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RESEARCH ARTICLE

Molecular cloning of three pyranose dehydrogenase-encoding genes from *Agaricus meleagris* and analysis of their expression by real-time RT-PCR

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Abstract Sugar oxidoreductases such as cellobiose dehydrogenase or pyranose oxidase are widespread enzymes among fungi, whose biological function is largely speculative. We investigated a similar gene family in the mushroom *Agaricus meleagris* and its expression under various conditions. Three genes (named *pdh1*, *pdh2* and *pdh3*) putatively encoding pyranose dehydrogenases were isolated. All three genes displayed a conserved structure and organization, and the respective cDNAs contained ORFs translating into polypeptides of 602 or 600 amino acids. The N-terminal sections of all three genes encode putative signal peptides consistent with the enzymes extracellular secretion. We cultivated the fungus on different carbon sources and analyzed the mRNA levels of all three genes over a period of several weeks using real-time RT-PCR. The glyceraldehyde-3-phosphate dehydrogenase gene from *A. meleagris* was also isolated and served as reference gene. *pdh2* and *pdh3* are essentially transcribed constitutively, whereas *pdh1* expression is upregulated upon exhaustion of the carbon source; *pdh1* appears to be additionally regulated under conditions of oxygen limitation. These data are consistent with an assumed role in lignocellulose degradation.

Keywords Pyranose dehydrogenase · GMC-family of flavin-containing enzymes · Lignocellulose degradation · *Agaricus* spp. · Transcription analysis

Introduction

Oxidoreductases of free, non-phosphorylated sugars are very common in fungi and are investigated mainly for their technological potential. Their biological function, however, is largely unknown and speculative and ranges from roles in lignin degradation to functions in defense or pathogenesis. Pyranose dehydrogenases are comparably novel representatives, and knowledge was thus far mostly limited to catalytic properties.

Glucose 1-oxidase (EC 1.1.3.4) catalyzes the oxidation of D-glucose at position C-1 and is typical for a wide range of Ascomycetes and Fungi Imperfecti. Pyranose 2-oxidase (P2O, EC 1.1.3.10) is described as a tetrameric protein containing one FAD molecule per subunit that catalyzes the oxidation of aldopyranoses at C-2 to the corresponding ketoaldoses. It was characterized from *Trametes multicolor* (Leitner et al. 2001) and other white-rot Basidiomycetes (Giffhorn 2000), and genes putatively encoding P2O (but lacking experimental confirmation) are also found in the

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genomes of non-ligninolytic species such as *Aspergillus nidulans*. The hemoflavoprotein cellobiose dehydrogenase (CDH, EC 1.1.99.18) oxidizes cellobiose (Glc-β-1,4-Glc) and other β-1,4-linked di- or oligosaccharides at C-1 to the corresponding lactones and was likewise described in a number of Basidiomycetes (white- and brown-rot fungi) as well as (mostly thermophilic) Ascomycetes (Zamocky et al. 2006), and a number of hypothetic proteins in genome annotations are classified as CDH.

The catalytically related pyranose dehydrogenase (PDH, EC 1.1.99.29) was first isolated from *Agaricus bisporus* (Volc et al. 1997). Originally mistaken for CDH due to low activity with cellobiose (Morrison et al. 1999), it is a monomeric protein that is unable to utilize dioxygen as electron acceptor and relies on (substituted) quinones or complexed metal ions as alternatives. All major constituents of (hemi)cellulose such as D-glucose, D-xylose, D-galactose and L-arabinose yield comparable activities (Volc et al. 1997; Kujawa et al. 2007). Depending on enzyme source and sugar substrate, oxidation occurs at various positions, with C-2 and/or C-3 predominating.

Current knowledge about the biological function of these enzymes is very limited. P2O is implicated to provide H_2O_2, an essential cofactor to ligninolytic peroxidases (Daniel et al. 1994); a function that is impossible for PDH or CDH, which do not produce H_2O_2. Other functions suggested for P2O and CDH (and conceivable for PDH) are the reduction of quinone or radical intermediates formed during lignin degradation, preventing their repolymerization (Ander and Marzullo 1997; Giffhorn 2000), as well as the production of Fenton's reagent through reduction of metal ions (Kerem et al. 1999), which may degrade cellulose, xylan and lignin (Henriksson et al. 1995). The only experimental evidence for a function of any of these enzymes is the reduced ability of fungal hyphae to penetrate wood, observed for a *T. versicolor cdh* disruptant strain (Dumonceaux et al. 2001). Despite the increasing number of cloned or postulated genes encoding CDH or P2O found in recent years, studies on their expression are scarce. It is interesting to note that a common occurrence of P2O and PDH in the same species is unknown to date: among 76 lignocellulose-degrading basidiomycetes, white-rot fungi such as *Phanerochate chrysosporium*, *Phlebia radiata*, and *Trametes* spp. generally produced P2O (and often CDH) but not PDH, whereas production of PDH was limited to Agaricaceae and Lycoperdaceae, which do not produce P2O (Volc et al. 2001). Common occurrence of CDH and PDH is also not observed to date. Ecologically, PDH-producing species are referred to as "litter-decomposing" fungi. They mostly grow on lignocellulose-rich forest litter but not on compact lignocellulose such as tree trunks or branches (Dix and Webster 1995). Many are able to mineralize lignin, albeit with less efficiency than white-rot fungi (Steffen et al. 2000).

Pyranose dehydrogenase from *A. xanthoderma* and *A. meleagris* was recently characterized in detail (Kujawa et al. 2007; Sygmund et al. 2007). Here we present the cloning and analysis of three genes from *A. meleagris* encoding PDH and two other, as yet unknown, proteins with great similarities to PDH, as well as data on their expression.

Materials and methods

Organisms and culture conditions

All chemicals and media components were purchased from Sigma (Steinheim, GER) unless stated otherwise, and were of the highest purity available. *Agaricus meleagris* SCHAEff (strain CCBAS 907) was obtained from the Culture Collection of Basidiomycetes of the Academy of Sciences (Prague, Czech Republic). For DNA isolation, 1 l-Roux flasks containing 150 ml of glucose–casein medium were inoculated with mycelial fragments from freshly grown malt extract agar plates (1.5% malt extract, 0.15% peptone from soy bean, 2% agar), which were cut in small pieces and gently homogenized under sterile conditions, and incubated at 28°C without agitation. For transcript analysis, Roux-flask cultures were harvested after 1 week and washed three times in Basal Salts Medium (Manning and Wood 1983) without carbon source. One milliliter of mycelial suspension was used to inoculate 9 ml of liquid minimal medium with 1% (w/v) carbon source as indicated. These cultivations were performed in petri dishes over 24 days, the contents of a whole petri dish were harvested at indicated time points by filtration through a paper filter, and pyranose dehydrogenase activity and residual sugar concentration were measured in the filtrate. Pre-weighed filter paper discs with filtered mycelium were dried over night at 105°C and weighed on a Sartorius analytical balance.

Peptide sequencing by µHPLC-nano ESI mass spectrometry

The purification and biochemical characterization of *A. meleagris* PDH were described recently (Sygmund et al. 2007). Coomassie-Blue-stained protein bands were excised from the gel and destained in 10 mM dithiotreitol (DTT), 0.1 M 4-ethylmorpholine acetate (pH 8.1) in aqueous 50% acetonitrile. The gel slices were washed with water, acetonitrile and again in water, partly dried in a SpeedVac concentrator and rehydrated in a cleavage buffer containing 0.01% 2-mercaptoethanol, 50 mM 4-ethylmorpholine acetate, 10% acetonitrile, 1 µl of protease solution, and 50% of $H_2{}^{18}O$ water. Trypsin (Promega, Madison, WI, USA) and Lys-C protease (Roche Diagnostics, Mannheim, Germany)

at 50 ng μl^{-1}, and Asp-N and Arg-C protease (Roche) at 10 ng μl^{-1} (all sequencing grade) were used for overnight digestion at 37°C, then the peptide mixtures were acidified with 5% acetic acid and purified and concentrated using C18 ZipTips™ (Millipore). Peptide fragments were loaded onto a homemade capillary column (0.18 × 100 mm) packed with MAGIC C18 (5 μm, 200 Å) reversed phase resin (Michrom BioResources, Auburn, CA, USA) and separated using a gradient from 5% MeCN/0.5% acetic acid to 40% MeCN/0.5% acetic acid for 50 min. The column was connected to an LCQ^{DECA} ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with a nanoelectrospray ion source. The spray voltage was held at 1.2 kV and the tube lens potential was 10 V. The capillary was kept at 175°C with a voltage of 30 V. Full scan spectra were recorded in positive mode over the mass range 350–2,000 Da. MS/MS sequencing data were automatically acquired on the two most intense precursor ions in each full scan spectrum and interpreted manually.

Isolation of PDH- and glyceraldehydes-3-phosphate dehydrogenase-encoding (*gpd*) genomic DNA and cDNA

Agaricus meleagris mycelium was harvested from growing cultures (for RNA isolation, cultures with detectable PDH activity were selected), squeezed dry between filter paper and shock-frozen in liquid nitrogen. Nucleic acid isolations were done using the DNeasy Plant System, Lambda Midi Kit and RNeasy Plant System (Qiagen, Valencia, CA, USA), respectively. Restriction enzymes, polymerases and DNA modifying enzymes were obtained from Fermentas (St. Leon-Rot, GER) and used as recommended. Nucleic acid amplifications were done using thermostable *Taq* DNA polymerase or (for amplification of full-length cDNA) *Pfu* proof-reading polymerase, dNTP mix, oligonucleotide-primers (VBC Biotech, Vienna, Austria) and a Biometra TRIO thermocycler (Biometra, Göttingen, Germany). cDNA first strand synthesis was done using the First Strand cDNA Synthesis Kit (Fermentas) and an anchor primer (see Table 1). A genomic library of *A. meleagris* was constructed according to standard protocols (Ausubel et al. 1990) using *Sau*3A-partially digested genomic DNA and λGEM11 phage vector arms (Promega, Madison, WI, USA). Ligation assays were packed into bacteriophage heads using a Packagene Gold Extract (Stratagene, La Jolla, CA, USA) according to the manufacturers instructions and *Escherichia coli* LE392 as host strain. Library screening was done according to standard procedures (Ausubel et al. 1990) using positively charged nylon membranes, the DIG PCR Probe Synthesis Kit, DIG EasyHyb Granules for hybridization and the DIG Luminescent Detection Kit (all from Roche Diagnostics). Southern Hybridizations were performed by digesting fungal genomic DNA (or isolated λ-bacteriophage DNA for mapping of phage clone inserts) using various restriction enzymes, followed by electrophoresis, capillary transfer to nylon membranes and hybridization with labeled probes according to standard protocols (Ausubel et al. 1990).

Table 1 Oligonucleotide primers used for cloning experiments

	Sequence 5′–3′	Derived from/specific for
Csrk1	atcacctaccagca(c/t)ga(c/t)ga	ITYQHPDD, N-terminus
Csrk2	ttgaagaa(c/t)tc(a/c/g/t)gc(a/g)tt	NAEFPFK, sequence tag 8
Csrk3	cacaactagcccacggttcc	5′-RAGE, gene specific
Csrk4	ggaaacgtaccgccagcgacg	5′-RAGE, nested primer
Csrk5	gttgtgtggaattgtgagcgg	5′-RAGE, plasmid-specific
Csrk6	acgccaagcgcgcaattaacc	5′-RAGE, nested primer
anchor	ggccacgcgtcgactagtacttttttttttttt	Reverse transcription
Csrk7	gtggcgatatttaaggctttcgg	*pdh1* cDNA amplification
universal	ggccacgcgtcgactagtac	cDNA amplification
Csrk8	catagcatgcaacaacgtgagac	*pdh1* genomic DNA amplification
Csrk9	actatggccttatgctttagc	*pdh2* cDNA amplification
Csrk10	accttatggcttatgctacgg	*pdh3* cDNA amplification
Csrk11	aagcttaggcatagctctttgc	*pdh3* genomic DNA amplification
Csrk12	atggt(c/t)aa(c/t)gt(c/t)gg(a/c/t)at(c/t)aa(c/t)gg	MVNVGING, conserved N-terminus of GPD
Csrk13	ta(a/g/t)cccca(c/t)tc(a/g)tt(a/g)tc(a/g)tacc	WYDNEWGY, conserved region of GPD
Csrk14	gcctacatgttcaagtatgactc	*gpd* cDNA amplification
Csrk15	cgagatcaatgaacgggtcg	5′-RAGE, gene specific
Csrk16	ggagagcattacggagcacg	5′-RAGE, nested primer
Csrk17	cctctccgttttcttcctgtac	*gpd* genomic DNA amplification
Csrk18	aagcaatcacggaacaacgc	*gpd* genomic DNA amplification

Rapid Amplification of Genomic Ends (RAGE) PCR (Mizobuchi and Frohman 1993) was performed using genomic DNA and pBluescript SK+ (Stratagene), digested with the same restriction enzymes (plasmid DNA was dephosphorylated with Shrimp Alkaline Phosphatase) and ligated with T4 DNA Ligase, and primers as listed in Table 1. Amplicons were cloned into the pCR 2.1 or the pCR Blunt II vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturers instructions and sequenced by a commercial service provider (VBC Biotech).

Sequence analysis

The translated amino acid sequences of the obtained cDNAs were analyzed using the programs Compute pI/mW, SignalP 3.0, and NetNGlyc 1.0 at http://www.cbs.dtu.dk/services/ (Nielsen et al. 1997; Gasteiger et al. 2003; Bendtsen et al. 2004; Blom et al. 2004). Multiple sequence alignments were done with Clustal W 1.83 (http://clustalw.genome.jp/; gap opening penalty 8.0, gap extension penalty 0.3 and Gonet series protein weight matrix). The phylogeny of 70 GMC oxidoreductases was reconstructed with the MEGA package, version 3.1 (Nei and Kumar 2000) using the Neighbor-joining method with following parameters: pairwise deletion of gaps, Poisson correction for amino acid substitutions, all substitutions included, homogenous pattern among lineages and uniform rates of distribution among sites. The reconstruction was tested by using 1,000 bootstrap replicates and depicted as unrooted phylogenetic tree.

Transcript quantification of *pdh* genes

One micrograms of RNA aliquots extracted from 100 mg mycelial samples were reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) using random hexamers as primers. The reaction was incubated for 10 min at 25°C (annealing), 120 min at 37°C (elongation), and 20 s at 85°C (reverse transcriptase inactivation).

Specific primers for the *pdh* genes and the *gpd* gene of *A. meleagris* were designed to generate amplicons between 259 and 335 bp (Table 2), all spanning an intron, to distinguish amplicons arising from trace amounts of genomic DNA. High similarities between the *pdh* genes required placing the reverse primers downstream of the stop codons to achieve sufficient specificity. PCR assays and gel electrophoresis were performed to verify specific amplification, and melting point analysis was performed after each real-time PCR run to confirm the amplification of a unique product for each well. The annealing temperature of the primer pairs was tested using a gradient PCR from 54 to 64°C. The highest temperature fitting all pairs was 60.4°C. 1:10, 1:100 and 1:1,000 dilutions of some templates were run in parallel to check the efficiency of the primers and for the presence of PCR inhibiting substances.

Table 2 Oligonucleotide primers used for real-time PCR transcript analysis

	Sequence 5′–3′	Specific for
Csrk19	catggtgtgggaacgttgtcg	5′-Real time primer *pdh1*
Csrk20	catagcatgcaacaacgtgagac	3′-Real time primer *pdh1*
Csrk21	ggctggcgttacctctgatgaa	5′-Real time primer *pdh2*
Csrk22	cggataattctcgggtactc	3′-Real time primer *pdh2*
Csrk23	aggacgttgtcaatggaacagt	5′-Real time primer *pdh3*
Csrk24	tctacacgctcgctgatgag	3′-Real time primer *pdh3*
Csrk25	actgttcatgccaccactgc	5′-Real time primer *gpd*
Csrk26	atgcccctgtattcgccctc	3′-Real time primer *gpd*

Ratio of 1:10 dilutions were directly used as templates for quantitative real-time PCR in 96-well plates using a MyIQ Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Fluorescein Calibration Dye (Bio-Rad) was added to a final concentration of 10 nM as an internal reference for normalization of the reporter fluorescence. The reaction mixture was prepared using 12.75 µl Power SYBR Green PCR Master Mix (Applied Biosystems) including the Fluorescein Calibration Dye, 0.4 µl of each primer (6.25 µM), 8.95 µl nuclease-free DEPC-treated water (Fermentas) and 2.5 µl of the cDNA sample (1:10 dilution). Amplification conditions were 10 min at 95°C to activate the hot-start *Taq* DNA polymerase, followed by 50 cycles of 95, 60.4 and 72°C (20 s each). Three technical replicates were conducted for each sample, and appropriate controls containing no template were included.

Baseline range and threshold values were calculated using the MyIQ v2.0 software (Bio-Rad), amplification efficiency was estimated for each reaction (Tichopad et al. 2003) and was generally over 0.9. The *gpd* gene was included for every sample for normalization of expression levels of the genes of interest. Changes of *pdh* transcripts in the samples versus control (i.e., the sample of the time point 0) were calculated using the formula: ratio = $[(E_{\text{target}})^{\Delta Ct}\text{target}^{(\text{control–sample})}]/[(E_{\text{ref}})^{\Delta Ct}\text{ref}^{(\text{control–sample})}]$, where E_{target} is the real-time PCR efficiency of a *pdh* transcript, E_{ref} is the real-time PCR efficiency of the *gpd* transcript, $\Delta Ct_{\text{target}}$ is the Ct deviation of control–sample of the target gene, and ΔCt_{ref} is the deviation of control–sample of *gpd* (Pfaffl 2001).

GenBank accession numbers

The GenBank accession numbers for the genes in this work are AY753306, AY753308, DQ117577 and EU090910 (genes), AY753307, AY753309, DQ117578 and EU090911

(mRNAs), and AAW82996, AAW82999 and AAZ94875 (proteins).

Results

Isolation of PDH-encoding genomic DNA and cDNAs

During growth on a glucose/casein medium *A. meleagris* was found to secret one major protein with PDH activity, which was purified to apparent homogeneity (Sygmund et al. 2007). Internal peptide sequences (sequence tags) were determined by μHPLC-nano ESI/MS (Table 3). Oligonucleotide-primers were designed from the N-terminus of the purified protein (AITYQHPDDL; forward primer Csrk1) and of sequence tags containing few amino acids encoded by four or more codons (e.g. numbers 4 and 8, Table 3) using the programme CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/codehop.html; Rose et al. 1998), and used to amplify an 804 bp long fragment of genomic DNA (reverse primer Csrk2, Table 1). Sequence analysis revealed significant similarity to fungal dehydrogenases and oxidases as well as additional experimentally determined sequence tags, and confirmed that the amplified fragment represented a portion of the gene encoding the purified enzyme. The fragment was used to screen a genomic library of *A. meleagris*. A positive phage clone was obtained and found to contain the entire coding region of a putative dehydrogenase gene which was highly similar (approx. 85%) but not identical to the PCR fragment, and none of the peptide sequence tags was found in the conceptual translations. We tentatively named this gene *pdh2*, assuming it encodes an enzyme with a likely similar activity as PDH. Repeated screening of the library did not turn up further clones containing a full-length copy of (the gene now named) *pdh1* nor other, similar sequences, probably due to a low complexity of the primary library. Therefore, the gene fragment amplified earlier was extended by 5′-RAGE-PCR using the gene-specific primers Csrk3 and Csrk4 and the plasmid-specific primers Csrk5 and Csrk6 to obtain a DNA fragment containing the 5′-portion of *pdh1* and 5′-untranslated regions. First strand cDNA was reverse-transcribed from RNA obtained from cultures of *A. meleagris* with significant PDH activity, and a full-length cDNA encoding PDH was amplified using a forward primer binding upstream of the putative start codon (Csrk7) and a universal primer specific for the multiple cloning site of the anchor primer. Subsequently the full-length *pdh1* gene was amplified from genomic DNA using Csrk7 and a reverse primer (Csrk8) annealing shortly downstream of the stop codon. Interestingly, during RAGE experiments, an additional similar sequence was amplified, representing a third gene possibly encoding an enzyme with PDH-activity. Using the same strategy as for *pdh1*, a full-length copy of both the coding sequence (primers Csrk10 and Csrk11) as well as the cDNA (Csrk10 and universal) was obtained for this gene, tentatively named *pdh3*, and eventually a full-length cDNA clone of *pdh2* (Csrk9 and universal; all primers see Table 1). Southern Hybridization of digested genomic DNA of *A. meleagris* with each of the three genes separately revealed identical patterns due to cross-hybridization but allowed assignment of all bands to the three genes (not shown). Thus there is no indication of additional genes encoding PDH-like enzymes.

Table 3 Internal peptide sequence tags of PDH determined by μHPLC-nano ESI MS

No.	Protease	Sequence tag
1	Trypsin, Lys-C	**KVLxVLxEAG**PSN**K**
2	Trypsin, Lys-C	**K**xx**SWGVVNPDFK**
3	Trypsin	(ED/DE)**LxDLxALxLxR**
4	Trypsin, Lys-C	**K**FT**QDFTDQ**(W/SV/VS)**K**
5	Trypsin, Lys-C	**K**(VE/EV/DLx/LxD)**FAVDAN**SP**K**
6	Trypsin	**QAPLxPAA**xxx**R**
7	Trypsin, Lys-C	**K**xxx**AVGLxDTLxLxDN**xxx**K**
8	Trypsin, Lys-C	**KELxNAEFPFK**

Lx: Leu or Ile; bold: certain; in regular print: highly probable; (in brackets): different possibilities; x: unknown. W and SV/VS in Nr. 4 and VE/EV and DLx/LxD in No. 5, respectively, have the same mass and cannot be distinguished

Isolation of *gpd* cDNA and genomic DNA of *A. meleagris*

In order to provide a reference gene for real-time RT-PCR, we also isolated a GPD-encoding gene from *A. meleagris*. The amino acid sequences of the GPD proteins of *Coprinopsis cinerea*, *Crinipellis perniciosa*, *Schizophyllum commune*, *Pycnoporus coccineus* and *A. bisporus* were aligned using the program Clustal W 1.83 (http://clustalw.genome.jp). Primers Csrk12 and Csrk13 were derived from conserved regions lacking amino acids encoded by six codons. A 972 bp long fragment was amplified by PCR using *A. meleagris* cDNA as template. The 5′-region was amplified by RAGE-PCR using the gene-specific primers Csrk15 and Csrk16 and the plasmid-specific primers Csrk5 and Csrk6 to obtain the 5′-portion of the gene including upstream regions. The 3′ part of the *gpd*-cDNA was amplified using the primers Csrk14 and universal and a cDNA reverse transcribed with an anchor primer as template. The genomic DNA of *gpd* was amplified with primers Csrk17 and Csrk18.

Analysis of gene and protein sequences

All three *pdh* genes consisted of ten exons separated by nine introns, 51–60 bp in length, as established by comparison

of genomic with cDNA sequences. Positions and lengths of the introns and exons, respectively, are identical in *pdh2* and *pdh3*, with only minor deviations in *pdh1*—additional three nucleotides in exons 3 and 6. Hence the mRNA of *pdh1* contains an ORF of 1,809 bp translating into 602 amino acids, as compared to 1,803 bp and 600 amino acids for *pdh2* and *pdh3*. The N-terminal amino acid sequence of the purified mature protein (PDH1) is found in all three sequences, starting at position 26, the internal peptide sequence tags, however, are all exclusively found in the translation of *pdh1* (Fig. 1), and respective regions in the other sequences are distinctly different. Moreover, MS data obtained on tryptic, Lys-C, Arg-C and Asp-N digests of the protein cover approximately 95% of PDH1 sequence (not shown). The N-terminal sequence is preceded by a signal peptidase cleavage site between amino acids 25 and 26 (Ala–Arg–Gly–Ala–Ile, cleavage between Gly and Ala), and the protein is classified as a secretory protein with a probability of 0.762 (SignalP; PDH2 and PDH3: probabilities 0.591 and 0.831, respectively). The calculated molecular masses of the mature proteins minus the signal sequence are 61 967, 62 536 and 62 269 Da, respectively, and the theoretical p*I*s are 5.42, 4.80 and 4.82 (compute p*I*/Mw). PDH1 contains five potential N-glycosylation sites conforming to the consensus N-X-S/T, asparagine residues 100, 200, 277, 338 and 344 (numbering includes the signal sequence). N338 should be rendered non-functional by the presence of proline at position X, and N344 shows a low probability of actually being glycosylated (NetNGlyc 1.0). PDH2 has potential glycosylation sites in almost identical positions—99, 199, 275, 336 and 342 plus an additional

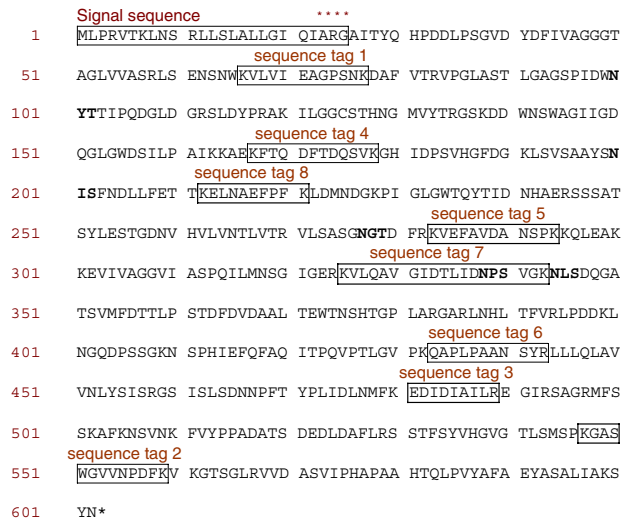

Fig. 1 Amino acid sequence of *Agaricus meleagris* PDH1. The signal sequence and sequence tags obtained by μHPLC-nano ESI MS peptide sequencing are *boxed*, putative *N*-glycosylation sites are in *bold* print. The signal sequence cleavage site (amino acid −3 to +1 of the mature protein) is marked with *asterisks*

site (N114), also with N336 likely ruled out (proline at position X) and a low probability for N342. PDH3 contains additional three sites with high (N173, N399, N507) and one with low probability (N546).

PDH1 and PDH2 share 75% identical and 85% conserved amino acids, PDH1 and PDH3 76% (85%), and PDH2 and PDH3 84% identical and 92% conserved amino acids upon comparison using the BLAST algorithm (Altschul et al. 1997). Interestingly, similarity with the recently described amino acid sequence of PDH from *A. xanthoderma* (Kujawa et al. 2007) is also highest with PDH2 (89 and 94%) and PDH3 (90 and 95%) and lower with PDH1 (75 and 86%). All three proteins display similarity in the range of 25–35% with various oxidoreductases found in GenBank, namely aryl alcohol oxidase from *Pleurotus pulmonarius* and *Pleurotus eryngii* (accession numbers AAF31169 and AAC72747), cellobiose dehydrogenase flavoprotein-domains from various Basidiomycetes as well as a number of hypothetical proteins from fungal genomes, among them the white-rot-fungi *P. chrysosporium* and *Laccaria bicolor* (http://genome.jgi-psf.org), *C. cinerea* but also *Aspergillus* spp., *Neurospora crassa*, *Fusarium graminearum*, *Magnaporthe grisea* and *Ustilago maydis* (http://www.broad.mit.edu/annotation/fgi). Similarities with catalytically related pyranose oxidases, e.g., from *P. chrysosporium*, *T. multicolor* and *Tricholoma matsutake* (accession numbers AAS93628, AAX09279 and BAC24805, respectively) were considerably lower (data not shown).

The amino acid sequence translated from the *gpd* cDNA was 94% identical with the respective protein from *A. bisporus*. The genetic organization is highly similar to the one described for *A. bisporus gpd2* (Harmsen et al. 1992): the start codon ATG is immediately followed by an intron, exon 3 consists of only five nucleotides and the positions of all nine introns are strictly conserved. The upstream sequence of the gene showed high similarity to the *A. bisporus* gene *gpd1*, situated upstream and in tandem of *gpd2*. Unlike in *A. bisporus* this similarity only extends over two stretches of less than 200 bp each, and no proper gene structure that can be transcribed into a cDNA containing an ORF could be detected (data not shown).

Phylogeny of PDH genes

A reconstructed phylogram is presented in Fig. 2. All major clades of the GMC-superfamily of flavoproteins defined in previous works (Zamocky et al. 2004) are clearly discernible. Cholesterol oxidases as a distinct clade reveal bacterial origin and are closely related to a newly defined clade of fungal pyranose 2-oxidases. In the case of CBQ (fifth clade in the previous work) new members from recently sequenced genomes are included. Basidiomycete *pdh*

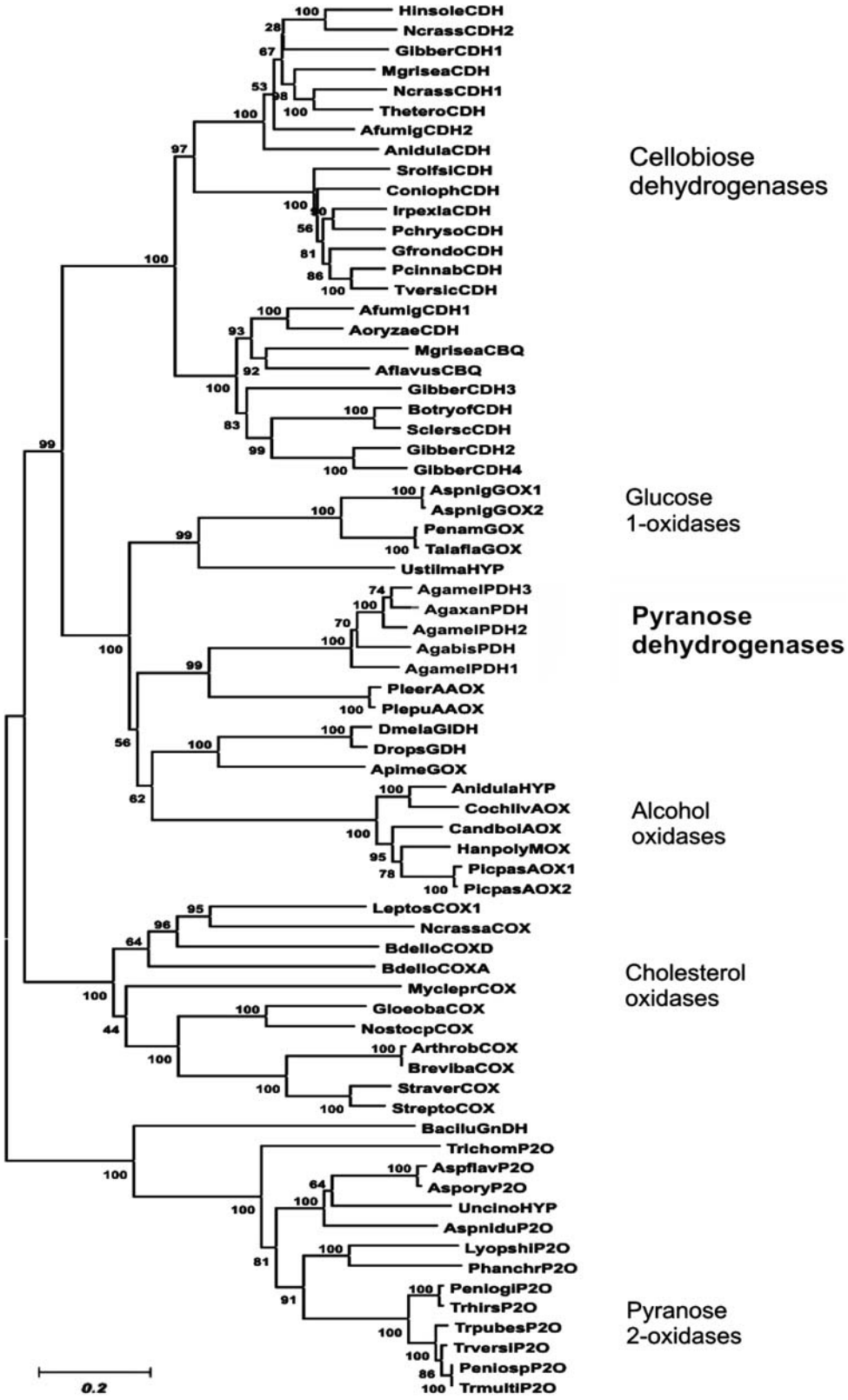

Fig. 2 Reconstructed unrooted phylogenetic tree of the GMC superfamily. *Numbers* on the branches represent bootstrap values obtained from 1,000 replications. The *scale bar* represents 20% of the estimated sequence divergence. Pyranose dehydrogenase genes are in *bold*

genes—focus of this work—form a well-separated clade in the first and largest subfamily represented by alcohol oxidases, glucose 1-oxidases and glucose dehydrogenases of fungal and metazoan origin.

Transcript analysis of *A. meleagris pdh* genes

Agaricus meleagris was cultivated as described in "Materials and methods" on minimal medium containing

D-glucose, cellobiose, D-fructose, D-xylose, lactose, xylan, carboxy-methyl-cellulose and glycerol as carbon sources respectively. The fungus only showed growth on D-glucose, cellobiose, D-fructose and to a very limited extent on glycerol. Samples were taken over 24 days of cultivation. During the first 2–3 days of cultivation the mycelial fragments were in a lag phase, showing no increase in biomass. The fungus entered the growth phase approximately on day 3. The growth curves on all three carbon sources are similar, only cellobiose causes an elongation of the growth phase when biomass on the other carbon sources is already decreasing (Fig. 3). Cellobiose is hydrolyzed to glucose, which is consumed very slowly during the stationary phase. PDH production was highest on cellobiose and very low on fructose.

Changes of the mRNA levels are compared to the first sample taken of the washed mycelium on day 0 of the cultivation. An up to 80-fold rise in the mRNA level of *pdh1* could be detected during the lag phase on cellobiose, more than 30-fold on glucose and approximately 16-fold on fructose (Fig. 4). Later, a significant increase occurred on glucose when the carbon source was almost depleted during the transition into the stationary phase (day 12, Fig. 5a). This is mirrored in the slow increase in PDH activity starting at a time when the carbon source was almost consumed. Both mRNA-formation and PDH-activity continued to increase for several days, until biomass started to decrease. On cellobiose the mRNA level never fell to the low levels observed on glucose and fructose. The increase around day 12, at a point when almost no residual cellobiose was detected, but glucose (derived from cellobiose through hydrolysis by β-glucosidase) was still present, is significantly weaker. Late in the cultivation the mRNA levels on cellobiose were always higher than on glucose and lowest on fructose.

pdh2 and *pdh3* are transcribed on a much lower level (Fig. 5b, c). On average over all measured samples *pdh3* shows only 4.8% of the amount of transcript of *pdh1*, and *pdh2* only 0.2%. Both genes neither show the increase in mRNA levels during the lag phase, nor the sharp increase in transcription around day 12 as for *pdh1*. Transcription levels only vary within a range of about 200% throughout the cultivation and are thus almost constant.

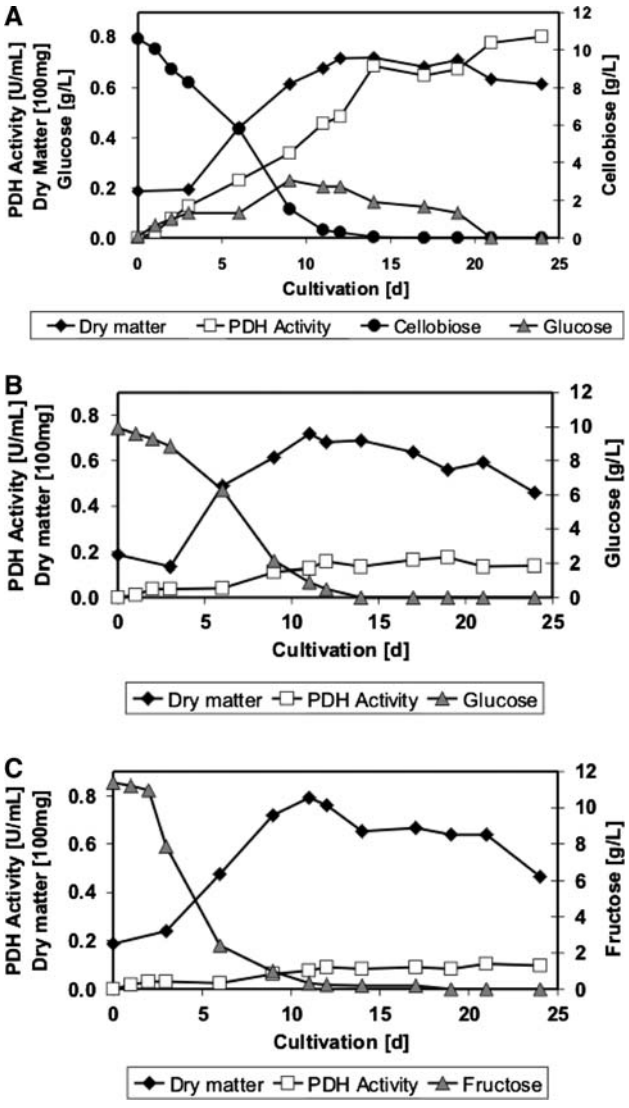

Fig. 3 Biomass production, carbon source consumption and PDH activity of *A. meleagris* on three carbon sources. **a** cellobiose; **b** D-glucose; **c** D-fructose

Discussion

This work reports on the isolation and transcript analysis of several genes encoding pyranose dehydrogenase from the litter-decomposing mushroom *A. meleagris*. Starting from peptide sequence tags obtained from a purified protein, the

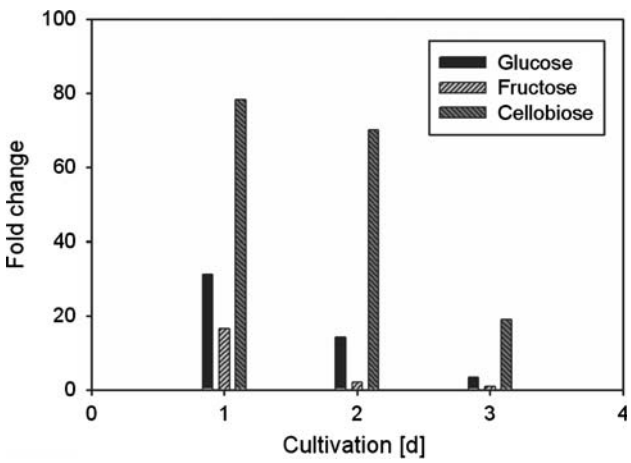

Fig. 4 Transcript levels of *pdh1* during the first 5 days following transfer of the mycelia to fresh medium containing the three indicated carbon sources. Levels of mRNA are related to the control sample taken at Day 0 immediately after transfer

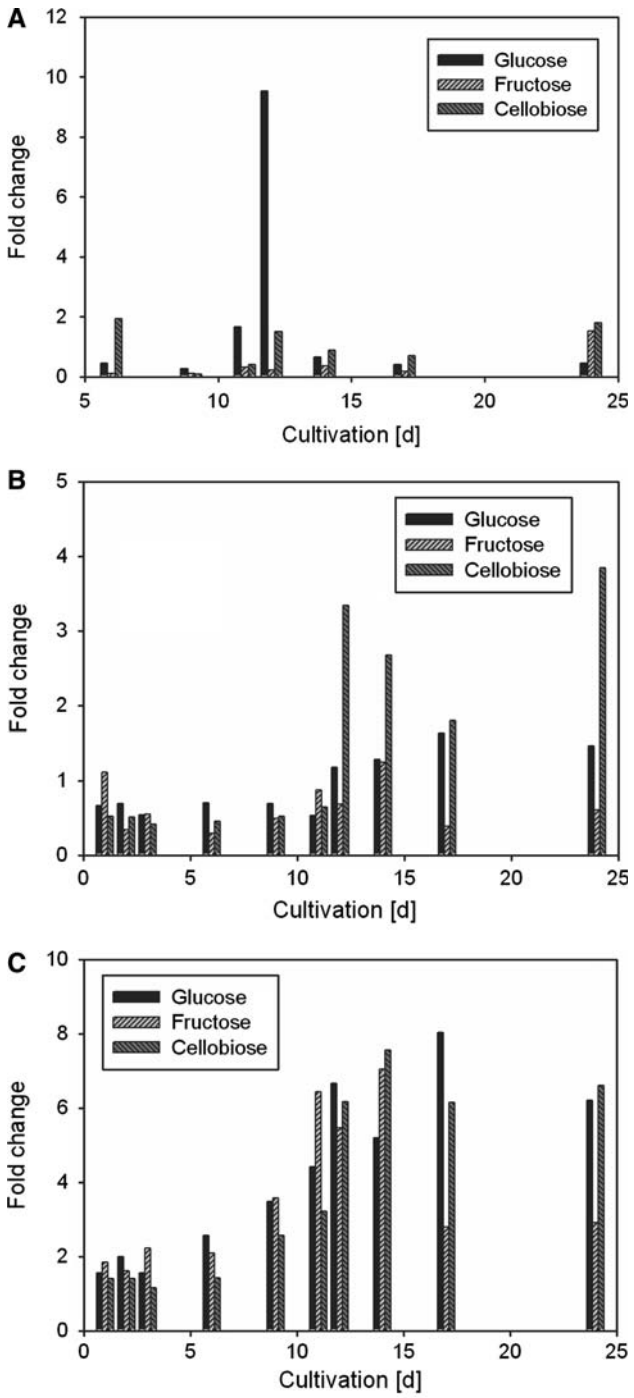

Fig. 5 Transcript analysis of PDH-encoding genes of *A. meleagris* during the growth phase on three carbon sources. **a** *pdh1*, the first 5 days are displayed in Fig. 4 to keep the scale manageable; **b** *pdh2;* **c** *pdh3*. Levels of mRNA are related to the transcription level in the control sample taken at Day 0 immediately after transfer of the mycelia to fresh medium containing the three indicated carbon sources

gene encoding PDH was isolated as described in "Results" together with two related sequences, which putatively encode highly similar, previously undescribed proteins. There is to date no experimental evidence as to the catalytic properties of these proteins, and the naming of *pdh2* and *pdh3* is therefore tentative. The high similarity between these genes and with the *pdh* gene from *A. xanthoderma* suggests that they all encode enzymes with PDH activities. Attempts to confirm this by heterologous expression of the genes have thus far been unsuccessful, as no active protein could be obtained in sufficient amounts in *E. coli*, *P. pastoris* and *Trichoderma reesei* (our unpublished information). This matter is the subject of ongoing studies. When the amino acid sequences of the three *pdh* genes, or a cellobiose dehydrogenase sequence from *T. versicolor* (GenBank accession no. AAO32063), are compared with the genome of *C. cinerea* (the most closely related mushroom to *A. meleagris* where a complete genome is available), a high number of putative proteins are returned. It is interesting to note that the similarities of PDH to pyranose 2-oxidases from white-rot fungi are considerably lower than those to (flavoprotein domains of) cellobiose dehydrogenases even from less closely related ascomycetous species. All known *p2o* genes are phylogenetic descendants of a *Firmicutes* gene encoding a 2-keto-gluconate dehydrogenase (*BaciluGnDH* in Fig. 2, NCBI accession AAY60294), suggesting gene transfer from *Firmicutes* into the kingdom Fungi. The closest phylogenetic neighbour of pyranose dehydrogenases is aryl alcohol oxidases of the genus *Pleurotus* (order Agaricales). As all currently known PDH genes are found in Agaricales, a common ancestor for aryl alcohol oxidases and pyranose dehydrogenases can be supposed.

We also isolated the complete *A. meleagris gpd* gene as well as the corresponding cDNA, to use it as a reference gene for transcript analysis. *A. bisporus* contains two genes encoding GPD, *gpd1* and *gpd2*, arranged in tandem and highly conserved in sequence and gene structure. The former was described as either being a pseudogene or only transcribed in certain developmental stages (Harmsen et al. 1992). In *A. meleagris*, sequences upstream of the *gpd* gene (which is therefore homologous to *gpd2*) show similarity to *A. bisporus gpd1*, but do not constitute an intact gene. It appears that GPD1 possibly retained some (limited) function in *A. bisporus* but not in *A. meleagris*, thus allowing the encoding gene to accumulate mutations, deletions and rearrangements.

We analyzed growth rate, PDH production and transcript levels of the three PDH-encoding genes on various carbon sources. Only glucose, cellobiose and fructose (but not xylose, which is an excellent substrate of PDH) supported substantial growth under the conditions used. Growth of *Agaricus* spp. on various cellulose preparations was reported, but not tested here, as it did not result in formation of significant levels of PDH acitivity (Morrison et al. 1999). Growth curves on glucose, cellobiose and fructose are remarkably similar. On cellobiose the growth phase is extended for several days, during which glucose—derived

from extracellular hydrolysis of cellobiose—is consumed very slowly. This could indicate a growth limitation by another factor than the availability of the carbon source. *pdh1* mRNA formation increases sharply (but briefly) upon depletion of glucose, but not on fructose, suggesting a regulation at least partially influenced by glucose derepression. On cellobiose, carbohydrate levels do not fall to zero until 6 days later compared to glucose and fructose, and only towards the end of the cultivation a (very limited) response in mRNA synthesis is observed. This, however, happens at a time when biomass is already decreasing as a result of starvation and autolysis. These data are in agreement with the expression patterns of *p2o* in *P. chrysosporium*, where high transcript levels in carbon-limited cultures were detected compared to very low levels in the logarithmic growth phase or on medium containing cellulose as the only carbon source (de Koker et al. 2004). In contrast, the cellobiose-dehydrogenase-encoding *cdh* gene in *T. versicolor* is transcribed on cellulose-containing medium but can be repressed by addition of glucose or other monosaccharides, suggesting carbon catabolite repression not limited to glucose (Stapleton et al. 2004). High levels of P2O activity were also observed in *T. versicolor* on cellulose media and on glucose before carbon exhaustion (Oliveira et al. 1992).

Additionally we observed an increase in *pdh1* (but not *pdh2* and *pdh3*) transcript levels during the first days following transfer to fresh medium containing the various carbon sources. This increase was more pronounced than the one following carbon depletion, and again was highest on cellobiose and lowest on fructose. At this stage of cultivation the mycelial fragments are submerged in the liquid medium and do not show measurable growth (although no nutritional limitations apply) until they rise to the surface and start to form a dense mycelial mat. This sharp increase in *pdh1* mRNA does not result in an equally strong increase in PDH activity—probably due to the inhibition of growth and metabolic processes in this stage. On cellobiose, higher mRNA levels are sustained into the growth phase, resulting in higher PDH-activity levels on cellobiose throughout the cultivation. These observations may indicate a regulatory mechanism connected to oxygen supply, which is limited as long as the fungus is submerged. An up-regulation of *pdh* transcription in reaction to limited oxygen supply appears logical, as PDH does not utilize molecular oxygen as electron acceptor, and can therefore stand in for oxygen-dependant oxidases. P2O would be a candidate for such an enzyme, but is not found in fungi producing PDH (Volc et al. 2001). Regulation of gene transcription in response to oxygen limitation is mainly investigated in fermentative yeasts (Fredlund et al. 2004), no data are available for filamentous fungi and mushrooms. The significance of our observation as well as of the special behavior on cellobiose therefore requires further studies.

Multigenicity is common among fungal enzyme systems (Kilaru et al. 2006) and often serves the purpose of providing certain enzymatic activities for varying environmental conditions or different developmental stages. The three investigated *pdh* genes are regulated differentially: *pdh2* and *pdh3* are transcribed on a very low level and rather constantly throughout the cultivations, the largest observed (fourfold) increase does not indicate major derepression or induction events. The much higher transcription of *pdh1* as well as the upregulation during the lag phase and upon glucose depletion, however, suggests a differentiation of the regulatory systems, although the significance is not yet clear. No such observations have been made for P2O or CDH to date, and PDH is thus far the only representative of this enzyme family where multiple genes are reported. Given the vastly different transcription levels of *pdh1*, *pdh2* and *pdh3*, the respective proteins could, however, easily remain unnoticed, as purification experiments invariably turn out the dominant PDH1 protein. This may well be the case for other fungi harboring P2O and/or CDH as well. Speculations that the oxidative half-reaction (reduction of electron acceptors) actually represents the in vivo function in lignocellulose degradation were recently discussed for PDH (Kujawa et al. 2007) but are valid for P2O and CDH as well, although the degradative abilities towards wood are considerably lower for *Agaricus* spp. and related mushrooms.

The results discussed in this study, together with the catalytic properties of PDH, indicate a role in the degradation of high molecular weight organic substrates such as lignocellulose and its derivatives. Varying regulatory patterns suggest that highly similar enzymes may fulfil different functions in different fungi, whereas the occurrence of large numbers of highly similar, evolutionarily and possibly catalytically related enzymes suggests a certain redundancy within such enzyme systems, making classical studies of knock-out mutants laborious and difficult. The increase in available genome data on other fungi as well as advances in heterologous expression, however, will facilitate studies on both biochemistry and evolution of these enzymes.

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