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Argininosuccinate synthetase and argininosuccinate lyase: two ornithine cycle enzymes from *Agaricus bisporus*

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ABSTRACT

Accumulation of high quantities of urea in fruiting bodies is a known feature of larger basidiomycetes. Argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) are two ornithine cycle enzymes catalysing the last two steps in the arginine biosynthetic pathway. Arginine is the main precursor for urea formation. In this work the nucleotide sequences of the genes and corresponding cDNAs encoding argininosuccinate synthetase (*ass*) and argininosuccinate lyase (*asl*) from *Agaricus bisporus* were determined. Eight and six introns were present in the *ass* and *asl* gene, respectively. The location of four introns in the *asl* gene were conserved among vertebrate *asl* genes. Deduced amino acid sequences, representing the first homobasidiomycete ASS and ASL protein sequences, were analysed and compared with their counterparts in other organisms. The *ass* ORF encoded for a protein of 425 amino acids with a calculated molecular mass of 47 266 Da. An alignment with ASS proteins from other organisms revealed high similarity with fungal and mammalian ASS proteins, 61–63 % and 51–55 % identity, respectively. The *asl* open reading frame (ORF) encoded a protein of 464 amino acids with an calculated mass of 52 337 Da and similar to ASS shared the highest similarity with fungal ASL proteins, 59–60 % identity.
Northern analyses of *ass* and *asl* during fruiting body formation and post-harvest development revealed that expression was significantly up-regulated from developmental stage 3 on for all the tissues studied. The expression reached a maximum at the later stages of fruiting body growth, stages 6 and 7. Both *ass* and *asl* genes were up-regulated within 3 h after harvest showing that the induction mechanism is very sensitive to the harvest event and emphasizes the importance of the arginine biosynthetic pathway/ornithine cycle in post-harvest physiology.

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Introduction

Argininosuccinate synthetase (ASS; EC 6.3.4.5) and argininosuccinate lyase (ASL; EC 4.3.2.1) represent the last two steps in the arginine biosynthetic pathway catalysing the conversion of citrulline and aspartate into argininosuccinate and then to arginine and fumarate successively. Arginine is a major precursor for protein synthesis and is involved in production of creatine,

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polyamines and proline. Being part of the ornithine cycle, ASS and ASL are involved in the detoxification of ammonia through the production of urea in ureotelic species. In mammals, deficiency in ASL and ASS leads to argininosuccinic aciduria (Allan et al. 1958; Levy et al. 1980) and classical citrullinemia (Ratner & Petrack 1951; McMurray et al. 1962), respectively.

In a reversible two-step reaction argininosuccinate is generated by action of ASS. First an activated intermediate, AMP–citrulline is formed by addition of $MgATP^{2-}$, then ligation with aspartate forms argininosuccinate in the second part of the reaction (Ratner 1973). The subsequent reversible conversion of argininosuccinate to fumarate and arginine is catalysed by ASL. The fumarate synthesized links the urea cycle and the citric acid cycle.

ASL belongs to a superfamily of homotetrameric enzymes, including fumarases, aspartases, adenylosuccinate lyases, 3-carboxy-cis,cis-muconate lactonizing enzyme and δ-crystallin, the major structural component of avian and reptilian eye lenses. These enzymes share similar three-dimensional structures (Abu-Abed et al. 1997; Shi et al. 1997; Simpson et al. 1994; Weaver et al. 1995) and catalyse homologous reactions with the formation of fumarate as one of the products. Although the sequence similarity is low, three highly conserved regions, denoted C1, C2 and C3, have been identified. The signatures are spatially distant in the monomer but cluster together in the tetramer to form a multisubunit active site in which the three regions are joined by a different monomer (Sampaleanu et al. 2001). This accounts for the observed phenomenon of intragenic complementation (McInnes et al. 1984), which occurs when a multimeric protein is formed from subunits produced by two different mutated alleles of a gene. An additional interest for the two enzymes comes from their participation in the citrulline–nitric oxide (NO) cycle (Hattori et al. 1994; Nussler et al. 1994) in which ASS was found to have a rate-limiting role in high output NO synthesis (Xie & Gross 1997).

For ASS a number of highly conserved regions have been identified. The presence of a glycine-rich motif common to a subset of ATP pyrophosphatases defines ASS as the fourth structurally defined member of the N-type ATP pyrophosphatases (Lemke & Howell 2001). Three other conserved regions were involved in aspartate and citrulline binding.

Few data are available on the arginine biosynthetic pathway in *Agaricus bisporus*. Heavy isotope tracer experiments with precursors and intermediate metabolites of the ornithine cycle (urea cycle) have proven the existence of the pathway in *A. bisporus* and revealed the presence of orithine carbamoyltransferase, ASS, and arginase by activity measurements (Reinbothe et al. 1969; Wasternack & Reinbothe 1967). β-Methylaspartate and δ-N-acetylornithine were found to block ASS activity and caused ^{14}C-citrulline to accumulate. The ornithine cycle has been demonstrated to be the main route for urea synthesis (Reinbothe et al. 1967), although the contribution of the purine degradative pathway could not be excluded (Hammond 1979; de Windt et al. 2002). It is known for members of the family *Agaricaceae* that they accumulate substantial amounts of urea in their fruiting body. Hammond (1979) reported for *A. bisporus* a urea content of 0.1 % (g/g of dry matter) at harvest, which increased tenfold during storage at room temperature for 4 d. Beside its function as waste nitrogen stored as urea after ammonium overflow through arginine, it was proposed that urea may act as an osmotic solute and may drive fruiting body expansion (Cochrane 1958; Donker & van As 1999; Wagemaker et al. 2005, 2006). The product quality of harvested mushrooms is lost during storage because the mushroom maintains its function to produce and release spores. The processes, described as post-harvest senescence, are under genetic control (King & O'Donoghue 1995; Eastwood et al. 2001; Kingsnorth et al. 2001). The continued development occurs despite the mushroom being nutritionally isolated. It was shown that stored carbohydrate levels fall to almost zero within days of post-harvest storage (Hammond & Nichols 1975). Therefore, the mushroom must obtain its nutrition from other sources and it was hypothesized that nitrogen metabolizing pathways would be crucial (Kingsnorth et al. 2001). Protein is the main source of nitrogen available for redistribution after harvest. However, it is likely that protein is also used as a carbon source post-harvest as *A. bisporus* has been previously shown to use protein as a sole source of carbon (Kalisz et al. 1986). Increased protease levels were found in *A. bisporus* fruiting bodies releasing large amounts of amino acids (Burton 1988; Burton et al. 1997). Understanding the regulation of genes involved in the ornithine cycle after harvest would help breeding programmes to improve mushroom quality and would elucidate how fungal morphogenesis is maintained under low carbon conditions.

In this study, we describe the isolation and characterization of the *A. bisporus* ASS and ASL genes and cDNA clones. Both represent the first fully reported homobasidiomycete sequence in their family. Expression studies have also been performed during fruiting body development and post-harvest senescence.

Experimental procedures

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* fruiting bodies were grown and sampled as described before (Wagemaker et al. 2005). Post-harvest development was studied as described by Eastwood et al. (2001). Harvested mushrooms, stage 2 (classification of Hammond & Nichols 1975), were either frozen immediately under liquid nitrogen, termed time 0, or stored in a controlled environment, 18 °C and 95–98 % relative humidity. Samples from the stored mushrooms were taken at: (1) 3 h intervals for the first 24 h post-harvest; (2) 24 h intervals for 5 d post-harvest; and (3) 24 h intervals for 2 d post-harvest were mushrooms were dissected into stipe, cap and gill tissue. Frozen samples were stored at −80 °C.

Escherichia coli strains, recombinant DNA techniques and enzymes

Escherichia coli XL1-blue MRF' and SOLR (Stratagene, Amsterdam) were used for plating the cDNA library and *in vivo* excision of cloned cDNA inserts. *E. coli* LE392 (Promega, Leiden) 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 (St Leon-Rot). Plasmid pUC19 (Yanisch-Perron *et al.* 1985) was used as cloning vector for genomic DNA fragments. The pGEM-T vector system (Promega) was used for cloning PCR products. Synthetic oligonucleotide primers were purchased from Biolegio (Malden) and Isogen Bioscience (Maarsden).

Cloning of the Agaricus bisporus ass *gene*

For amplification of *Agaricus bisporus* DNA encoding a part of the *ass* gene, synthetic degenerate inosine-containing deoxyoligonucleotide primers were designed from conserved regions in the corresponding *Escherichia coli* (*arg*G: P22767), human (*ass*: P33280) and *Saccharomyces cerevisiae* (*arg*1: P22768) genes. The primers were used in a PCR with strain Horst® U1 genomic DNA as a template. Primer 1 (5′-ACI GGI AAR GGN AAY GAY C-3′) encodes amino acids 118–124 of the *S. cerevisiae* ASS amino acid sequence and primer 2 (5′-TTY TCR TCI GTI SWC CAN GG-3′) encodes the antisense codons of amino acids 177–183. 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 58 °C and 2 min 72 °C. The amplified 252 bp product was cloned into pGEM-T (Promega) and sequenced.

Isolation of the *ass* gene from a λEMBL3 genomic library of strain Horst H39 and a mixed primordial small fruiting body λ-ZAP-cDNA library (de Groot *et al.* 1996), subsequent sequencing and characterization were basically performed as described previously (Wagemaker *et al.* 2005). The isolation of the *ass* 5′-end was established with a *ass*-specific forward primer (*ass*Mf: 5′-ACT TAC TCT TAA GAA TGC CTG-3′) and reverse primer (*ass*2r: 5′-GCA ACA GTT TGT TGG ACA GG-3′) based on the *ass*-gDNA nucleotide sequence.

Cloning of the Agaricus bisporus asl *gene*

The *asl* gene was isolated from an *Agaricus bisporus* C54-carb8 genomic Lawrist cosmid library (Yague *et al.* 1996) combined with direct PCR cloning of a fragment, encoding the 5′ region of the *asl* gene. A first screening of the cosmid library was performed on 87 pooled batches comprising DNA from 96 *Escherichia coli* clones containing Lawrist cosmids by means of a PCR with specific ASL primers designed to the *asl* cDNA sequence (Eastwood *et al.* 2001). PCR products of the correct size were sequenced and identified by BlastX analysis. A second screening by colony hybridization was performed with the 96 *E. coli* clones from the positive batch. The [^{32}P]-CTP labelled *asl* cDNA was used as a probe. The *asl* gene sequence was obtained from the isolated cosmid, containing the *asl* gene, by the primer walking method. The PCR fragment encoding the 5′ of the *asl* gene was obtained with specific primers from *A. bisporus* C54-carb8 total DNA. The forward primer (*asl*1f: 5′-CCC ACA ACG TGC CAG CTA T-3′), ranging base pair −17 to +2 of the *asl* gene sequence, was designed to the *asl* gene promoter sequence isolated, as described before (Wagemaker *et al.* 2006). The [^{32}P]-CTP labelled *asl* cDNA was used as probe. The reverse primer was complementary to the *asl* cDNA base pair 268–289 (*asl*2r: 5′-GAC GTT CAT TGG CAG TGT GTA T-3′).

Phylogenetic analysis

Protein sequences listed were extracted from the SwissProt and TrEMBL protein databases. The sequences were aligned with the align utility of Vector NTI Suite software (Informax, Frederick) and a tree was constructed with Treeview.

Northern analysis

Total RNA was isolated from *Agaricus bisporus* fruit bodies and analysed as described previously (Wagemaker *et al.* 2005). Post-harvest development was studied as described by Eastwood *et al.* (2001). The PCR product obtained using forward primer 5′-CAT TCA TGG CAG ATG TAG GC-3′ (*ass*1f) and reverse primer *ass*2r, was labelled with [^{32}P]-CTP as described (Wagemaker *et al.* 2005) and used as *ass* probe. The [^{32}P]-CTP labelled *asl cDNA* (Eastwood *et al.* 2001) was used as *asl* probe. Northern blots were also probed with an *A. bisporus* 28S ribosomal DNA fragment (Schaap *et al.* 1996) as a loading control.

Isolation of total genomic DNA, Southern blot analysis

Total DNA from strains H39 and H97 was isolated (de Graaff *et al.* 1988), digested with various restriction enzymes, separated on 0.8 % agarose gels and transferred onto Nytran® Super Charge membranes by vacuum blotting and hybridized with ^{32}P-labelled *ass* and *asl* probes as described previously. Southern blots were washed at 65 °C to a final stringency of 0.1× SSC (15 mM NaCl; 15 mM sodium citrate), 0.1 % sodium dodecyl sulphate (SDS).

Results

The ass *gene*

For the isolation of the *ass* gene, degenerate primers were designed based on conserved amino acid residues identified by alignment of the ASS amino acid sequences from *Escherichia coli*, human, and *Saccharomyces cerevisiae*. PCR with these primers and Horst U1 genomic DNA as a template resulted in a 252 bp fragment, which was cloned and sequenced. BlastX analysis of the sequence identified the fragment as a part of the *ass* gene sharing 72 % and 71 % identity with the *S. cerevisiae* ASS protein over two regions interrupted by a putative intron in the *Agaricus bisporus* gene fragment. After labelling the fragment was used as a probe to screen a strain H39 genomic library and positive λ-clones were isolated and used as template in an inverted-PCR with specific primers based on the *ass* gene fragment for isolation of the complete gene. The same specific primers were used for isolation of the *ass* cDNA from a Lambda Zap cDNA library by PCR techniques.

The 2240 bp isolated *ass* gene region comprised 190 bp promoter region, 1275 bp ORF interrupted by eight introns and 394 bp 3′-UTR. All introns, ranging in size from 51 to 73 bp, were confirmed by cDNA analysis and appeared to have

normal fungal splicing sites (Unkles 1992). The nucleotide sequences reported here have been deposited in the Genbank/EMBL databases under the accession numbers AJ864863 and AJ864864 for the cDNA and the gDNA sequence, respectively.

The 190 bp *ass* promoter region (Fig 1A) contained one TATA box at position −115 bp upstream from the translation start. A putative CAAT-box followed by a CT-rich stretch described for the CCAAT regulatory factor, CTF/NF-I, binding domain (Unkles 1992; Nussinov 1992) was found at position −79. Another CAAT-box was found at position −142, but was not followed by a CT-rich stretch. Three putative GATA-factor binding sites at positions −149, −95 and −88 appeared to be present. Furthermore, a CTCA repeat was found repeating itself seven times starting at position −56 bp from the translation start. No known consensus sequence for eukaryotic polyadenylation recognition motif was found in the region surrounding the polyadenylation site.

ASS amino acid sequence analysis

The codon usage of the *A. bisporus ass gene* shows a bias towards a thymine in the third position of a codon (37 %) and 66 % of the codons use a pyrimidine in the third position. The *ass* gene encodes a 425 amino acid protein with a calculated molecular mass of 47,266 Da and an isoelectric point of 5.2. The deduced amino acid sequence was aligned with corresponding ASS sequences from a selection of distantly related organisms (Fig 2). The alignment clearly showed the highly conserved regions documented in Prosite as ASS consensus sequences. Both a glycine rich motif A ([A/S]-[F/Y]-S-G-G-[L/V]-D-T-[S/T]) common to a subset of N-type ATP pyrophosphatases (Tesmer *et al.* 1996) and motif B (G-x-T-x-K-G-N-D-x(2)-R-F) the site involved in aspartate binding (Lemke & Howell 2001) were observed. Two regions, S-x-D-x-N-x(6)-E and E-[N/D]-R-x(4)-K-x(4)-Y-E (motifs C and D, respectively) found to be responsible for citrulline binding by forming β-hairpins enabling formation of a four-stranded β-sheet were also conserved in our alignment.

The asl *gene*

The 5′ truncated *asl* cDNA was isolated from a λ-ZAP cDNA library by differential screening (Eastwood *et al.* 2001). The translated sequence showed 80 % similarity to the *Saccharomyces pombe* (P40369) ASL protein sequence. Full length *asl* gene sequence was determined from analysis of *Agaricus bisporus* genomic DNA isolated from an *A. bisporus* C54-carb8 genomic Lawrist cosmid library. Exon/intron boundaries were identified by the determination of conserved *gtnng* and *yag* intron boundaries (Unkles 1992). However, the cosmid sequence showed an unfortunate artificial *Hind*III ligation at position 195 in the *asl* gene sequence published in the EMBL database. The missing N-terminal coding region was isolated from total *A. bisporus* DNA by PCR using specific primers derived from an *asl*-positive λ-clone from *A. bisporus* H39.

The *asl* gene sequence revealed an ORF of 1392 bp interrupted by six introns of which five were confirmed by cDNA sequence analysis. The sixth intron, base pairs 66-114 of the *asl* gene, was deduced from the *asl* gene sequence as described above for the *ass* gene. All introns appear to have normal splicing sites and range in size from 45 to 62 bp. The additional nucleotide sequences reported here have been deposited in the GenBank/EMBL databases as updates of the *asl* genomic and cDNA sequences (AJ277474 and AJ271691). The 358 bp of the promoter region (Fig 1B) was deposited in the GenBank/EMBL databases under the accession number AJ864965. One putative TATA-box, position −115, and two putative CAAT-boxes were found at positions −189 and −47 from the translation start. Furthermore, one putative GATA-factor binding site and many GC-rich regions were found. The GC-box at position −288 matches the SYGGRG-motif described as CREA consensus binding site (Cubero & Scazzocchio 1994). In the 3′-UTR region of the *asl* gene a polyadenylation signal (Humphrey & Proudfoot 1988) was found in the last Lys and stop codon of the *asl* sequence.

ASL amino acid sequence analysis

The codon usage of the *Agaricus bisporus asl gene* shows a limited bias towards a thymine in the third position of a codon (29 %). Fifty-eight percent of the codons use a pyrimidine in the third position corresponding to what was found for the arginase and urease gene (Wagemaker *et al.* 2005, 2006). The *asl gene* encodes a 464 aa protein with a calculated molecular weight of 52,337 Da and an isoelectric point of 5.96. The deduced amino acid sequence was aligned with representative

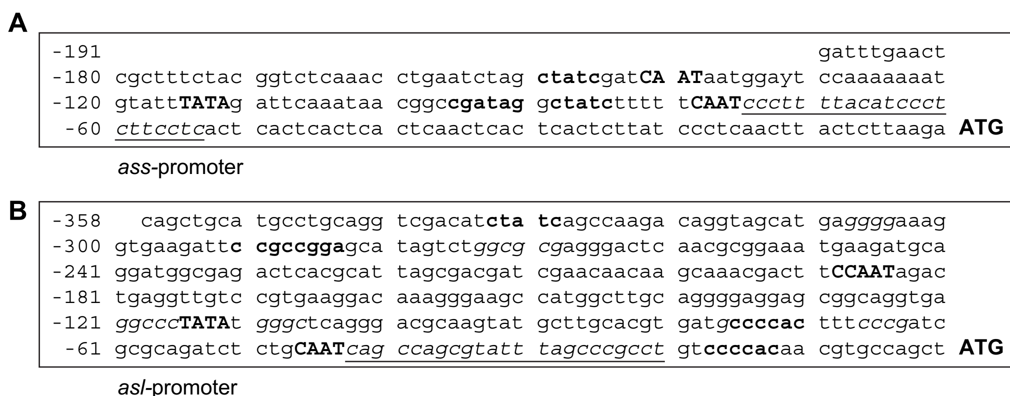

Fig 1 – Promoter region of (A) the *ass* gene and (B) the *asl* gene from *Agaricus bisporus* H39. TATA boxes and CAAT motifs are in bold capitals; CT-rich regions are underlined. Putative GATA factor binding sites are marked in bold. GC-rich regions are in italics.

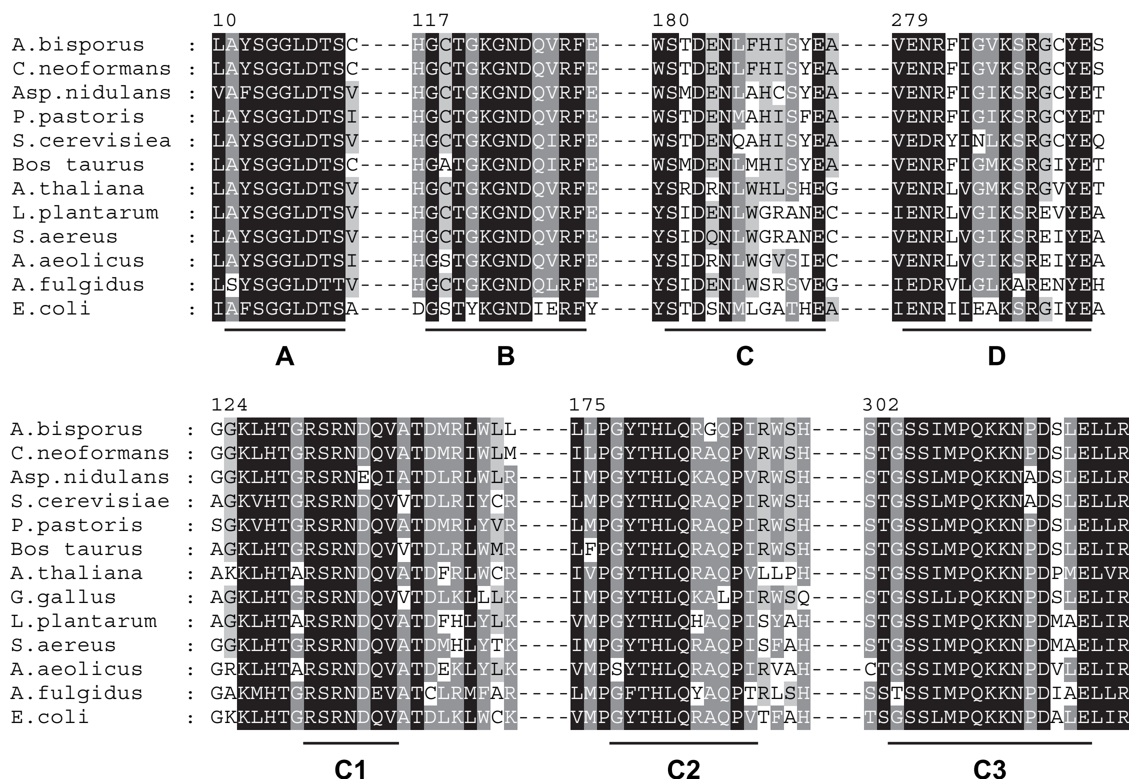

Fig 2 – Alignment of amino acid residues from selected highly conserved regions (A–D and C1–C3) of ASS proteins (top) and ASL proteins (bottom) from distantly related species (for accession numbers see Fig 3). Numbers indicate amino acid position in the *Agaricus bisporus* proteins.

ASL sequences from a selection of distantly related organisms (Fig 2). The alignment clearly shows the conservation of the three regions, denoted C1, C2 and C3, conserved throughout the ASL superfamily (Sampaleanu *et al.* 2001).

Phylogenetic analysis

The alignments of both the deduced sequences (ASS and ASL) were used to calculate a NJ tree (Fig 3). The ASS alignment showed striking similarity between *A. bisporus* ASS and the ASS amino acid sequences derived from the genomes of the heterobasidiomycete *Cryptococcus neoformans* and the ustilaginomycete *Ustilago maydis* showing 76 and 72 % identity, respectively. The three fungi fit into the fungal group in the phylogenetic tree. The fungal ASS cluster together with the eukaryotic ASS enzymes from mammalian species, showing identities ranging from 51 to 55 %. The fungal ASS are less related to the plant (43 % identity), *Archaea* (37–42 % identity) and bacteria (21–48 % identity) ASS.

The *A. bisporus* ASL clusters with the fungal ASL enzymes sharing the highest identities again with *Cryptococcus neoformans* (70 %) and the *Ustilago maydis* (67 %). Vertebrate ASL cluster close to delta-crystallin proteins from bird eye lenses showing identities ranging from 53–60 % with the *A. bisporus* ASL. The diverse group of bacteria (actinobacteria, proteobacteria, firmicutes and cyanobacteria) have identities ranging from 43–48 %. The *Archaea* form their own group with identities ranging from 37–42 %. Comparison of the eukaryotic *asl* gene structure, with respect to intron position in the coding region, revealed the conservation of all introns among the vertebrate species including the delta-crystallin proteins. The *A. bisporus asl* gene shared four introns with the vertebrate *asl* gene sequences, which were all located in the 3′ region of the gene (Fig 4). The *Chlamydomonas reinhardtii asl* gene was stated to have four intron positions at the same place as the vertebrate *asl* genes (Auchincloss *et al.* 1999), but only three could be identified in our alignment.

Southern analysis

Southern analysis of genomic DNA of the dikaryon *Agaricus bisporus* Horst U1 and its homokaryotic parents, strains H39 and H97, using a PCR product of primers *ass*1f and *ass*2r as an *ass*-probe and the *asl* cDNA as the *asl*-probe, indicated that a single copy of both of these genes is present in the genome of *A. bisporus* (data not shown).

Regulation of the ass *and* asl *genes*

To study the expression of the *ass* and *asl* genes, during fruiting body formation, stipe, gills and cap tissue were sampled from fruiting bodies at different developmental stages and mRNA was extracted for Northern analysis. Both genes were expressed in all tissues studied but expression in gill and cap tissue is slightly higher than in stipe tissue. Fig 5 shows that the expression of the *asl* has a constant level during

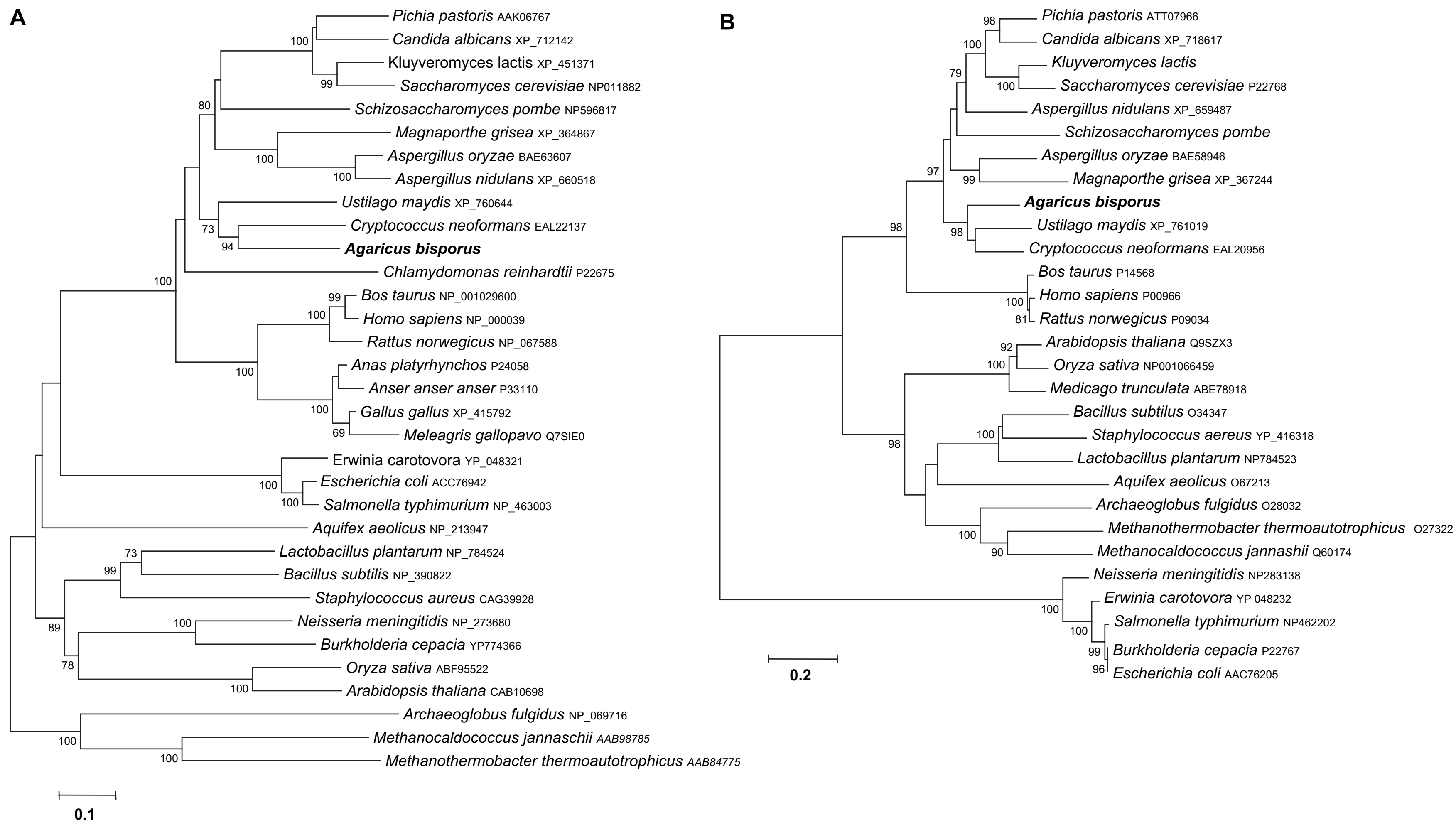

Fig 3 – NJ phylogenetic trees of selected ASS (A) and ASL (B) proteins. Accession numbers are given after the names of the organisms. BS values higher than 75 % (500 replicates) are given at the nodes. Bar = ten substitutions per 100 amino acid residues.

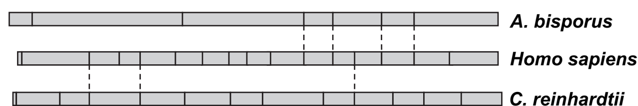

Fig 4 – cDNA map illustrating the location of introns in the ASL encoding genes of *Agaricus bisporus*, *Homo sapiens* and *Chlamydomonas reinhardtii*.

developmental stages 1 to 4 and a strong increase is seen from stages 5 to 7. Although the signal is lower for the *ass* gene the same trend is observed with the highest expression at stages 5 to 7. Gill tissue of stage 7 showed a lower expression for *ass* compared with the other tissues.

Expression of the *ass*- and *asl* genes during post-harvest senescence was studied using fruiting bodies harvested at stage 2. Expression of both genes was up-regulated between days 0 and 1 (Fig 6A). A finer time course shows this increase to occur within the first 3 h (Fig 6B) indicating a rapid detection and response mechanism by *A. bisporus* of the changes associated with the harvest event. Unlike natural growth, there is considerable increase in all three tissues (including the stipe) after 1 d of post-harvest storage (Fig 6C).

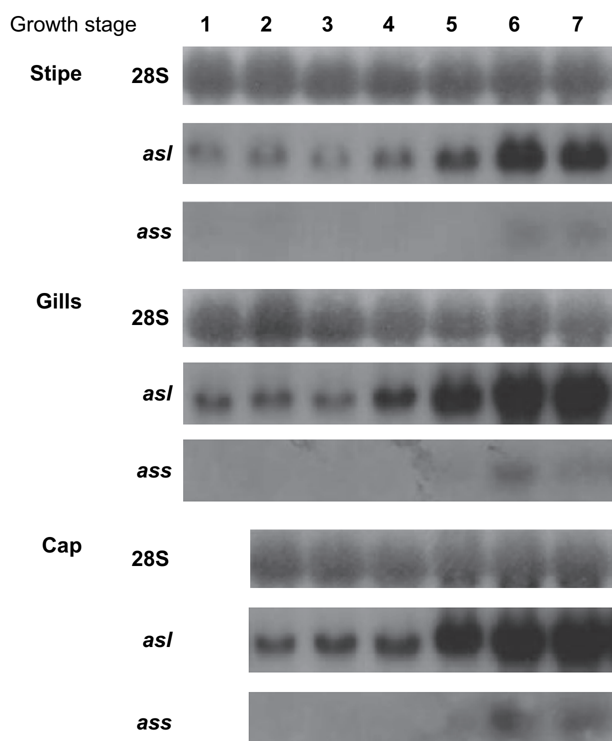

Fig 5 – Northern analysis of total RNA isolated from pooled *Agaricus bisporus* Horst U1 stipe and cap tissue cut from fruiting bodies at different stage of development. Before RNA isolation, cut samples were pooled according to the following criteria: stage 1, $d_{cap}/d_{stipe} \leq 1$ and no lamella visible; stage 2, β-angle = 75–97°, $d_{cap}/d_{stipe} = 0.95–1.5$; stage 3, β-angle = 40–75°, $d_{cap}/d_{stipe} = 1.4–1.9$; stage 4, β-angle = 25–40°, $d_{cap}/d_{stipe} = 1.85–2.5$; stage 5, 6 and 7 normal growth as defined before (Hammond & Nichols 1975; Wagemaker *et al.* 2005). All stages except stage 1 were dissected into stipe, gill and cap tissue. Ten micrograms of total RNA was size-fractionated and hybridized with an *ass*, *asl* or 28S fragment.

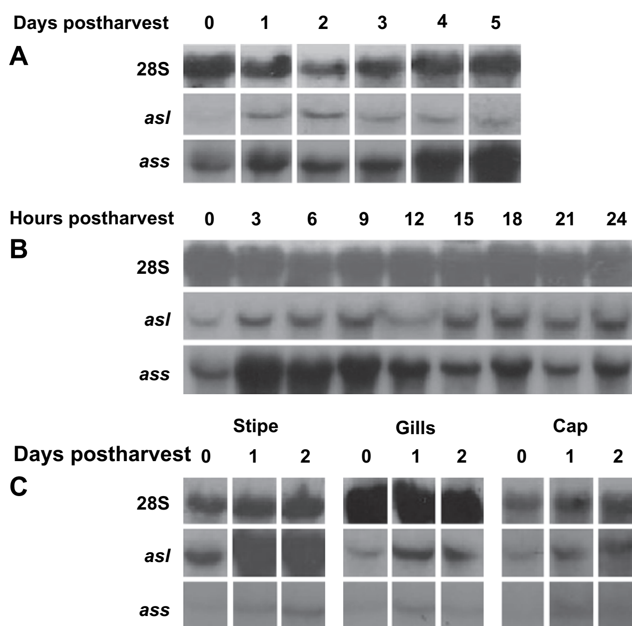

Fig 6 – Northern analysis of the *ass* and *asl* gene in *Agaricus bisporus* Horst U1 total RNA isolated from: (A) whole fruiting bodies, lanes indicate fruiting bodies sampled after 0–5 d post-harvest storage. (B) Whole fruiting bodies, lanes indicate fruiting bodies sampled after 0–24 h post-harvest storage. (C) Stipe, cap and gill tissue of *A. bisporus* Horst U1 fruiting bodies stored for 0, 1 and 2 d after harvest.

Discussion

Studies on the ASS and ASL amino acid sequences from diverse species revealed both enzymes to be highly conserved. The sequences presented in this work are the first homobasidiomycete *ass* and *asl* gene sequences reported. Our alignment clearly shows the conservation of the three regions, denoted C1, C2 and C3, conserved throughout the ASL superfamily (Sampaleanu *et al.* 2001). Forty-nine residues were conserved over all organisms aligned. Comparative analysis of our alignment with an ASL/fumarase superfamily alignment with adenylosuccinate lysase, fumarase, aspartase and 3-carboxy-cis,cis-muconate lactonizing enzyme (Sampaleanu *et al.* 2002) revealed seven conserved residues in the C3 region, indicating that this region is the best conserved region throughout the ASL/fumarase superfamily. A conformational change in the loop of residues 284–294 and a rigid body movement of the C-terminal small domain result in sequestration of the substrate from the solvent (Fujii *et al.* 2003; Sampaleanu *et al.* 2001). A mutation of the residue corresponding to S285 in the duck δ2-crystallin resulted in the complete loss of ASL activity (Chakraborty *et al.* 1999) and a catalytic role was suggested in serving as a proton donor for releasing the α-amino group (Sampaleanu *et al.* 2001). Three residues, D33, H91 and

D117, of which the first two are involved in anchoring the amino end of the substrate arginine moiety and the latter resembles a very high conserved residue of the C3 region, were not conserved in δ1-crystallin, which were rendered to have no ASL activity (Barbosa *et al.* 1991; Chiou *et al.* 1992; Kondoh *et al.* 1991). Residues D33 and H91 were not conserved when the ASL/fumarase superfamily was taken into account. However, residue D117 was conserved throughout the superfamily, which makes this amino acid a reasonable candidate for being involved in loss of activity of the δ1-crystallins.

Our alignment revealed high similarity of the *Agaricus bisporus* ASL with the *Chlamydomonas reinhardtii* ASL, sharing 59 % identity, which is high considered the latter organism belongs to the kingdom *Viridiplantae*. Alignment with a deduced ASL amino acid sequence from the annotated genome of *Arabidopsis thaliana* (http://pedant.gsf.de/index.html) revealed 41 % and 40 % identity to the *A. bisporus* and the *C. reinhardtii* ASL, respectively. The previously reported conservation of four intron positions in *C. reinhardtii* ASL with the human *asl* gene (Auchincloss *et al.* 1999) could not be confirmed in our alignment in which only three intron positions were shared with the vertebrate *asl* genes. The high identity together with the strange pattern of intron conservation (Fig 3) suggests a gene exchange between the pre-ancestral species in an early stage of evolution of the eukaryotes.

Regulation of the ass *and* asl *genes*

The expression of the *ass* and *asl* genes during fruiting body development generally follows the same pattern as the urea cycle gene arginase (Wagemaker *et al.* 2005). Levels of the arginase and urease gene expression correlated well with the urea content of the fruiting body, i.e. the accumulation of urea, especially in cap tissue during late stages of development, as a result of increased arginase gene expression. The absence of significant urea accumulation in stipe tissue may result from a higher urease gene expression and a lower arginase gene expression compared with other tissues. It was suggested that arginase transcript abundance would precede urea accumulation (Wagemaker *et al.* 2005). The increased expression of the *ass* and *asl* genes in stage 7 compared with the arginase gene could result from the need for arginine for various metabolic processes, such as protein synthesis in spore production. The continued high expression of the *asl* gene might be evoked by a general amino acid control mechanism as a result of amino acid depletion.

A good comparison can be made to *Coprinopsis cinerea* (syn. *Coprinus cinereus*) where a causal relationship between urea accumulation and amplification of the arginine biosynthesis and arginase activity during cap development was suggested (Ewaze *et al.* 1978). Four enzymes, NADP-glutamate dehydrogenase, glutamine synthetase, ornithine acetyltransferase and ornithine carbamoyltransferase, all involved in ornithine synthesis, were considerably derepressed in developing caps while remaining low (or declining) in the stipes supporting those caps.

The higher expression of the *ass* and *asl* genes during post-harvest senescence correlates very well with the expression of the arginase gene seen in fruiting bodies of *A. bisporus* both in normal development and post-harvest senescence and with the accumulation of urea in post-harvest senescence (Wagemaker *et al.* 2005). The greater and quicker accumulation of urea during post-harvest storage compared with normal growth can be explained by the more pronounced up-regulation of the genes involved in the harvested mushrooms. The *ass* and *asl* gene were up-regulated immediately following the harvest event, which emphasizes the importance of the arginine synthetic pathway in post-harvest senescence. The regulatory system involved is probably the cross-pathway control of filamentous fungi (Hinnebusch 1992; Sachs 1996) also known as the general amino acid control system in yeast (Mesenguy & Dubois 2000). This system is the only known system to control the expression of the *asl* gene in these fungi. The *ass* gene is regulated by the same system and in addition by the UASarg and URSarg promoter elements (Dzikowska *et al.* 2003; Empel *et al.* 2001). Interestingly, *ass* transcripts appear to increase to a greater level in the post-harvest mushroom, while in the undetached mushroom the expression of *asl* is greater, which may suggest a different mechanism controlling the expression of the two genes. The reason for this observation is not known, although different regulatory elements are described between *asl* and *ass* (Dzikowska *et al.* 2003; Empel *et al.* 2001). The harvested mushroom is affected by both damage stress and nutritional limitation. However, it is not clear how these factors could influence the expression of adjacent enzymes on the same pathway and it is not known whether ASL enzyme activity is rate limiting to ASS, or *vice versa*.

After harvest, the fruiting body is deprived from the import of nutrients and is restricted to its own resources. As the fruiting body develops further to the fully opened state, a sharp decrease in protein content is observed (Hammond 1979). Protease activity is present during post-harvest senescence and the continuous breakdown of protein results in the restoration of amino acid availability (Burton 1988; Burton *et al.* 1994; Foret 1990) and results in a reduction in the general amino acid control activation system.

Analysis of the promoter region of the *ass* and *asl* genes revealed the presence of several putative GATA sites for binding a general nitrogen regulatory protein (Merika & Orkin 1993; Ravagnini *et al.* 1997) and a SYGGRG sequence motif known to bind the CREA protein (Cubero & Scazzocchio 1994). The latter protein is involved in general carbon catabolite repression in *Aspergillus nidulans*. General carbon and nitrogen regulation of arginine synthesis has yet to be described in fungi and further study would be required to determine whether these promoter elements regulate the expression of the *ass* and *asl* genes in *A. bisporus*. However, the stop in transported carbon metabolites from the mycelium at harvest will likely trigger the immediate use of carbon supplies stored in the fruiting body and use of protein as an alternative source of carbon, requiring increased activity of the urea cycle enzymes.

The expression of the genes encoding arginine-synthesizing enzymes is compatible with phenomena seen in fruiting body development, as well as post-harvest senescence, and is compliant with regulatory mechanisms found in other fungi. In several cases (including yeast *asl*) where genes are acting under the control of amino acids, the correlation between the level of mRNA and enzyme activity is proportional (Flint & Wilkening 1986; Messenguy & Dubois 1983). This indicates that mRNA levels are a good measure for enzyme

activity in arginine synthesis. Coordinated regulation of the arginine biosynthetic enzymes of *C. cinerea* could also be demonstrated in vegetative mycelium subjected to particular synthetic growth media and to some extent metabolic changes that occur naturally in the sporophore cap could be reproduced. This together with the presumption that good correlation between level of expression and enzyme activity also exists in *A. bisporus* makes the control system suitable for experimental study in pure culture. The isolation and characterization of the genes encoding the ornithine cycle enzymes provides a good tool for such a study.

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