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Expression of the urease gene of *Agaricus bisporus*: a tool for studying fruit body formation and post-harvest development

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Abstract Fruit body initials of *Agaricus bisporus* contain high levels of urea, which decrease in the following developmental stages until stage 4 (harvest) when urea levels increase again. At storage, the high urea content may affect the quality of the mushroom, i.e. by the formation of ammonia from urea through the action of urease (EC 3.5.1.5). Despite the abundance of urea in the edible mushroom *A. bisporus*, little is known about its physiological role. The urease gene of *A. bisporus* and its promoter region were identified and cloned. The coding part of the genomic DNA was interrupted by nine introns as confirmed by cDNA analysis. The first full homobasidiomycete urease protein sequence obtained comprised 838 amino acids (molecular mass 90,694 Da, *pI* 5.8). An alignment with fungal, plant and bacterial ureases revealed a high conservation. The expression of the urease gene, measured by Northern analyses, was studied both during normal development of fruit bodies and during post-harvest senescence. Expression in normal development was significantly up-regulated in developmental stages 5 and 6. During post-harvest senescence, the expression of urease was mainly observed in the stipe tissue; expression decreased on the first day and remained at a basal level through the remaining sampling period.

Introduction

Higher fungi of the family Agaricaceae, including *Agaricus bisporus*, accumulate substantial amounts of urea in their fruit bodies (Hammond 1979; Wagemaker et al. 2005). Fruit body initials of *A. bisporus* were found to contain high levels of urea, which decrease in the following developmental stages until stage 4 where urea levels increase again, with the exception of stipe tissue where no significant changes were observed. Mushrooms are harvested at stage 4. High amounts of urea have a negative effect on the taste of the mushroom. Furthermore, the ammonia formed when urea is hydrolysed stimulates post-harvest bacterial attack and affects crop quality. Despite the abundance of urea in the edible mushroom *A. bisporus*, little is known about its physiological role. Urea is chemically inert and soluble to high concentration. Therefore, urea may serve as an osmotically favourable form of fungal nitrogen reserve, especially when mannitol, an osmotically favourable form of carbon and energy reserve stored during active growth, is used as a respiratory substrate in post-harvest senescence (Wood 1985).

Urea formation is a result of purine catabolism and ornithine cycle activity, and urea release accompanies the biodegradation of nitrogenous compounds like arginine, agmatine, allantoin and allantoic acid (Vogels and van der Drift 1976). Two pathways are encountered for urea degradation: hydrolysis via urease (EC 3.5.1.5) or via an ATP-hydrolyzing urease (EC 3.5.1.45). In higher plants, some fungi and many prokaryotes, urea is hydrolyzed by urease. Urease is a nickel-containing enzyme that catalyses urea hydrolysis into ammonia and carbamate. Carbamate is unstable and hydrolyzes spontaneously to carbonic acid and a second ammonia molecule. In other fungi and in green algae, urea is first carboxylated to yield allophanate by an ATP-hydrolyzing urease. The allophanate in turn is hydrolyzed into two molecules each of ammonia and carbon dioxide (Lefley and Syrett 1973; Roon and Levenberg 1972). So far, no data on the presence of a degradation pathway through allophanate in *A. bisporus* exist. No influence on the ammonia formation was found after addition

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of ATP to the urease reaction (unpublished data). Urea was shown to be a good nitrogen source for mycelial growth of *A. bisporus* (Baars et al. 1994), and urease activity was demonstrated in fruit bodies (de Windt et al. 2002). Besides urease, evidence for the presence of a urease inhibitor in cell-free extracts has been presented (Reinbothe et al. 1967; de Windt et al. 2002). Urease allows micro-organisms to use externally and internally generated urea as a source of nitrogen (Mobley and Hausinger 1989).

Expression of a catalytically active urease in bacteria is usually directed by at least seven genes, which in general are arranged in operons (Collins and Dorazio 1993; Koper et al. 2004). Activation of the urease apoenzyme by incorporation of nickel ions into the multimeric molecule is best understood in the bacterium *Klebsiella aerogenes* (Hausinger et al. 2001). It is accomplished by the coordinated action of four accessory proteins, encoded by *ureD*, *ureE*, *ureF* and *ureG*. In bacteria, three genes (*ureA*, *ureB* and *ureC*) encode the structural subunits (γ , β and α , respectively) of urease, which associate in an $(\alpha\beta\gamma)_n$ stoichiometry to form the urease apoenzyme (Jabri et al. 1995). In plants and fungi, the structural urease protein is encoded by one gene that comprises homologues of the three bacterial genes. In this article, we report the isolation and characterization of the first homobasidiomycete urease gene. The *A. bisporus* urease gene, promoter region and cDNA were isolated and characterized. Moreover, the correlation between the expression of urease at mRNA level and the urea content of fruit bodies at different developmental stages and in post-harvest senescence is discussed.

Materials and methods

Agaricus bisporus strains and culture conditions

Agaricus bisporus Horst U1 and its homokaryotic parents, strains H39 and H97, were obtained from the collection of the Mushroom Experimental Station, Horst, the Netherlands.

A. bisporus fruit bodies were grown and sampled as described before (Wagemaker et al. 2005). Mushrooms for post-harvest studies were sampled and stored as described by Eastwood et al. (2001).

Escherichia coli strains, recombinants DNA techniques and enzymes

Escherichia coli LE392 (Promega) and *E. coli* XL1 blue (Stratagene) were used for phage amplification/ λ DNA isolation and plasmid transformation/propagation, respectively. Standard DNA manipulations were carried out as described in Sambrook et al. (1989). Restriction enzymes and other enzymes used for DNA manipulations were purchased from MBI Fermentas. Plasmid pUC19 (Yanisch-Perron et al. 1985) was used as cloning vector for genomic DNA (gDNA) fragments. The pGEM-T vector system (Promega) was used for cloning polymerase chain reaction (PCR) products. Synthetic oligonucleotide primers were

purchased from Biolegio BV and Isogen Bioscience BV. FlexiPrep Kit (Pharmacia Biotech) was used for plasmid isolation.

Cloning of the *Agaricus bisporus* gene encoding urease

For amplification of *A. bisporus* DNA encoding a part of the urease gene, synthetic degenerate inosine-containing deoxyoligonucleotide primers, designed from aligned regions conserved in *Filobasidiella neoformans* (O13465), *Coccidioides immitis* (O14420) and *Schizosaccharomyces pombe* (O00084), were used in a PCR performed on a Tgradient Thermocycler (Biometra) with strain Horst U1 gDNA as a template. PCR primer 1 (5'-GGI CAY GCI CCI GAY ATY ATY-3') encodes urease amino acids 547–554 of the *S. pombe* sequence, and primer 2 (5'-CCA IAR IAC IAR RTC NGC-3') encodes the antisense codons of amino acids 701–706. A nested PCR was performed on 0.1 μ l of the first PCR product mix: primer 3 (5'-TIG AYA TGY TIA TGR TNT GYC A-3') encodes urease amino acids 583–590 and primer 4 (5'-ATO GCO GGR TTD ATO GTR TAU TT-3') encodes the antisense codons of amino acids 677–684. The initial denaturation step of 10 min at 95°C was used to add the Taq polymerase enzyme as a hot-start and was followed by 30 cycles of 1 min at 95°C, 1 min at 52°C, 2 min 72°C and a final extension step of 5 min at 72°C. The amplified 301-bp product was cloned into pGEM-T (Promega), and four clones were sequenced. Sequences obtained were 100% identical. The fragment was labelled using [α -³²P]dCTP by random oligonucleotide priming (HexaLabel DNA Labeling Kit, MBI Fermentas) and used as a probe for the screening of a λ EMBL3 genomic library of strain Horst H39 using conditions described previously (Schaap et al. 1996). Plaques giving a positive hybridization signal were purified, and the urease gene was isolated from the λ DNA by ligation of *SalI* fragments into pUC19 and subsequent sequence analysis of the subclones.

Isolation, sequencing and characterization of *ureA* cDNA from a mixed primordial small fruit body λ -ZAP-cDNA library (de Groot et al. 1996) were performed as described before (Wagemaker et al. 2005).

Amino acid sequence analysis

Relevant protein sequences were extracted from the Swiss-Prot or the TrEMBL protein databases. The sequences were aligned with the align utility of the Vector NTI Suite software (Informax) and the alignment was edited with GeneDoc.

Northern analysis

Total RNA was isolated from *A. bisporus* fruit bodies and analyzed as described before (Wagemaker et al. 2005). The PCR product of forward primer 5'-GGC GAC GAA GTC

AAA TTT GG-3' (ure1f) and reverse primer 5'-TCG TTG AGA GTG TCG GTA TG-3' (ure2r) was labelled with [³²P]CTP and used as a probe (Wagemaker et al. 2005). Northern blots were also probed with an *A. bisporus* 28S ribosomal DNA fragment as loading control and with probes specific for the genes *gdhA*, *gdhB* and *glnA* encoding enzymes of the primary nitrogen metabolism (Kersten et al. 1997, 1999; Schaap et al. 1996).

To study post-harvest development, mushrooms were sampled and stored, and total RNA was isolated as described by Eastwood et al. (2001). The PCR product of the ure1f and ure2r primers was used as probe through the incorporation of [α -³²P]dCTP-labelled molecules via the Redi-Prime random-labelling system (Amersham International).

Isolation of total genomic DNA, Southern blot analysis

Total DNA from strains H39 and H97 was isolated, digested with various restriction enzymes, separated on 0.8% agarose gels and transferred onto Nytran Super Charge membranes by vacuum blotting. DNA blots were washed at 65°C to a final stringency of 0.1× SSC, 0.1% SDS.

The gDNA and cDNA sequences of the urease gene from *A. bisporus* have been deposited in the GenBank/EMBL databases under the accession numbers AJ748112 and AJ748113, respectively.

Results

The *Agaricus bisporus* urease gene

The work on urease was initiated by isolating a gene fragment from *A. bisporus* strain H39 gDNA by a nested PCR approach using degenerate primers. The degenerated primers were designed from conserved regions in the primary structure of urease sequences from the heterobasidiomycete *F. neoformans* (Cox et al. 2000) and the ascomycetes *C. immitis* (Yu et al. 1997) and *S. pombe* (Tange and Niwa 1997). A nested PCR was performed on the first amplification product, and this resulted in a specific product of 296 bp. Blast search revealed identities of 74 and 75% with

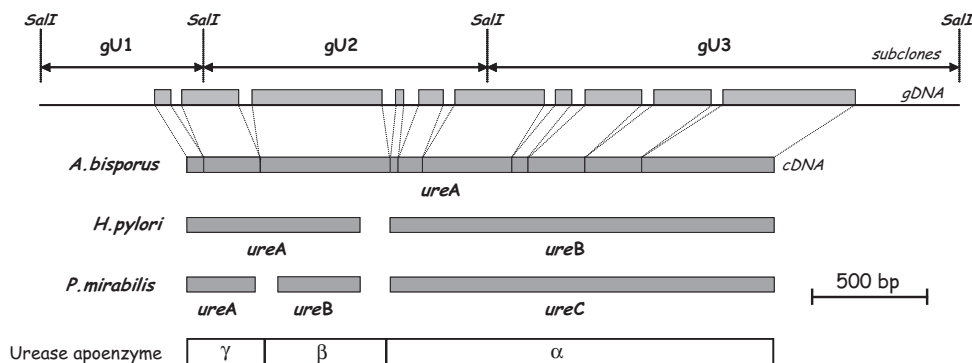
F. neoformans and *Glycine max* urease genes, respectively. The fragment was used as a probe for screening the strain H39 λ EMBL3 genomic library. Urease-positive λ DNA was isolated and digested with *SalI* restriction endonuclease and ligated in pUC19. The isolated promoter region and gene sequence comprised about 3.5 kb distributed over three *SalI* fragments: clones gU1, gU2 and gU3 (Fig. 1); the full sequence was obtained by the primer walking method. The specific primers were also used in combination with pBlue-script vector primers for isolation of the urease cDNA by a PCR on a cDNA library and subsequent sequencing.

The 3,761 bp gDNA sequence contains an open reading frame (ORF) of 2,514 bp, interrupted by nine introns of 47–71 bp in length. The introns were confirmed by cDNA analysis and all appear to have normal fungal splicing sites (Unkles 1992), except for intron 6, which has *gtatt* instead of *gtmng* at its start. The 3'-end of the cDNA was located 50 bp downstream of the TAA stop codon just 2 bp upstream of a putative polyadenylation site (Humphrey and Proudfoot 1988). Seven putative GATA factor binding motifs were found in the 471-bp urease promoter sequence. Furthermore, a putative TATA box, a GC box (box) and a CAAT box involved in transcription initiation could be identified.

Southern analysis of gDNA of strains U1 and its homokaryotic constituents H39 and H97, using a PCR product of primers ure1f and ure2r as a probe, indicated that a single copy of this gene is present in the genome of *A. bisporus* (Fig. 2). The Southern blot of the H97 strain shows two hybridization products for the *ClaI* digest instead of one as seen for H39. This is the result of a sequence polymorphism between the two parental strains of Horst U1 located in the wobble base and thus not resulting in a different amino acid (data not shown).

The codon usage of the *A. bisporus* gene showed a limited bias towards a thymine in the third position of a codon (33.6%), similar to the nicotinamide adenine dinucleotide phosphate (NADP)-dependent glutamate dehydrogenase (GDH) (Schaap et al. 1996) and arginase (Wagemaker et al. 2005), whereas in 55.8% of the codons, a pyrimidine was used in the third position of a codon. This is significantly lower compared to the NADP-dependent GDH gene, the arginase gene and the codon usage reported for other *A. bisporus* genes by Schaap et al. (1999).

Fig. 1 Genomic map of the gene *ureA* encoding the urease of *A. bisporus* strain H39 based on subcloning of the urease positive lambda phage. gU1, gU2 and gU3 are the subclones of the urease gDNA obtained after digestion with *SalI*. The gaps in the gDNA sequence indicate the position of introns. For comparison, two bacterial type ureases are included together with the organisation of the apoenzyme



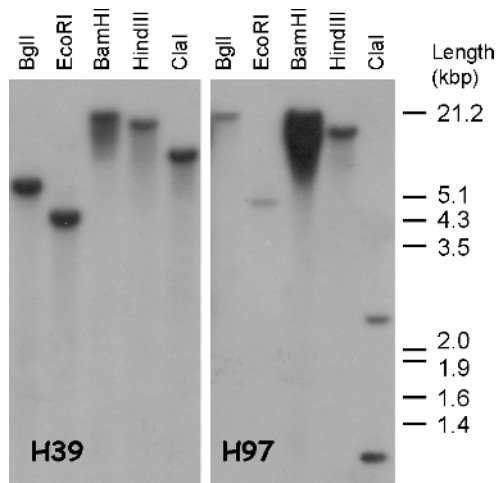


Fig. 2 Southern analysis of gDNA of *A. bisporus* strains H39 and H97 probed with a urease gene PCR product obtained with primers ure1f and ure2r after digestion with different restriction enzymes

Amino acid sequence analysis

The deduced protein sequence of 838 amino acids has a calculated molecular mass of 90,694 Da with a theoretical *pI* of 5.8. The encoded protein resembles the γ , β and α subunits of the bacterial ureases fused into one protein (Fig. 1) as described before for other eukaryotic ureases. The *A. bisporus* urease gene provides the first full homobasidiomycete urease protein sequence. An alignment with fungal, plant and bacterial ureases revealed a high similarity among all representative species included. The alignment was used to create a phylogenetic tree (Fig. 3). For comparison, the homobasidiomycete urease DNA sequences of *Coprinus cinereus* and *Phanerochaete chrysosporium* were extracted from the genome data provided by the Joint Genome Institute (JGI; <http://genome.jgi-psf.org/>) and the Broad Institute (<http://www.broad.mit.edu/>). The translated sequences, included in the alignment and tree, showed high identity (75–77%) with the *A. bisporus* urease amino acid

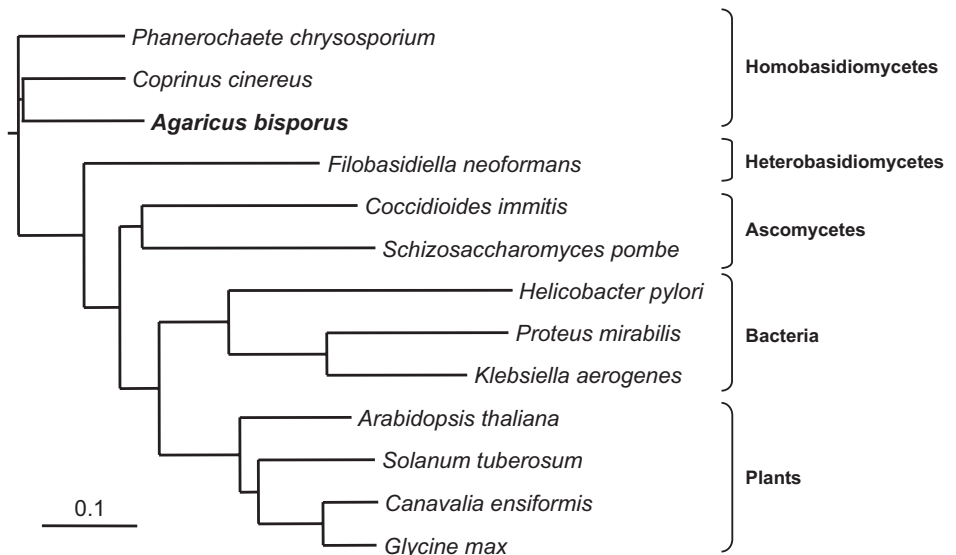
sequence. High conservation of intron positioning was observed among the homobasidiomycetes. Eight introns were conserved in all three species; one intron was conserved only with *C. cinereus* and one intron was conserved between *C. cinereus* and *P. chrysosporium*. *C. cinereus* possessed one unique intron. The fungal ureases from *F. neoformans* (O13465), *C. immitis* (O14420) and *S. pombe* (O00084) revealed identities of 63, 60 and 60%, respectively, with the *A. bisporus* urease. For the plant urease from *Canavalia ensiformis* (P07374) and the bacterial ureases, 60% and 52–55%, respectively, identity was found.

Regulation of urease expression

Transcriptional regulation of the urease gene was investigated by determining the mRNA levels in various parts of the *A. bisporus* fruit body during sporophore formation. Tissue samples from developing fruit bodies were used for RNA isolation. Figure 4a shows that the urease gene in normal growing sporophores has a low basal level of expression, which was up-regulated in stages 5, 6 and 7 for stipe and stage 6 for cap tissue. The expression in stipe tissue was much higher when compared to cap tissue and was still increasing in stage 7. The expression in cap tissue decreased in stage 7.

If RNA was isolated from the complete fruit body, high expression was observed at harvest ($t=0$). Expression of the urease gene in post-harvest senescence revealed a down-regulation in the first 24 h post-harvest (Fig. 4b). The slight increase at 15–18 h is not significant when the signals are corrected for the 28S rRNA loading control. For comparison, the blot was also hybridized with probes specific for the NADP-dependent GDHs (*gdhA*, *gdhB*) and the glutamine synthetase (*glnA*) of *A. bisporus* (Kersten et al. 1997, 1999; Schaap et al. 1996). These enzymes of the primary nitrogen metabolism showed the same expression pattern (Fig. 4b). Studied in more detail, the post-harvest expression of urease appeared to be mainly situated in stipe tissue

Fig. 3 Phylogenetic tree of representative urease family members. Organisms and their accession numbers are *A. bisporus* (AJ748113), *F. neoformans* (O13465), *C. immitis* (O14420), *S. pombe* (O00084), *C. ensiformis* (M65260), *Solanum tuberosum* (Q93WI8), *G. max* (Q7XAC5), *Arabidopsis thaliana* (A96699), *Helicobacter pylori* (X17079), *P. mirabilis* (M31834) and *K. aerogenes* (M36068). Urease protein sequences from *P. chrysosporium* and *C. cinereus* were extracted from the genome data provided by the JGI and the Broad Institute, respectively. Bar = 10 substitutions per 10 amino acid residues



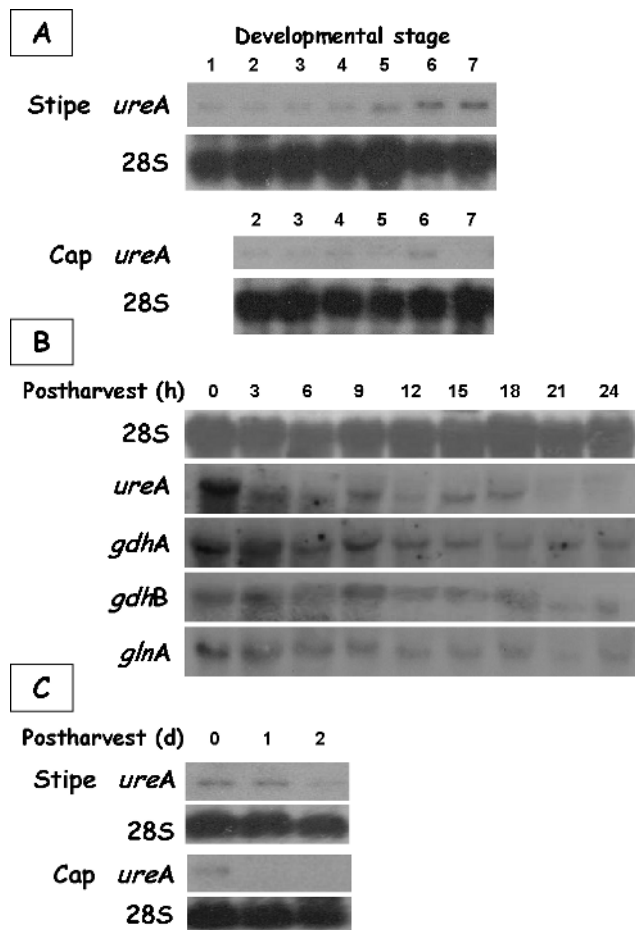


Fig. 4 Northern analyses of genes in *A. bisporus* Horst U1. *a* Total RNA isolated from stipe and cap tissues cut from fruit bodies in different stages of development (indicated by the numbers). *b* Total RNA isolated from whole fruit bodies (stage 4) after post-harvest storage for 0 to 24h. *c* Total RNA isolated from stipe and cap tissues cut from fruit bodies (stage 4) after post-harvest storage for 0 to 2 (days). Ten micrograms of total RNA was size-fractionated and hybridized with a urease (*ureA*), a NADP-dependent GDH (*gdhA*), a NAD-dependent GDH (*gdhB*), a glutamine synthetase (*glnA*) gene fragment or a 28S rRNA gene fragment

(Fig. 4c). Expression decreased on the first day, but after another day a basal expression level was still visible, whereas in cap tissue at harvest, a lower expression was observed decreasing below detection level within one day. For gill tissue, no post-harvest mRNA expression was detected.

Discussion

During fruit body development of *A. bisporus*, urea levels first decrease, but at the stage of harvest (stage 4), urea levels increase again. Upon storage, the high urea content may affect the quality of the mushroom, i.e. by the formation of ammonia from urea through the action of urease. Since little is known about the physiological role of urea, this paper focusses on the characterization and expression of the urease gene and the correlation with the urea content

of fruit bodies at different developmental stages and in post-harvest senescence.

Ureases found in bacteria, fungi and higher plants (Mobley et al. 1995) are highly conserved. In most bacterial species, the urease structural enzyme is encoded by three genes: a large catalytic subunit (α), encoded by the *ureC* gene, and two smaller subunits (β and γ), which are the products of *ureB* and *ureA*, respectively. The three subunits can associate as a heteromultimer with $(\alpha\beta\gamma)_n$ stoichiometry to form the urease apoenzyme. Whereas the ureases of most bacterial species have been found to possess three distinct subunits, *Helicobacter* species produce ureases with only two distinct subunits (Clayton et al. 1990). Here the subunits β and γ are encoded by one gene *ureaA*, which is a fusion of the *ureA* and *ureB* genes from the bacterial system. In higher plants and fungi, the enzyme is made up of only one unit comprising the three subunits encoded by a single gene (Takashima et al. 1988). The urease of the fungus *A. bisporus* presented in this work also is the product of one single gene and shows high similarity to the eukaryotic ureases and less to the bacterial enzymes. A complete description of the secondary structure elements and an indication of which residues are orientated to the interior of the $(\alpha\beta\gamma)$ trimer of *K. aerogenes* urease were presented by Jabri et al. (1995) and Jabri and Karplus (1996). Their alignment of 14 bacterial ureases along with jack bean urease showed 189 residues to be identical. Six residues were involved in nickel ligation and five (His²¹⁹, His³²⁰, Gly²⁷⁷, Ala³⁶³ and Met³⁶⁴ of the *K. aerogenes* urease α subunit) were implicated either by biochemical means or by the structure in substrate binding or catalysis. Also in our alignment of the fungal species *P. chrysosporium*, *C. cinereus*, *F. neoformans*, *C. immitis* and *S. pombe* along with plant ureases and three bacterial ureases, those residues are conserved. A total of 178 residues were proposed to be important in hydrogen bonding and hydrophobic interactions within the $\alpha\beta\gamma$ unit or in interactions to build the $(\alpha\beta\gamma)_3$ trimer; 27 of these residues were not identical in our alignment.

The clear correspondence between the amino acid sequence of the fungal and plant ureases that consist of single units and the two or three subunit bacterial ureases indicates the occurrence of gene fusion or disruption events during evolution (Fig. 3; Hausinger 1993). Sequences that are very similar to the intron splice acceptor consensus sequences were found in the DNA sequence between the *ureA* and *ureB* ORFs of *Proteus mirabilis*. On the basis of this, it was suggested that the origin of *ureA* and *ureB* may have resulted from an intron site that is not functional in prokaryotes (Mobley et al. 1995). Such a scenario is consistent with a eukaryote-to-prokaryote urease gene exchange during evolution. However, the absence of conservation of one or more introns among the eukaryotic ureases while they share higher identity than with *P. mirabilis* or other bacterial urease sequences and the presence of a set of accessory genes in one operon in bacteria make a gene fusion more likely.

The accumulation of urea in the fruit body of *A. bisporus* was proposed to facilitate the translocation of water and

metabolites in fruit bodies (Donker and van As 1999), which is required for the production of spores. Urea could be used as an osmoticum to drive cell expansion and, therefore, cap expansion. Expansion is more important to cap opening than growth. Data suggest that sugars drive expansion during natural development (Wood 1985), but in post-harvest most sugars and soluble proteins are metabolised (Hammond and Nichols 1979). Therefore, urea could be involved in continued cap expansion during post-harvest development. Secondly, in post-harvest development, urea was proposed to be the end product of catabolic pathways (Reinbothe et al. 1967; Foret 1990). The observed difference in urea accumulation between the stipe and the rest of the fruit body (Wagemaker et al. 2005) illustrates the dynamics of the translocation process of water and metabolites. The expression of the urease gene is in accordance with this hypothesis. The expression of urease during normal growth was higher in stipe tissue compared to other tissues and prolonged to stage 7, where also no accumulation of urea was observed (Wagemaker et al. 2005). The expression in cap tissue stopped at stage 6 when the urea content increased. For cap tissue, the higher accumulation of urea in post-harvest senescence when compared to normal development stages 5, 6 and 7 did not correlate with a higher expression of the arginase gene (Wagemaker et al. 2005) but can be explained by an earlier stop in urease expression.

In *C. cinereus*, high urease activity in mycelium and stipe tissue was demonstrated but no detectable activity in cap tissue was found, which correlated with the accumulation of urea in cap tissues (Ewaze et al. 1978). A causal relationship between urea accumulation and water influx into developing cap tissues was proposed, identical to the situation in *A. bisporus*. The up-regulation of ammonium-scavenging enzymes, NADP-linked GDH (NADP-GDH) and glutamine synthetase in cap tissue was linked with the start of karyogamy (Moore et al. 1987a). It was suggested that the role of the glutamine synthetase and NADP-GDH ammonium scavenging is to maintain an ammonium-free environment in a cell during sporulation, since such processes are inhibited by the presence of ammonium (Moore et al. 1987b; Pinon 1977). An expression study of *gdhA*, *gdhB* and *glnA* and activity measurements of the corresponding enzymes during sporophore formation and post-harvest development will unravel the scavenging role of the primary ammonia-assimilating enzymes in *A. bisporus*. A preliminary Northern analysis (Fig. 4b) provides evidence that expression of these genes is correlated with urease expression in this fungus.

The urea decrease in the premature stages of sporophore formation indicates that urea might serve as a nitrogen reserve used for synthesis of protein, cell wall and other nitrogenous compounds needed in the development of the fruit body. The expression of the urease gene presented in this paper is in line with the observed urea levels (Wagemaker et al. 2005) and activities measured (Reinbothe et al. 1967). The decrease in urea content of the growing sporophore was thus not only the result of the dilution effect as the fruit body develops and grows to a larger volume. The urease activity

demonstrated indicates that ammonium is produced and can be reassimilated by the primary ammonium assimilation pathways addressed by Baars et al. (1996) and Kersten et al. (1997, 1998, 1999). For mature fruit bodies of *A. bisporus*, however, no reassimilation of urea-ammonia was determined as was shown for puffballs of *Lycoperdon pyriforme* (Reinbothe et al. 1967). An increase in alanine and proline concentration after urea administration suggested assimilation of the urea nitrogen. Because urease activity is evident and high levels of urea are present, a different cell localization of the urease enzyme and the high level of urea might be speculated. Sequence data on the arginase (Wagemaker et al. 2005) and urease genes indicate that production of urea as well as the degradation are cytosolic activities, since the encoded protein sequences do not show translocation signals. Identification and localization of the previously reported inhibitor (Reinbothe et al. 1967; de Windt et al. 2002) and its role in controlling the urease enzyme activity will give a better understanding of the various functions that urea accumulation fulfils in *A. bisporus* fruit body formation. The expression of the urease enzyme as a reporter-fusion protein would enable one to study the enzyme and its localization in more detail.

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