

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INTRODUCTION

Oxalic acid is the predominant organic acid produced by wood-rotting fungi when they are cultivated on defined liquid media or on solid lignocelluloses (Kuan & Tien, 1993; Shimada *et al.*, 1997; Galkin *et al.*, 1998; Urzúa *et al.*, 1998; Hofrichter *et al.*, 1999; Mäkelä *et al.*, 2002). According to the type of decay that they cause on wood, these organisms may be classified as white-, brown- and soft-rot fungi (Kuan & Tien, 1993; Hatakka, 2001).

Fungi synthesize oxalic acid in their mitochondria as a waste compound from the tricarboxylic acid cycle, and by the glyoxylate cycle that operates in the glyoxysomes (Espejo & Agosin, 1991; Dutton & Evans, 1996; Munir *et al.*, 2001). Typically, brown-rot fungi produce high quantities of extracellular oxalic acid (Dutton *et al.*, 1993;

Espejo & Agosin, 1991), although it has been shown that both white- and brown-rot fungi express specific oxalate-degrading enzymes (Mehta & Datta, 1991; Dutton *et al.*, 1994; Micales, 1997; Aguilar *et al.*, 1999; Mäkelä *et al.*, 2002).

Three types of oxalate-degrading enzymes have been described in microbes and plants: oxalate decarboxylases (ODC, EC 4.1.1.2), oxalate oxidases (OXO, EC 1.2.3.4) and oxalyl-CoA decarboxylases (OXC, EC 4.1.1.8) (Svedružić *et al.*, 2005). ODC, isolated from fungi and bacteria, is a Mn-containing enzyme that decomposes oxalic acid to formic acid and CO₂ in a reaction that requires O₂ (Reinhardt *et al.*, 2003). ODCs belong to the cupin protein superfamily, characterized by a conserved metal-ion-binding cupin motif with an overall β -barrel fold (Dunwell *et al.*, 2000, 2004), and are further classified as bicupins, as ODCs possess two cupin motifs, probably due to an evolutionary gene duplication event (Dunwell *et al.*, 2004). The evolutionarily related monocupin enzyme OXO similarly requires O₂ but cleaves oxalic acid to two CO₂ with generation of H₂O₂. OXO is expressed mainly in plants; only one fungal OXO has been reported so far (Aguilar *et al.*, 1999). The third enzyme, OXC, is a bacterial enzyme, which converts activated oxalyl-CoA to formyl-

Abbreviations: FDH, formate dehydrogenase; FPLC, fast protein liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ODC, oxalate decarboxylase; OXC, oxalyl-CoA decarboxylase; OXO, oxalate oxidase; qRT-PCR, quantitative RT-PCR; UTR, untranslated region.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are FM946037, FM955140, FM946036, and FM954981 for *odc*, cDNA of *odc*, partial *gapdh* of *D. squalens* FBCC312, and *odc* of *D. squalens* FBCC184, respectively.

CoA and CO₂, and is linked to oxalate-dependent synthesis of ATP, at least in *Oxalobacter formigenes* (Anantharam *et al.*, 1989).

The best-characterized ODC enzyme is from *Bacillus subtilis*, with the crystallized protein structure available (Emiliani & Bekes, 1964; Anand *et al.*, 2002). ODCs from several species of basidiomycetous and ascomycetous fungi have been reported (Emiliani & Bekes, 1964; Magro *et al.*, 1988; Mehta & Datta, 1991; Dutton *et al.*, 1994; Micales, 1997; Kathiara *et al.*, 2000; Mäkelä *et al.*, 2002), but so far only one basidiomycetous ODC enzyme, from the white-rot fungus *Flammulina (Collybia) velutipes* has been thoroughly characterized, at both the gene and protein level (Mehta & Datta, 1991; Kesarwani *et al.*, 2000).

Fungal ODCs are considered mainly as intracellular enzymes since only small amounts of extracellular ODC activities have been detected, secreted either to the culture medium or to the fungal cell wall and extracellular polysaccharide layer (Dutton *et al.*, 1994; Kathiara *et al.*, 2000). It is assumed that the major role of ODC in fungi is to prevent too high intracellular levels of oxalic acid, and thereby to control excess secretion of oxalic acid (Micales, 1997; Mäkelä *et al.*, 2002). Secondly, it has been suggested that ODC decomposes extracellular oxalic acid to keep steady levels of pH and oxalate anions outside the fungal hyphae, as supplementation of oxalic acid or a change to more acidic environmental pH levels often promotes ODC activities (Mehta & Datta, 1991; Dutton *et al.*, 1994; Micales, 1997; Mäkelä *et al.*, 2002).

ODC enzymes have several potential and established biotechnological applications. In the pulp and paper industry, oxalate salt deposits have been prevented by enzymic degradation of oxalic acid from the bleaching filtrates of pulping processes (Sjöde *et al.*, 2008). Other applications have utilized ODC in, for example, assays of oxalic acid concentration in clinical and food samples. The construction of transgenic *odc*-expressing crop plants can (i) improve their resistance against certain oxalic-acid-secreting plant-pathogenic fungi and (ii) by reducing their oxalic acid content, make them less toxic to humans and herbivores (Kesarwani *et al.*, 2000; Dias *et al.*, 2006). In a search for a treatment for excessive excretion of urinary oxalate (hyperoxaluria), oral therapy with a crystalline cross-linked formulation of ODC has been shown to reduce symptoms in experiments with mice (Grujic *et al.*, 2009).

In our previous study, the white-rot basidiomycete *Dichomitus squalens* (isolate FBCC184, formerly PO114) secreted oxalic acid during growth on wood and demonstrated high levels of mycelial ODC activity after supplementation with excess oxalic acid (Mäkelä *et al.*, 2002). This strain and another isolate of *D. squalens* (CBS 1000.73) are efficient in decaying spruce wood with selective lignin degradation (Hakala *et al.*, 2004; Fackler *et al.*, 2006). Others have reported that *D. squalens* (CBS 432.34) expresses multiple laccases and manganese perox-

idases (Périé & Gold, 1991; Périé *et al.*, 1998), employing a typical lignin-modifying enzyme machinery (Hatakka, 2001; Hammel & Cullen, 2008).

In this work, we cloned and sequenced a novel ODC-encoding gene (*Ds-odc*) and identified the ODC enzyme in mycelial extracts from two distinct wild-type isolates of *D. squalens*. The unique primary structure of Ds-ODC is described; it shows the longest polypeptide main chain (493 aa) for any cloned or isolated ODC enzyme characterized so far. Expression of the *Ds-odc* gene was studied by real-time quantitative RT-PCR in submerged liquid cultures and during solid-state cultivation on spruce wood sticks. On wood, transcript quantities diminished in the course of cultivation. In the submerged cultures, oxalic acid supplementation caused no increase in transcript amounts. Our results point to a constitutive role of the novel *Ds-odc* gene, principally operating during primary metabolism.

METHODS

Fungal cultures. *Dichomitus squalens* FBCC184 (formerly *D. squalens* PO114) and FBCC312 (formerly *D. squalens* A-670) from the Fungal Biotechnology Culture Collection, University of Helsinki, Helsinki, Finland (fbcc@helsinki.fi), were maintained on malt agar plates [2 % (w/v) malt extract, 2 % (w/v) agar agar]. Fungi were cultivated in 75 ml 2 % (w/v) liquid malt extract medium (submerged cultures) or on 2 g (dry weight) Norway spruce (*Picea abies*) wood sticks (solid-state wood cultures) (Mäkelä *et al.*, 2006). The stationary submerged cultures were inoculated with 4 ml mycelial suspension from 7 day cultures as described previously (Mäkelä *et al.*, 2002) and incubated at 28 °C. Oxalic acid (Sigma-Aldrich) was added to the submerged cultures on day 8 to give 2.5 mM or 5 mM final concentration. For extraction of RNA and total protein, the mycelia were harvested after 1 day and 2 days exposure to oxalic acid, respectively.

Protein extraction and chromatofocusing. After treatment with 5 mM oxalic acid, mycelia from submerged cultures of *D. squalens* FBCC184 and FBCC312 were filtered through Miracloth and stored at -20 °C. For extraction of proteins, the mycelia were ground under liquid N₂ with a mortar and pestle and extracted with cold 0.1 M potassium citrate buffer (pH 3.0). The suspensions were agitated on a magnetic stirrer for several hours at 4 °C, and centrifuged for 30 min at 30 000 g at 4 °C. The supernatants were concentrated in an Amicon ultrafiltration unit with a 10 kDa cut-off Omega membrane filter (Filtron) at 4 °C.

The concentrated protein extracts from *D. squalens* FBCC184 and FBCC312 were dialysed against 40 mM L-histidine buffer (pH 4.5) containing 10 mM NaCl. The protein sample was transferred into a 4 ml MonoP HR 5/20 chromatofocusing column (Pharmacia) using an FPLC (fast protein liquid chromatography) system (Pharmacia). Proteins were eluted with a linear Polybuffer 74 (Amersham Pharmacia Biotech) gradient from 0 to 10 % (from pH 4.5 to 2.3) by collecting 1 ml fractions. The pH and ODC activity of the collected fractions were determined. For measurement of ODC activity, the NADH-generating method described previously was used (Mäkelä *et al.*, 2002). This is a modification of the method of a commercially available kit (Boehringer Mannheim, Cat. No. 755 699). The effect of added oxalic acid or acidity in the samples was subtracted by using a sample blank for each measurement (Mäkelä *et al.*, 2002). The fractions showing ODC activity were pooled and concentrated.

Western blotting. The concentrated protein pools from chromatofocusing of *D. squalens* FBCC184 and FBCC312 were separated by SDS-PAGE and electroblotted to nitrocellulose membrane. IgG fraction of polyclonal rabbit antiserum against ODC from *Aspergillus* sp. (Nordic Immunology) was used as the primary antibody. The immunoreacted proteins were detected by alkaline phosphatase conjugated to goat anti-rabbit IgG (Bio-Rad) as the secondary antibody (Hakala *et al.*, 2005; Mäkelä *et al.*, 2006), and visualized with a BCIP/NBT colorimetric assay (Bio-Rad). ODC from *Aspergillus* sp. (Sigma-Aldrich) was used as positive control. Pre-stained PageRuler protein size standard (Fermentas) was used for the estimation of protein transfer efficiency and determination of molecular mass.

Extraction of nucleic acids. From the submerged fungal cultures, total DNA and RNA were extracted from ground mycelia frozen under liquid N₂ using the methods previously described (Hildén *et al.*, 2005). From the spruce wood cultures, total RNA was extracted by the method described by Chang *et al.* (1993). Prior to extraction, 2 g (dry weight) of the fungal-colonized wood sticks was milled in liquid N₂ with a Polymix Analysenmühle A10 (Kinematica). DNA was removed by RQ1 RNase-free DNase (Promega). Amount and quality of total RNA was determined by absorbance at 260 nm and 260/280 nm, respectively.

cDNA synthesis. A Smart RACE cDNA Amplification kit (Clontech) was used for the cDNA synthesis. The 20 µl reactions, containing 1 µg total RNA, 200 U SuperScript III reverse transcriptase (Invitrogen), 4 µl 5 × first strand buffer, 10 mM dithiothreitol, 0.5 µM 3'-RACE cDNA synthesis primer, 0.5 µM SMART II oligonucleotide and 0.5 mM dNTP mixture (Finnzymes), were carried out according to the instructions of the manufacturer (Clontech).

Amplification of *Ds-odc*. The genomic 915 bp fragment was amplified from total DNA of *D. squalens* FBCC312 with *odc* sense and antisense primers (Table 1) designed according to the cupin 1 and 2 motifs, respectively, of the *Flammulina velutipes odc* sequence (EMBL accession no. AF200683). The 25 µl PCR mixture contained 0.5 µl DNA template, 0.3 mM dNTP mixture (Finnzymes), 0.4 µM 5' and 3' primers, 1 × Phusion HF buffer (Finnzymes), 3 % DMSO and 0.8 U Phusion Hot Start DNA polymerase (Finnzymes). PCR was performed with initial denaturation at 98 °C for 30 s; then 45 cycles of (1) denaturation at 98 °C for 10 s, (2) annealing at 57 °C for 30 s,

(3) elongation at 72 °C for 15 s; and final extension at 72 °C for 10 min.

The 3' end of the *Ds-odc* gene was amplified using the Universal Genome Walker kit (Clontech) according to the instructions of the manufacturer. The nested PCR amplification strategy was conducted with a gene-specific primer (GSP sense) in the first round of PCR, followed by the second PCR with a nested gene-specific primer (nGSP sense) (Table 1). PCR conditions were as described by Hildén *et al.* (2005).

The 5' end of *Ds-odc* was amplified by the inverse PCR approach. One microgram of total DNA from *D. squalens* FBCC312 was digested with 1 U *Eco*RI restriction enzyme (Fermentas). The restricted DNA batch was circularized with 5 U T4 ligase (Fermentas) in a reaction containing 0.5 mM ATP. Circularized DNA templates for inverse PCR were purified with Microcon centrifugal tubes (Millipore). In the first round of PCR amplification, the gene-specific primers (nGSP sense, I-PCR antisense) were used, and in the second PCR round, gene-specific nested primers (nI-PCR sense, nI-PCR antisense) were used (Table 1). PCRs were conducted as described above.

The full-length genomic *odc* gene was amplified from the total DNA of both *D. squalens* isolates (FBCC184 and FBCC312), and the ORF fragment was amplified from the cDNA of *D. squalens* FBCC312 with primers designed according to the nucleotide sequence data from the genome walking and inverse PCR products (start, end; Table 1). PCR conditions were as described above.

Cloning and sequencing. The PCR amplification products were run on 1 % agarose gels and stained with ethidium bromide. The gels were inspected under UV light and the PCR products of correct size were cut out of the gels, purified with the GeneClean Turbo kit (MP Biomedicals), and cloned into the pJET1.2/blunt vector (Fermentas) according to the instructions of the manufacturers. Double-stranded plasmid DNA was extracted with the GeneJET Plasmid Miniprep kit (Fermentas) and used for sequencing (Magrogen Ltd, Republic of Korea).

Real-time quantitative RT-PCR (qRT-PCR). Real-time qRT-PCR was used to measure relative levels of expression of the *Ds-odc* gene. The *Ds-gapdh* gene encoding the glyceraldehyde-3-phosphate dehydrogenase of *D. squalens* was selected as a constantly expressed endogenous control gene to normalize quantification of cDNA in all qRT-PCRs. Gene-specific primer pairs were designed for *Ds-odc* (*odc* qPCR sense, *odc* qPCR antisense) to amplify one intron overlapping,

Table 1. Primers used for cloning and expression studies of the *D. squalens odc* gene

Primer description*	Nucleotide sequence (5'-3')	Application
<i>odc</i> sense	GGCGCTATCAGGGAGCTGCA	Genomic PCR
<i>odc</i> antisense	AAAGCACCAGGCTCAACTGT	Genomic PCR
GSP sense	CACAGTCTTCAAGCGACCAA	Genome walking PCR
nGSP sense	TTCCCGGCTTCGACCCAGAT	Genome walking and inverse PCR
I-PCR antisense	AGCAGTCACTTGGACGGAAC	Inverse PCR
nI-PCR sense	GAGACACCGGTAACGACACG	Inverse PCR
nI-PCR antisense	CTCAGCGTCTTGTGCCAAT	Inverse PCR
Start sense	ATGGTCCGCGCACTCCTCTCTCT	Genomic and RT-PCR
End antisense	TCACTGGGACGGGCTACGA	Genomic and RT-PCR
<i>odc</i> qPCR sense	CTCTTCCCTCTGGCATTGT	Real-time qRT-PCR
<i>odc</i> qPCR antisense	GCCTACGACGAATTCCTTTG	Real-time qRT-PCR
<i>gapdh</i> qPCR sense	GCTACCGGTGTCTTACCAC	Real-time qRT-PCR
<i>gapdh</i> qPCR antisense	TTGACACCGCAGACAAACAT	Real-time qRT-PCR

*GSP, gene-specific primer; nGSP, nested gene-specific primer; I-PCR, inverse PCR; nI-PCR, nested inverse PCR.

147 bp and 263 bp products from cDNA and genomic DNA amplicons, respectively (Table 1). For *Ds-gapdh*, 129 bp and 178 bp (one intron containing) products were amplified from cDNA and genomic DNA, respectively, using the gene-specific primers (*gapdh* qPCR sense, *gapdh* qPCR antisense; Table 1).

For each time point and treatment, two biological replicates, i.e. cDNA templates synthesized from RNA extractions from two separate cultivations, were used for the generation of cDNA template, and three replicate PCRs were conducted with each cDNA template. The amplification efficiencies for *Ds-odc* and *Ds-gapdh* were determined to be equal by using five serial dilutions, and fold-differences between the samples were calculated by the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). The 20 μ l qRT-PCRs contained 0.3 μ l of the cDNA template, 0.5 μ M 5' and 3' primers and 1 $\times$ Maxima SYBR Green qPCR Master Mix (Fermentas). The qRT-PCRs were performed in an ABI 7300 instrument (Applied Biosystems) using the following cycling parameters: initial denaturation at 95 °C for 15 min; then 40 cycles of (1) denaturation at 94 °C for 60 s, (2) annealing at 58 °C for 30 s, and (3) elongation at 72 °C for 30 s; and for melting curve analysis, initial denaturation was performed at 95 °C for 15 s, hybridization at 60 °C for 30 s, and final denaturation at 95 °C for 15 s. Fluorescence was measured during the elongation step of qRT-PCR.

Phylogenetic sequence analysis. Translated amino acid ORF sequences of the *Ds-odc* cDNA clone from isolate FBCC312 and of the full-length genomic DNA clone from isolate FBCC184 were identified and compared to other ODC and OXO sequences with BLAST (<http://www.ncbi.nih.gov/blast>) using the BLASTP search algorithm. Nucleotide and Uniprot translated sequences of ODC and OXO-encoding genes at EBI-EMBL were retrieved with SRS (<http://www.ebi.ac.uk>) and the annotated *odc* sequences from the whole genome websites of *Phanerochaete chrysosporium* (<http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>) and *Postia placenta* (<http://genome.jgi-psf.org/Pospl1/Pospl1.home.html>). With *P. placenta*, one allelic sequence variant of each three putative *odc* gene was used (Martinez *et al.*, 2009). Maximum-parsimony and minimum-evolution neighbour-joining trees with a bootstrapping value of 1000 were created for 31 amino acid sequences with the MEGA 4.0 software (<http://www.megasoftware.net/>) (Tamura *et al.*, 2007).

RESULTS

ODC activity and pH in submerged cultures

In the submerged cultures of *D. squalens* FBCC184 incubated without additional oxalic acid, most of the ODC activity was found in the mycelial fraction (Fig. 1a). ODC activity was hardly detectable in the culture fluids [0.4 μ mol oxalic acid decarboxylated (mg protein) $^{-1}$ min $^{-1}$], corresponding to only 2.5 % of the total activity. Addition of 5 mM oxalic acid promoted the highest ODC activity levels, and extracellular ODC activity was increased [9.3 μ mol oxalic acid decarboxylated (mg protein) $^{-1}$ min $^{-1}$]. However, up to 87 % of the ODC activity was in the mycelial extracts [62 μ mol oxalic acid decarboxylated (mg protein) $^{-1}$ min $^{-1}$, a 30-fold increase in comparison to non-induced conditions]. With 10 mM oxalic acid, mycelial ODC activity dropped to a lower level than was observed in non-induced cultures (Fig. 1a).

Supplementation with oxalic acid quickly acidified the culture fluids: with 5 and 10 mM oxalic acid the

Fig. 1. Effect of supplementing submerged cultures of *D. squalens* FBCC184 with oxalic acid. (a) ODC activity [μ mol oxalic acid decarboxylated (mg protein) $^{-1}$ min $^{-1}$] in mycelial extracts and culture liquids 2 days after addition of 5 mM (light grey columns) and 10 mM (dark grey column) oxalic acid. Non-induced mycelium was cultivated without addition of oxalic acid (white columns). (b) Effect on the extracellular pH values immediately (0 days), 1 day and 2 days after addition of 2.5, 5 or 10 mM oxalic acid. Error bars indicate the standard deviation between three parallel cultures.

extracellular pH immediately declined below 3 (Fig. 1b). Within 2 days, extracellular acidity settled to about pH 4 with 2.5 and 5 mM oxalic acid, whereas with 10 mM oxalic acid, the pH stayed lower, at 3.4. In non-induced cultures the extracellular pH remained at 4.6 (Fig. 1b).

Characterization of *D. squalens* ODC protein

Mycelial protein fractions with the highest ODC activities eluted at pH 4.2–4.25 of the chromatofocusing elution buffer, which indicates that the pI value of the Ds-ODC enzyme is within this range (Fig. 2a). A minor amount of enzyme activity was also detected at pH 2.6, which suggests marginal production of another ODC isozyme.

Western blotting with ODC antibody detection showed two protein bands, of 55 and 95 kDa, in the 5 mM oxalic acid induced mycelial extracts from *D. squalens* FBCC312, whereas one ODC protein of 52 kDa was observed in isolate FBCC184 (Fig. 2b). In the non-induced mycelial extract of *D. squalens* FBCC312, only the 95 kDa ODC protein band was detected, possibly due to the lower

Fig. 2. (a) Separation of *D. squalens* FBCC184 ODC isoforms using MonoP chromatofocusing fractionation. ■, ODC activity; ◆, pH of the fractions. (b) Immunoblotting of SDS-PAGE-separated ODC from 5 mM oxalic acid induced mycelial extract of *D. squalens* FBCC184 (lane 1) and FBCC312 (lane 2), and from non-induced mycelium of *D. squalens* FBCC312 (lane 3). *Aspergillus* sp. ODC (Sigma-Aldrich) was used as a positive control (lane 4).

protein concentration of the sample. The 52–55 kDa size is in accordance with the predicted molecular mass of the translated mature amino acid sequence of the *Ds-odc* ORF (see below), accounting for one bicupin monomeric subunit of active Ds-ODC. The larger protein band may represent a dimeric complex containing two bicupin subunits, even though the proteins were electrophoresed under denaturing conditions (Fig. 2b). Another possible explanation is that the commercial antibody (raised against *Aspergillus* sp. ODC) may have reacted with another cellular protein of *D. squalens*.

Characterization of the *D. squalens odc* gene

One *Ds-odc* ORF of 1479 bp was amplified from the cDNAs originating from mycelia obtained from both

Fig. 3. The full-length *D. squalens* FBCC184 *odc* sequence amplified from DNA (lane 1) and the full-length *D. squalens* FBCC312 *odc* sequences amplified from DNA (lane 2), cDNA from submerged liquid culture (lane 3) and cDNA from solid-state wood culture (lane 4). Sizes of the PCR products are shown on the left.

submerged liquid and solid-state wood cultures of *D. squalens* FBCC312 (Fig. 3). The nucleotide sequences of the two *Ds-odc* genomic clones obtained from isolates FBCC184 and FBCC312 are both 2537 bp in length and no differences were detected in their nucleotide sequences within the ORF coding regions (exons). The coding regions are also identically interrupted with 17 introns that were similar in length between the isolate clones (Fig. 4). The genomic sequence contains one non-canonical 5' splice-site junction with a GC dinucleotide instead of GT at the beginning of intron XIV. The intron length varies from 49 nt to 91 nt in *Ds-odc*; the lengths of the exons range from only 10 nt up to 452 nt. A TATA box is found 62 bp upstream of the start codon (ATG). One putative metal response element (MRE), one CCAAT box, and two cyclic AMP responsive elements are found at 55 bp, 334 bp, 354 bp and 411 bp upstream of the start codon, respectively (Fig. 4).

Ds-odc ORF codes for a putative 493 aa polypeptide with the conserved bicupin primary structure as defined for one cupin motif as G(X)₅HXH(X)₃₋₄E(X)₆G followed by G(X)₅PXG(X)₂H(X)₃N, with 15–27 aa in the intermotif region (Dunwell & Gane, 1998; Dunwell *et al.*, 2000) (Fig. 5). The conserved residues of three histidines and one glutamate, which are known to bind one Mn²⁺ ion, are present in both of the cupin motifs. A putative secretion signal peptide of 20 aa at the 5' N-terminus is predicted with the SignalP 3.0 program (<http://www.cbs.dtu.dk/services/SignalP/>) (Fig. 4). According to the general eukaryotic rule for N-glycosylation (N-X-S/T, where X is not P), four potential glycosylation sites are found in the translated protein. Two of the predicted N-glycosylation sites reside in the N-terminus within an approximately 60 aa alanine-serine-rich stretch that is located in conjunction to

Fig. 4. Nucleotide and translated amino acid sequences of the *D. squalens odc* gene. Exons are in capital letters, and introns and 5' and 3' UTR sequences in small letters. The 3' UTR sequence amplified from the cDNA until poly(A) is in italics. Introns are numbered I–XVII. The non-canonical 5' splice site of intron XIV with a GC dinucleotide is in bold and double-underlined. The stop codon is marked with *, amino acids involved in Mn²⁺ binding with ▼, and putative N-glycosylation sites with ■. The 5' promoter sequence is numbered negatively upstream from the translational start codon (ATG). The TATA box is in bold and the CCAAT box is underlined. Predicted regulatory elements are underlined and marked as CREB (cyclic AMP responsive element binding protein) and MRE (metal response element). The putative N-terminal secretion signal peptide is highlighted in grey.

CREB
 cgacggcccggtgtgactcattccac -401
 CCAAT
 gtccacgcaatcacgogggttttggtcagcagcatgcttcgcaaaggtcgactccacgtcagcctcgaccctatcagggttggtgattgtttcaaat -301
 gcggtatttcgagatccgtgcccgtatggcccttcgacaacccacgaagtctagcccggaagtggttaactgatcgtagtaactggggcagcacacctg -201
 gcacctcaaggaagctcaccgtttgtgcaccggcggtgtaatccccatgctaggcgcaaggtgatctgtggcgtagacaggtgtgccgccaccatttct -101
 MRE
 tgcgcaggtgcttaggtgattttcgccgacggcggaaggtataaatgctcagctcaaatgcacatttctcaccgcccagggttcaccagcattctccgca - 1
 M V R A L L S L V A L S L Q L G S A L A A P A A S A A S A A D A S A 34
 ATGGTCCGCGCACTCTCTCTCTGCTGCTCTATCGTGCAGCTTGGGTCCGCCCTAGCTGCACCTGCGGCCAGCGCGGCTCTGCCGCTGACGCTTCGG 100
 A A S V S L A T A A S A V S S V A T A S L A T N A S P A A S A A P 67
 CCGCAGCGAGCGGTGTCCTCGCGACCGCCGATCTGCGGTATCTTCCTGAGTACTGCCTCCCTTGCAGCAACGCGTCCCCCGCGCCAGCGCAGCCCC 200
 N A S G S V S A A A R G T P S G T Q I S P P D A T V P P A S D D P 100
 CAACGCTAGCGGCTCTGTGTCTGCGGTGCACGAGGACCCCTTCGGGGACCCAGATTCTCCGCGGATGCCACTGTACCACCCGCGAGCGATGACCCC 300
 N N V I F Q P N A P D V N P Q P I R G Q L G A P T I T N E N I P V D 134
 AACCAAGTGTCTTCAGCCTAACGCCCCGAGCTAACCCCGAGCAATTCTGTGGGCGAGCTCGGCGCCCCACGATACCAACGAGAACATTCTGTGTG 400
 V Q N P D L L A P P S T D H G E F intron I 151
 ACGTGCAGAACCCAGATCTGTCTCGACCGCGCTCGACGGACCATGGGGAATTgtatgtgctccttgaaactctgtgtgtgctgaaagacctggagc 500
 Q N A K W P F S L S H N R L Q T G G W A R E Q N V 176
 cgtgttgacttgcggtacgaatatagCCAGAACGCAAGTGCGCTTCAGTCTCAGCCATAACCGTCTACAGACAGAGGCTGGGCCCGTGCAGCAGAACG 600
 intron II G V M 179
 gtatgtctcgtataggccttggtgagggcggtggggctatgccaccacagagtgctgtgactgagggctgacgaaaccttatcatcacagTCGGTGTCA 700
 P I S T S intron III M A G V N M R L E A 194
 TGCCATCTCAACCTgtgagtcataagcgttgctgctaactataggcagcagctcaagtccaatgcagCTATGGCCGGTGTGAATATGCGTCTGGAAGC 800
 G A V R intron IV E L H W H K T S E 207
 TGGGCGAGTTCGgtgagtgccaattacgccatcttcatccttgcacttgcacaaagggctattaggGAGCTGCATTGGCACAAGACTTCTGAGgtat 900
 intron V W A Y I L N 213
 agtaatcttcgtatccgtgacctccgatgttaccgaggttgctaatacgcatgtgtggcatctccaaagTGGGCATATATCTGAACgtatgtcaggttt 1000
 intron VI G S V Q V T A I N A D G Q N F I G H V 232
 acctcgtgaagcgcaaaccttgctgatatctcgtccagGGTTCGTCGAAGTACTGTATCAATGCGGATGGCCAGAACTTCATCGGGCAGCTGgtaagt 1100
 intron VII N P G D L W F F P A G M P H S L 248
 ccgtgctcaggtgcatgtaggagggttggaactcaccgactcacatcaccttagAACCCAGGGGACCTTTGGTTCTTCCCTGCTGGTATGCTCAGTCT 1200
 Q A T N E N P D G A E F V L intron VIII 262
 TCAAGCGACCAACGAGAATCCGGATGGTGCCGAATTCGTGCTGtaagtcagccatagcgggacagggaggttctgaacgcttgacaatgacgtcatctag 1300
 V F P D G S F S E D S T F L intron IX 276
 GTCTTCCCGCAGGCTCCTTCAGCGAAGACTCGACGTTCTGTgtagtccctcttaggcagtggtccacttgtatctctgatcgtgacaccacaactgc 1400
 L T D W L S H I P K E V I A K N F Q T S I S A F N N L P S Q E L 308
 acagCTGACTGATTGGTTGTGCGACATTCCCAAGGAAGTCATCGCCAAAACCTTCAGACAGCATCTCCGCTTCAACAACCTTCCAGCCAGGAACCTA 1500
 Y I F P S T intron X P P P V N Q 320
 TACATATCCCGAGTAgtagtggtgacatcagcttcacgctctccctttccacatatttactgacatatccgtagacagCTCCCCCTCCGGTCAACC 1600
 Q P P S D P Q G T I P Q P W T F A L S Q V K A T Q V Q G G T I K I 353
 AGCAACCTCTAGCGACCTCAAGGCACCATTCACAACCTGGAGCTTTGCCCTGTCGAGGTCAAGGCTACCCAGGTCAAGGAGGCACTATCAAGAT 1700
 V D Q T T F P A S T Q I S A I E V T V E P G A M R intron XI 378
 TGTGACCAAGACTACTTTCCCGGCTTCGACCCAGATCTCTGCGATCGAGGTGACAGCTGAGCCTGGTGTATGAGgttcgaccttggcttatatacaactt 1800
 E I H intron XII W H 383
 tcccctaacgcccactaacaccaccccgagGGAATTCATgtaagtttgcgatgatgcacgtgggcaggtatatactcacaggcatgcatgagcagTGGC 1900
 P T Q D E W T F Y I intron XIII 393
 ATCCCACTCAAGATGAATGGACTTCTATATgtgctggttcccttccccgctctccttgagcgctttatatgttcatgctgacgagggctttcccggaat 2000
 S G Q G R V T I F A G T S T S R T F D Y Q intron XIV 414
 acagTAGTGGGCAAGGCCGCTCAGATCTTCGCCGGAACGTCGACGCTTAGGACGTTTCGACTATCAGgacgttattctttagtgaacctcactaatc 2100
 A G D V G F V P A S M G intron XV 426
 ttgtggtcctgagcgtgcatatcggttccatagGCCGGTGATGTCGGTTTCGTCGCCGCTTCGATGGgtaagcagtagccctttaggtttggcatagcgtt 2200
 H F V E N T G N D T L H F L E L F P S G intron XVI 446
 gacctctctgagcagGTATTTCTGTGGAGAACACCGGTAACGACACGCTCCACTTCTTGGAGCTCTTCCCTCTGgtacgtttcaaccaccagcatcgg 2300
 I V Q D V S L R Q intron XVII 455
 ccaggatgtgactgcttttccacataaacctgtatagGCATTGTCCAGGACGTTAGCTTGAGACAGgtgtgtacgagttccccgggtccctgtctctact 2400
 W L A L T P P E L V Q V H L G I D Q D T L N Q L T S F 482
 aacacagcgacgatttgagTGGTGGCTTTGACGCCCGGAGCTGGTTCCAGTCCACCTGGGTATTGACCAGGACCCCTCAATCAGTGACTAGCTT 2500
 K T K E F V V G P S Q * 493
 CAAGACAAAGGAATTCGTCGATAGGCCGTCAGTGAATccgggcttttgaaggggtctcttgggggtatataattaagattattatgtttcaatgactgc 2600
 attctcggtgtacaatacagagactctcgaatccttacaactcactccttgcacaaaaaaaaaaaaaaaaa 2674

the secretion leader peptide, all encoded by an exceptionally long exon of 452 bp prior to the first intron (Fig. 4). The theoretical pI value and molecular mass of the mature Ds-ODC are 4.6 and 50 kDa, respectively, as determined by bioinformatic calculations (<http://au.expasy.org/>).

Ds-ODC shows the highest pairwise amino acid identity of 60% with two putative *Phanerochaete chrysosporium* ODCs encoded by potential genes annotated within the whole genome sequence (e_gwh2.16.56.1 and e_gwh2.5.232.1, <http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>) (Fig. 5). When the exon-intron structures of *odc* genes are compared, the 5' end of *Ds-odc* resembles the corresponding region of the *F. velutipes* *odc* while at the 3' end of *Ds-odc* the intron positioning is more similar to that of the putative *odc* gene annotated in the whole genome sequence of *Laccaria bicolor* (Fig. 6). The *D. squalens* and *F. velutipes* sequences contain 17 introns but four of the introns, nos

XII and XIV in *Ds-odc*, and nos X and XIV in *Fv-odc*, are found in non-equivalent positions (Fig. 6).

In the evolutionary tree of ODC and oxalate oxidase (OXO) amino acid sequences, the Ds-ODC groups within the main branch of ODCs from other basidiomycetous fungi (Fig. 7). The ODCs from ascomycetes and bacteria form two separate clusters or clades within the tree. Quite exceptionally, two of the seven putative ODC sequences from *P. chrysosporium* fall into the same branch with ascomycetous sequences. In fact, these two putative *P. chrysosporium* ODCs were significantly shorter as translated proteins. Also, the *Ceriporiopsis subvermispora* bicupin OXO clusters closest to the *Trametes versicolor* ODC, whereas the monocupin plant OXO from wheat (*Triticum aestivum*) was the shortest and most divergent in amino acid sequence, thus acting as a far-relative outgroup in the neighbour-joining tree.

Ds-ODC	1	--MVRALLSLVALSLQLGSALAAPAASAASAADASAAASVSLATAASAVSSVATASLATNASPAASAAPNASGSVSAAARGTPSG---	83
Pc-ODC1	1	<u>MPSAKLAKVVLCAVALASC</u> SLAPAGSSAPASSV-----ISSASASPALLSSAAASAATIAPGGSASSFLSIQSSLSRSPSTPSAS	83
Pc-ODC2	1	--MAHLKYSALLALSLSLFAHAAPAP-----LSSPTLT-----GTAPGPAP-----	40
Fv-ODC	1	--MFNNFQRLLTIVLLSGFTAGVPLAS-----TTTGTGT-----ATGTSTAAEP-----	42
Tv-ODC	1	--MGKFLATVLCVLYGSLAAAI PVGD-----VSASSASSSSIAEAATSTSGGASP-----	49
Ds-ODC	84	TQISPPDATVPPASDDPNVIFQPNAP--DVNPQPIRGQLGAPTITNENIPVDVQNPDLLAPPSTDHGEFQNAKWPFSLSHNRLQTGG	169
Pc-ODC1	84	LSAPPASETVPYASDDANTVVWSPGWD--GQPE-AIRGSLGSSVMGPQNDPIDLQNPDLLAPPTTDAGTVGNKWPFSLSHNRLQTGG	168
Pc-ODC2	41	-----TVPYASDDPNVEIYPPNINDPGYVPEPIRGKLSNLAQHNIPLALENPTLLAPPTTDHGSIPNAHWPFSLSHNRLQTGG	120
Fv-ODC	43	-----SATVPFASTDPNPVLWNETSD--PALVKPERNLQGATIQGPDNLPIDLQNPDLLAPPTTDHGFVGNKWPFSFSKQRLQTGG	123
Tv-ODC	50	-----SPTVPLASDDPNYWLWNETTT--TDPQ--PERGSLGANILGPQNVIAIDKQNPDIAPPTTDQGTGVGNKWPFSLSKQQLNTGG	128
CUPIN 1			
Ds-ODC	170	WAREQNVGVMPISTSMAGVNMRLGAVRELHWHKTSEWAYILNGSVQVTAINDGQNFIGHVNPGLWFFPAGMPHSLQATNENPDG	257
Pc-ODC1	169	WARQQNLAVLPMATEMAGVDMRLEVGAIRELHWHKTGEWAYVLQGSVQISAVNQNGQNFVSTVSQGLWYFPAGVPHSIQATDANPNG	256
Pc-ODC2	121	WARQQNVNEMPPFAKNLAGVDMRLEAGAIRELHWHSTAEWAYVLDGTMQITTVTPEGQNYIGTIHPGDLWYFPFGPQPHSLQATADNPQG	208
Fv-ODC	124	WARQQNEVVLPLATNLACTNMRLEAGAIRELHWHKNAEWAYVLKGSTQISAVDNEGRNYISTVGPGLWYFPFGPIPHSLQATADDPG	211
Tv-ODC	129	WVRQQNVQMPPIATAMAGVNMRLGSAIRELHWHQTAEWAYVLSGSTQISSVDQLGRNYVATVRQGLWYFPFGPIPHSLQATNDSSEG	216
▼			
Ds-ODC	258	AEFVLVFPDGSFSEDSTFLLTDWLSHIPKEVIAKNFQTS--ISAFNNLPSQELYIFPSTPPPVNQPPSPDQGTIPQFWTFALSQVKAT	344
Pc-ODC1	257	AEFLLVFDGNGFSEDDTFLLDWLAHVPKDVLAKNFQTS--MSTFDKLPKELYIFFAAPPGPDSDAPTSQGTVPNPFTFKLSGMPAT	343
Pc-ODC2	209	AEFLLVFDNGTFNEGATFLLTDWLAHVPEVIAKNFQTS--IAAFERIPDQELYIFPSNPPDDQPPSTSPYGTTPPEPTYEFYSKVPVT	295
Fv-ODC	212	SEFILVFDGSAFNDDGTFLLDWLHSHVPMVILKNFRANKPAAWSHIPAQQLYIFPSEPPADNPDPVSPQGTVPPLPYSFNFSSVEPT	299
Tv-ODC	217	TEFLLIFFDGNFNDDDTLLLDWLAHTPKVIAKNFQDN--IADWDDIPGSQLYIFPGVPPPDNQPPSTPAGEIPQFFSYAFSEITPT	303
CUPIN 2			
Ds-ODC	345	QVQGGTIKIVDQTFPASTQISAIEVTVPEPGAMREIHWHPTQDEWTFYISGQGRVTIFAGTSTSTRTFDYQAGDVGFVPASMGHFVENT	432
Pc-ODC1	344	QLQGGSVKIVDSNTFFKASKTIAAAEVTVPEPGAMRELHWHPTQDEWSYFISGNARVTLFAAQSSARTFDYQPGDVAYIPGSYGHYVENI	431
Pc-ODC2	296	QVPGGTFKIADSTTFKVSKTIAVAEFTVEPGALRELHWHPTFDEWTFYFLEGQARMTLFAAESNARTFDYQAGDIGVVPASVGHYIENT	383
Fv-ODC	300	QYSGGTAKIADSTTFNISVAIAVAEFTVEPGALRELHWHPTFDEWTFYFISGNARVTIFAAQSVASTFDYQGGDIAYVPASMGHYVENI	387
Tv-ODC	304	QYTGGTAKIADSTTFKVATKIAVAEFTVEPGAMREMHWHPTQSEWGFLEGRTARVTLFAGTAIAQTFDYQPGDISIYIPTAYGHYVENT	391
Ds-ODC	433	GNDTLHFLELFPFGIVQDVSLRQWLALTPELVQVHLGIDQDTLNQLTSFKTKFVVG--PSQ---	493
Pc-ODC1	432	GNDTLHYLEIFKTDTFQDVLSQWLALTPPDMVKATLNLDDALKHFSK--TKPVVVG--PSKGSS	493
Pc-ODC2	385	GNTTLKFLEIFKTDQDVLSQWLALTPELVKAHLGISDETVOHLSK--TKPMVVAPYTPAEN--	446
Fv-ODC	388	GNTTLTYLEVNTDRFADVLSQWLALTPPALVKQHLQLSDETLAELKQFATKATVVG--PVN---	447
Tv-ODC	392	GNTTLKFLEIFNSDVFQDVSLAQWLALTPPALVKQHLQLSDATISRFNR--TKGVVVGPGANVSS	455

Fig. 5. Comparison of translated amino acid sequences of white-rot fungal ODCs by CLUSTAL W (<http://www.ebi.ac.uk/Tools/clustalw/>) multiple alignment. Putative N-terminal signal peptide sequences are underlined, two cupin motifs are highlighted in grey, conserved amino acid residues involved in the binding of Mn^{2+} -ions are marked with *, and the amino acid position corresponding to Glu162 in *Bacillus subtilis* OxdC is marked with ▼. Fungal species, their abbreviations, and sequence accessions in the EMBL Nucleotide Sequence Data Bank or in Uniprot are: *Dichomitus squalens* FBCC312, Ds-ODC, FM946037 and FM955140; *Flammulina velutipes* Fv-ODC, Q9UVK4; and *Trametes versicolor* Tv-ODC, Q6UGB9. Translated sequences for two of the whole genome sequence annotated genes of *Phanerochaete chrysosporium* (Pc-ODC1, e_gwh2.5.232.1; Pc-ODC2, e_gwh2.16.56.1) were retrieved from the DOE Joint Genome Institute (<http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>).

Fig. 6. Exon-intron structure analysis of ODC-encoding genes from the basidiomycetous fungi *Dichomitus squalens* (this study), *Flammulina velutipes*, *Laccaria bicolor* and *Phanerochaete chrysosporium*. The *odc* genes cloned and characterized at the protein level are marked with *. Black, grey and white boxes represent putative secretion signal peptides, exons and introns, respectively. The scale bar shows sequence length in nucleotides. Introns in equivalent positions are connected with dashed lines. Gene sequence accession numbers are FM946037 for *D. squalens*, AF200683 for *F. velutipes* and DS547157 for the predicted protein annotated in the whole genome sequence of *L. bicolor*. *P. chrysosporium* annotated sequences e_gwh2.5.232.1 and e_gwh2.16.56.1 were retrieved from the DOE Joint Genome Institute (see the legend for Fig. 5).

Expression of *Ds-odc* in solid-state wood and submerged cultures

Real-time qRT-PCR showed that *Ds-odc* gene is expressed during the fungal growth on solid-state wood cultures after 3 and 4 weeks of cultivation (Fig. 8a). With the samples taken after 1 and 2 weeks of solid-state cultivation, cDNA synthesis did not succeed, most probably due to minor growth of fungus and too low a yield of total RNA. After 4 weeks, the amount of *Ds-odc* transcripts decreased to almost half of the amount detected after 3 weeks of cultivation. In the submerged cultures, addition of excess oxalic acid was not observed to induce expression of *Ds-odc* at the transcriptional level, as the highest amount of transcripts was observed in the cultures without supplementation of oxalic acid (Fig. 8b). In the submerged cultures where oxalic acid was added to give 5 mM final concentration, a decline in the quantity of *Ds-odc* transcripts was detected compared to the non-induced cultures (Fig. 8b). With 2.5 mM oxalic acid, the lowest amount of *Ds-odc* transcripts was obtained. The transcript-specific qRT-PCR primers designed for the *Ds-odc* and *Ds-gapdh* amplicons (Table 1) resulted in amplification of PCR products of the correct size, with a 116 bp and 49 bp DNA fragment length difference, respectively, from the corresponding genomic DNA products (Fig. 8c).

DISCUSSION

In this study, we cloned and performed preliminary characterization of a new oxalate decarboxylase (Ds-ODC) from two isolates of the lignin-degrading white-rot fungus *Dichomitus squalens*. ODC is a relevant enzyme for

biotechnological applications due to its ability to specifically decompose oxalic acid, with prospects from process industry to diagnostics. So far, only one fungal ODC has been completely cloned and enzymically described, from *Flammulina (Collybia) velutipes* (Mehta & Datta, 1991; Kesarwani *et al.*, 2000). To add to the pool of fungal ODCs, we focused on identifying the gene and enzyme from *D. squalens*, which showed high ODC activity in our previous screening study (Mäkelä *et al.*, 2002).

The primary characterization of Ds-ODC demonstrates a 493 aa protein of conserved bicupin core structure containing a unique alanine-serine-rich stretch of over 60 aa residues in the N-terminus directly after the putative secretion signal peptide. The ODC protein was also identified by immunohybridization in the mycelial extracts of the two *D. squalens* isolates. Most of the ODC activity was observed to be associated with the *D. squalens* mycelium while a small proportion of activity was detected in the extracellular culture liquid. Real-time qRT-PCR showed that the *Ds-odc* gene was expressed both on solid-state wood and in submerged liquid cultures, even without addition of excess oxalic acid, thus pointing to the importance of the ODC enzyme for general metabolism and growth of the fungus.

Ds-odc transcripts were detected and quantified during weeks 3 and 4 of cultivation of *D. squalens* on spruce wood sticks. This is consistent with our former results showing that *D. squalens* secretes oxalic acid together with production of manganese peroxidase after a similar cultivation period on spruce wood, with acidity staying at pH 4.1–4.4 (Mäkelä *et al.*, 2002). In the present study, the amount of *Ds-odc* transcripts was observed to decline in

Fig. 7. Evolutionary relations of selected and putative oxalate decarboxylase (ODC) and oxalate oxidase (OXO) amino acid sequences of microbial and plant origin depicted in a minimum-evolution neighbour-joining tree. Bootstrap values (1000 replications) higher than 50% are indicated for the nodes. The scale bar shows a distance equivalent to 0.2 amino acid substitutions per site. Species name and Uniprot or EMBL Nucleotide Sequence Data Bank sequence accessions are: *Aspergillus clavatus* putative ODC (A1C785), *A. fumigatus* putative ODC (Q4X060), *A. niger* contig An15c0140 from complete genome (A5ABZ4), *Bacillus* sp. ODC (A3IBX7), *B. cereus* ODC (Q81GZ6), *B. subtilis* OxD (O34767), *Ceriporiopsis subvermispura* OXO (Q5ZH56), *Coprinopsis cinerea* putative protein (A8NVA5), *Dichomitus squalens* FBCC184 ODC (FM954981), *D. squalens* FBCC312 ODC (FM946037), *Flammulina* sp. ODC (Q870M8), *F. velutipes* ODC (Q9UVK4), *Laccaria bicolor* predicted protein (B0DZS6), *Magnaporthe grisea* ODC-like protein (Q5EMV1), *Neosartorya fischeri* putative ODC (A1DI17), *Neurospora crassa* OxD (Q7SFG6), *Sclerotinia sclerotiorum* putative uncharacterized protein (A7EZM9), *Synechococcus elongatus* ODC precursor (Q31KK1), *Trametes versicolor* ODC (Q6UGB9), *Triticum aestivum* OXO (P15290). The following putative ODC sequences were retrieved from the DOE Joint Genome Institute (<http://genome.jgi-psf.org/>) whole genome sequence websites: *Phanerochaete chrysosporium* e_gww2.5.255.1, e_gwh2.5.232.1, e_gww2.7.295.1, e_gww2.9.251.1, e_gww2.9.269.1, e_gwh2.16.56.1 and e_gww2.7.301.1 and *Postia placenta* e_gwl.18.94.1, e_gwl.1.201.1 and e_gwl.1.217.1.

week 4, which coincides with the decrease in oxalic acid concentration on spruce wood (Mäkelä *et al.*, 2002). Previously, ODC was demonstrated after the growth of *Trametes* (*Coriolus*) *versicolor* on beech wood (Dutton *et al.*,

1994), and more recently *Phanerochaete chrysosporium* was reported to express ODC protein on oak wood cultures together with key enzymes involved in fungal metabolism of lignocellulose (Sato *et al.*, 2007).

Fig. 8. (a) Expression level of *D. squalens odc* in solid-state wood cultures after 3 and 4 weeks of incubation, as detected by real-time qRT-PCR. (b) Expression level of *D. squalens odc* in 2% liquid malt extract medium either without or after addition of 2.5 or 5 mM oxalic acid, as detected by real-time qRT-PCR. Two columns at each time point and treatment represent biological replicates. Error bars indicate the standard deviation between three replicate qPCRs. (c) Sizes of the PCR products from cDNA (lane 1) and genomic DNA (lane 2) amplified with gene-specific primer pairs designed to *D. squalens* ODC and GAPDH-encoding genes and used in real-time qRT-PCR.

To our surprise, *Ds-odc* was not upregulated at transcriptional level by addition of excess oxalic acid (2.5–5 mM), even though mycelial ODC activities were noticeably promoted by treatment with 5 mM oxalic acid. In contrast to the enzyme activities, the *Ds-odc* transcript levels were somewhat suppressed by these levels of extracellular oxalic acid, at least 1 day after the treatment in the submerged cultures. The highest level of transcripts was obtained with non-induced cultures where the extracellular acidity was kept at pH 4.4.

It is noteworthy that extracellular pH dropped to 2.7 immediately after introducing 5 mM oxalic acid. Our results differ from the data reported for the wood-colonizing basidiomycete *F. velutipes*, where the *odc* gene was induced at the transcriptional level at low pH (3.0)

without supplementation of exogenous oxalic acid (Azam *et al.*, 2002). The promoter region –287 to –278 bp upstream of the *F. velutipes odc* contains a low-pH responsive element, and a protein complex specifically binding to the promoter has been identified (Azam *et al.*, 2002). In our case, this type of element was not recognized within the 428 bp 5' promoter region of *D. squalens odc*. Likewise, the *Bacillus subtilis* ODC enzyme activity is inducible at acidic pH, independently of addition of oxalic acid (Tanner & Bornemann, 2000). The promoting effect of excess oxalic acid that was observed in our study for ODC activity in *D. squalens* may also indicate some kind of protein- or enzyme-level activation, which may be either due to low pH and extracellular acidity or specifically caused by oxalic acid.

The *Ds-odc* gene was cloned from the two isolates of *D. squalens*. The two genes were identical in sequence at the nucleotide level in the ORF coding regions (exons), and the introns were identically positioned and similar in length. *Ds-odc* shows a high number (17) of short introns, which vary in length from 49 to 91 nt. The ODC-encoding gene from *F. velutipes* also contains 17 small introns (Kesarwani *et al.*, 2000). Characteristically, the *Ds-odc* possesses two very short exons, only 10 and 18 nt in length. Of these, one is in the first cupin motif region, and the other is in the second cupin region (Fig. 4); this is again comparable with the *F. velutipes odc*, which contains two short exons of 18 and 21 nt in similar locations (Kesarwani *et al.*, 2000).

One of the striking features of the *Ds-odc* gene is the existence of one non-canonical 5' splice site in intron XIV with a GC dinucleotide at the beginning. The most common class of non-canonical intron splice sites in mammals is identically reported to consist of 5' splice sites with a GC dinucleotide (Wu & Krainer, 1999). In fungi, the percentage of introns with 5'-GC...AG-3' splice sites is reported to be 0.08 %, 0.86 %, 1.15 % and 1.19 % for the ascomycetes *Schizosaccharomyces pombe*, *Neurospora crassa*, *Aspergillus nidulans* and *Saccharomyces cerevisiae*, respectively, and even higher, 1.98 %, for the basidiomycetous yeast *Cryptococcus neoformans* (Kupfer *et al.*, 2004; Rep *et al.*, 2006). In the white-rot basidiomycete *Ceriporiopsis subvermispora*, one allele of its *oxo* gene has also been reported to contain one intron with GC instead of GT at the 5' splice site (Escutia *et al.*, 2005).

D. squalens was observed to produce two acidic ODC isozymes with pI values of 4.2 and 2.6, which is similar to the ODCs in the litter-decomposing basidiomycete *Agaricus bisporus* with pI values of 3.4 and 3.0 (Kathiara *et al.*, 2000), in *F. velutipes* with pI values of 3.3 and 2.5 (Mehta & Datta, 1991), and in *T. versicolor* with pI values of 3.0 and 2.3 (Dutton *et al.*, 1994). The molecular mass of the *D. squalens* ODC bicupin monomer, 52–55 kDa as estimated by SDS-PAGE, is slightly lower than those reported for the ODCs from *A. bisporus* (64 kDa) (Kathiara *et al.*, 2000), *F. velutipes* (64 kDa, deglycosylated enzyme 55 kDa) (Mehta & Datta, 1991), and *T. versicolor* (59 kDa)

(Dutton *et al.*, 1994). The functional *D. squalens* ODC is presumably a hexamer and approximately six times larger in terms of kilodaltons, as has been shown for the active *F. velutipes* ODC, with a molecular mass of 420 kDa under non-denaturing conditions (Chakraborty *et al.*, 2002).

With respect to the structural and functional similarities between ODC and OXO, and the first protein crystal structures available, their conserved Mn-binding sites have been compared (Svedružić *et al.*, 2005). Five amino acids, and especially Glu162 in *B. subtilis* ODC (OxdC), forming the so-called lid structure extending over one Mn-binding cupin site, seem to have a crucial role in binding of oxalic acid and the formic anion product, thus determining the specificity of oxalate oxidation and cleavage (Just *et al.*, 2004, 2007; Burrell *et al.*, 2007).

Burrell *et al.* (2007) showed with *B. subtilis* ODC that a mutation in Glu162 can convert the decarboxylase (ODC) activity into oxidase (OXO) activity. Ds-ODC also has a glutamate residue in the corresponding position (Glu271, Fig. 5), confirming its nature as a true ODC enzyme producing formic acid and CO₂ in the catalytic cycle. Multiple alignment of ODC sequences from basidiomycetous fungi shows that the *F. velutipes* and *T. versicolor* enzymes have a corresponding acidic residue, an aspartate, in this position (Fig. 5). Within the *B. subtilis* ODC, mutated aspartate in this position has been shown to lower the catalytic efficiency, but not the specificity of ODC (Svedružić *et al.*, 2007).

In spite of the N-terminal secretion signal peptide predicted in the Ds-ODC, most of the ODC activity was found in the mycelial extracts of *D. squalens* irrespective of the oxalic acid supplementation. This is consistent with observations for the ODC activities in *T. versicolor*, with 6–20 times lower activities detected in culture fluids than in the mycelial extracts (Dutton *et al.*, 1994). In *A. bisporus*, most of the ODC protein was located intracellularly (Kathiara *et al.*, 2000). In the brown-rot fungus *Postia placenta*, most of the ODC activity was observed on the surface of the fungal hyphae, while minor enzyme activities were detected intracellularly or in the culture filtrates (Micales, 1997).

These results raise a question regarding the location of ODC as a predominantly intracellular or fungal-cell-wall-associated enzyme, in spite of the existence of putative secretion signals in the white-rot fungal ODC amino acid sequences (Fig. 5), also present in the N-terminus of Ds-ODC. However, the possible targeting or signalling role of the ~60 aa alanine-serine-rich stretch in the N-terminus of the Ds-ODC is as yet unknown to us. A similar motif is present in some of the putative *P. chrysosporium* ODCs, and in a shorter form (~20 aa), it can be observed also in the *T. versicolor* ODC (Fig. 5).

As expected, Ds-ODC shows closest identity with the translated ODC protein sequences from other basidiomycetous fungi, with the highest pairwise identity (60 %) to

putative *P. chrysosporium* ODC sequences. In a previous study, when the conserved motif regions of proteins belonging to the cupin superfamily were analysed, bacterial and fungal ODCs formed one monophyletic clade (Khuri *et al.*, 2001). According to our phylogenetic analysis with the translated full-length ORF protein sequences, however, bacterial and fungal ODCs fall into several subclusters, indicating a diverse ODC enzyme family (Fig. 7). Most of the amino acid sequence dissimilarities are within the N-terminal regions.

Within our evolutionary tree, the basidiomycetous ODCs clustered within a main branch of their own (Fig. 7). The only exceptions to this were the two putative ODC-encoding sequences within the seven candidates recognized in the *P. chrysosporium* genome. The two exceptional sequences were the shortest, and clearly separated from the basidiomycetous and ascomycetous ODC branches. The basidiomycetous ODC cluster also includes the bicupin OXO cloned from *C. subvermispora*, thus confirming the very close evolutionary relationship of the Cs-OXO to the ODC proteins, which is in accordance with the previous suggestion of Escutia *et al.* (2005).

To our knowledge, *C. subvermispora* is the first fungus from which both ODC and OXO activities have been measured (Aguilar *et al.*, 1999; Watanabe *et al.*, 2005), and a scheme for the participation of both enzymes in the fungal metabolic reactions and lignin degradation has been proposed (Watanabe *et al.*, 2005). Furthermore in *C. subvermispora*, ODC is suggested to act sequentially with formate dehydrogenase (FDH). This will lead to complete conversion of oxalic acid to CO₂ with concomitant generation of NADH, which can be used as an electron source for ATP synthesis during vegetative growth of the fungus. A similar mechanism may also operate in all ODC-producing white-rot fungi, and FDH activity has in fact been detected in *F. velutipes*, *P. chrysosporium* and *T. versicolor* (Watanabe *et al.*, 2005). Very recently with the brown-rot fungus *Postia placenta*, transcripts of one putative ODC, three putative FDHs and one putative formate transporter-encoding gene were shown to be upregulated in cellulose media, and a physiological relationship between these genes was proposed (Martinez *et al.*, 2009). On the other hand, *C. subvermispora* OXO has been suggested to contribute more to lignin biodegradation by generating extracellular H₂O₂, which is needed for example in the catalysis of the lignin-modifying peroxidases during fungal secondary metabolism (Escutia *et al.*, 2005; Watanabe *et al.*, 2005).

Our data indicate that expression of the novel *Ds-odc* gene characterized in this work is not induced at the level of transcription by addition of oxalic acid or extracellular acidity. These findings do not exclude the possibility that the Ds-ODC enzyme may be activated at the protein level by oxalic acid or increasing acidity. These notions imply a constitutive metabolic role for the *D. squalens* ODC. The enzyme could operate in tight conjunction with formic

acid conversion and energy generation in fungi, as was by proposed Watanabe *et al.* (2005). It is predictable that more than one *odc* gene exists also in *D. squalens*. These results indicate that fungal ODCs have variant regulatory responses, and thereby diverse metabolic roles, for the wood-decaying fungi.

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