

Genome sequence of the button mushroom *Agaricus bisporus* reveals mechanisms governing adaptation to a humic-rich ecological niche

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Agaricus bisporus is the model fungus for the adaptation, persistence, and growth in the humic-rich leaf-litter environment. Aside from its ecological role, *A. bisporus* has been an important component of the human diet for over 200 y and worldwide cultivation of the “button mushroom” forms a multibillion dollar industry. We present two *A. bisporus* genomes, their gene repertoires and transcript profiles on compost and during mushroom formation. The genomes encode a full repertoire of polysaccharide-degrading enzymes similar to that of wood-decayers. Comparative transcriptomics of mycelium grown on defined medium, casing-soil, and compost revealed genes encoding enzymes involved in xylan, cellulose, pectin, and protein degradation are more highly expressed in compost. The striking expansion of heme-thiolate peroxidases and β -etherases is distinctive from Agaricomycotina wood-decayers and suggests a broad attack on decaying lignin and related metabolites found in humic acid-rich environment. Similarly, up-regulation of these genes together with a lignolytic manganese peroxidase, multiple copper radical oxidases, and cytochrome P450s is consistent with challenges posed by complex humic-rich substrates. The gene repertoire and expression of hydrolytic enzymes in *A. bisporus* is substantially different from the taxonomically related ectomycorrhizal symbiont *Laccaria bicolor*. A common promoter motif was also identified in genes very highly expressed in humic-rich substrates. These observations reveal genetic and enzymatic mechanisms governing adaptation to the humic-rich ecological niche formed during plant degradation, further defining the critical role such fungi contribute to soil structure and carbon sequestration in terrestrial ecosystems. Genome sequence will expedite mushroom breeding for improved agronomic characteristics.

carbohydrate-active enzymes | humic substances | litter decay | wood decay fungi

Lignocellulose is the most abundant organic compound in the terrestrial environment, consisting of three main components: cellulose, hemicellulose, and lignin (1). These polymers are decayed primarily by wood and litter decomposers from the Agaricomycotina

(mushroom-forming fungi). Comparative analyses of the “white-rot” fungi *Phanerochaete chrysosporium* and *Schizophyllum commune* (2, 3), the “brown-rots” *Postia placenta* and *Serpula lacrymans* (4, 5), and the coprophilous *Coprinopsis cinerea* (6) has provided considerable insight into the evolution of the wood-decomposition machinery in fungi. Much less is known about fungal decomposition of partially degraded plant material, particularly leaf litter, and about adaptation to humic-rich environments. Improving knowledge of processes governing this adaptation is critical to improving carbon management and predictive modeling of terrestrial carbon cycling.

The biomass of nonwoody litter in temperate woodlands can be four- to five times greater than that of woody litter (7). Concentrations of lignin and (hemi)cellulose in leaf litters of hardwood species range from 33–43% and 23–29%, respectively (8). After a succession of microbial colonisations, the litter is substantially modified by the removal of readily available carbon, nitrogen, and

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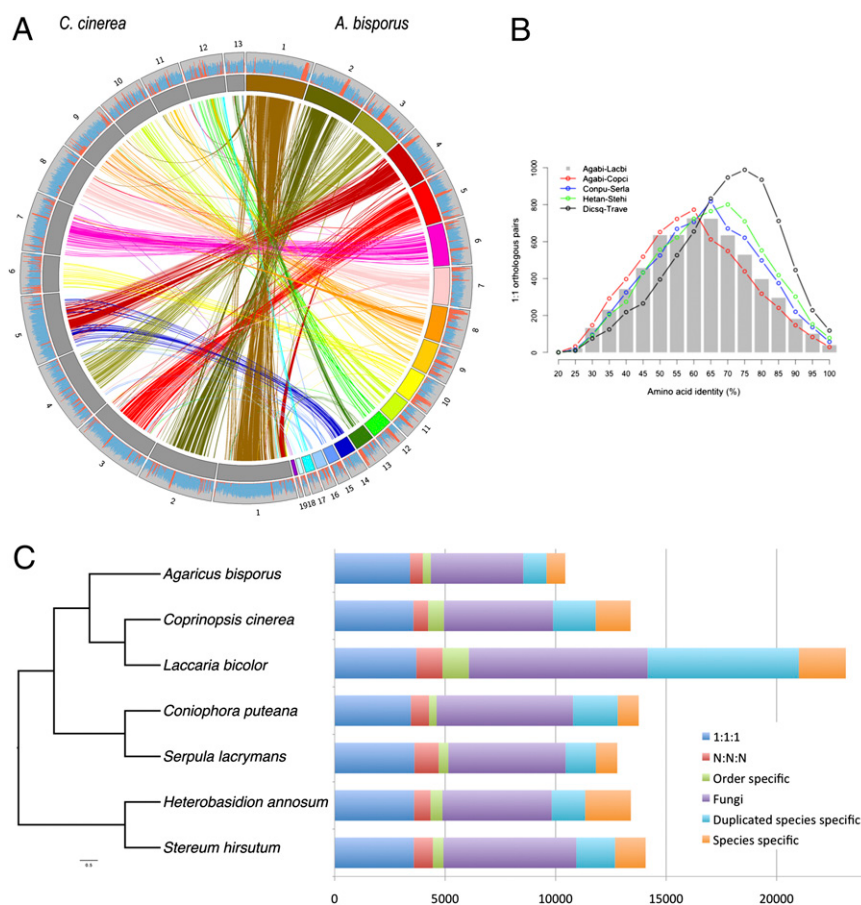


Fig. 2. Agaricomycete gene orthology and evolution. (A) Macro-synteny between *A. bisporus* var *bisporus* H97 scaffolds and *C. cinerea* Okayama7 chromosomes. *A. bisporus* scaffolds are depicted by the colored blocks and *C. cinerea* chromosomes are represented by gray blocks. Only regions larger than 5,000 bp are connected with links of colors matching those used for coloring *A. bisporus* scaffolds. The distributions of protein-coding regions and repetitive elements are shown in the outer circle, with protein-coding gene density in blue and repetitive sequences in red, with a window size of 0.1 Mb. Comparison between the two genomes sequences was performed with VISTA (<http://genome.lbl.gov/vista>). (B) The distribution of pair-wise amino acid identity. Histogram shows the distribution of sequence identity of 1:1 orthologs between *A. bisporus* and *L. bicolor* (diverged ~85 million y ago) (12). To highlight the similar level of molecular divergence, 1:1 orthologs between *A. bisporus* and *C. cinerea*, 1:1 orthologs of two Boletales (*S. lacrymans*/*Coniophora puteana*), two Russulales (*Heterobasidion annosum*/*Stereum hirsutum*), and two Polyporales (*Trametes versicolor*/*Dichomitus squalens*) were plotted in red, blue, green, and black, respectively. (C) Orthology assignment of seven Agaricomycete genomes. Bars are subdivided to represent different types of orthology relationships. 1:1:1 indicates universal single-copy genes; N:N:N indicates other universal genes, but absence in a single genome within the different orders is tolerated. "Order specific" indicates Polyporales-, Russulales-, Boletales-, or Agaricales-specific genes; "Fungi" indicates Fungi-specific orthologs; "Duplicated species-specific" indicates species-specific duplicated genes; "Species specific" indicates species-specific genes (orphans).

Agaricales Orthology and Evolution. Within the Agaricomycetes, the protein-coding genes of Agaricales have similar levels of divergence as in the Boletales, Russulales, and Polyporales (Fig. 2B). Based on the sequence divergence between conserved protein sequences from *A. bisporus* and other sequenced Agaricales (e.g., *L. bicolor*, *S. commune*, *C. cinerea*), the split between the ancestors of these species has been estimated to have occurred at least 100 million y ago (SI Appendix, Fig. S4) (13). Nearly 83% of the predicted *A. bisporus* genes had homology with those in the public and JGI Mycocosm (18) databases, mostly to Agaricomycetes (Fig. 2C and SI Appendix, Table S4). Clustering of *A. bisporus* H97 proteins with those of other sequenced basidiomycete fungi revealed 5,058 clusters containing at least 10 protein members and two fungal taxa (SI Appendix, Fig. S5). The number of gene families exhibiting expansion was similar to those of related Agaricomycetes, such as *C. cinerea*, *Pleurotus ostreatus*, or *S. commune*, but was lower than of *L. bicolor* (SI Appendix, Fig. S5).

The *A. bisporus* proteome specializations are illustrated by the over- and underrepresentation of protein family (PFAM) domains compared with other fungi (SI Appendix, Tables S5–S7). Several families of well-known detoxification enzymes were found among the protein families in expansion [e.g., heme-thiolate peroxidase (HTP), methylmalonate semialdehyde dehydrogenase, β -etherase (glutathione-S-transferases cleaving β -aryl ether linkages), and pyranose dehydrogenase], suggesting a higher ability to metabolize derivatives of lignin and other polymers abundant in humicolous habitats.

Genes Involved in Lignocellulose Decomposition. *A. bisporus* is a very poor competitor on fresh nondegraded plant wastes but competes well on partially decomposed plant litter on forest floors and grassland soils rich in humic substrates. *A. bisporus* is adapted to growing in this ecological niche, where it and other species of Agaricales can occur abundantly and even predominate based upon observed fructifications. To identify the genomic traits enabling *A. bisporus* to adapt to its biotope and to efficiently complete

its life cycle, we have identified the repertoire and expression of genes known to be involved in organic matter degradation [carbohydrate-acting enzymes (CAZymes), lignin-related oxidoreductases, secreted proteases] and compared this arsenal to that of the white and brown wood-rotters, the coprophilous *C. cinerea* and the mycorrhizal symbiont *L. bicolor* (2–6, 13, 19). We also used custom microarrays to compare gene expression at four developmental stages, defined by mycelial cultures on agar-medium, mycelium colonizing the casing-soil layer, or the compost (a proxy for humic-rich composted material) and mature fruiting bodies (Fig. 1). Among the most highly up-regulated transcripts found in mycelium grown on compost were CAZymes, cutinases, oxidoreductases, and secreted proteases (SI Appendix, Fig. S6 and Table S8).

Carbohydrate-acting enzymes. *A. bisporus* is a generalist with respect to polysaccharide degradation when grown in laboratory culture on minimal medium and specific carbon sources (SI Appendix, Fig. S7). *A. bisporus* grows better (relative to glucose) on xylan than do other basidiomycetes, such as *S. commune* and *L. bicolor*. Xylan is the second most abundant polysaccharide in plant cell walls (PCW), comprising 7–12% of plant dry mass (20), and in wheat straw (21, 22). Although *A. bisporus* grows well on cellulose, it is less efficient than *S. commune* and *C. cinerea*. This catabolic ability concurs with the presence of a large set of genes encoding CAZymes (23) acting on plant, fungal, and bacterial cell wall polysaccharides [including 188 glycoside hydrolases (GH), 59 polysaccharide lyases (PL), and 10 carbohydrate esterases (CE)]. Clustering of CAZyme profiles in a large set of sequenced fungi, including white- and brown-rot, plant and animal parasites, and an ectomycorrhizal symbiont showed that the CAZyme profiles deviate from species phylogeny. The total CAZyme repertoire for *A. bisporus* is similar to that of white- and brown-rot basidiomycetes (Fig. S8) rather than the more closely taxonomically related *C. cinerea* and *L. bicolor*. This pattern likely reflects

the adaptation of *A. bisporus* to PCW polysaccharide-rich leaf litters (8). The up-regulation of transcripts with high similarity to PCW-degrading GHs (e.g., GH5, GH6, GH7, GH12, GH61, GH105) from basidiomycetes implicated in wood decay confirms that *A. bisporus* has the generic potential to break down PCW polymers by deploying a complete suite of enzymes degrading crystalline cellulose and xylans (*SI Appendix, Fig. S8, S9, and Table S10*). Notably, families GH6 and GH7 (*SI Appendix, Fig. S9A*), which include cellobiohydrolases that are involved in the attack of crystalline cellulose (24, 25), are present in all white-rot lineages and *A. bisporus*, but they are absent in brown-rot lineages (except Boletales) and *L. bicolor* (*Fig. S8*).

Profiling of CAZyme transcripts from mycelium growing in compost demonstrated that 115 (51%) of GHs, PLs, and CEs present in *A. bisporus* were up-regulated (from 10- to 1,450-fold) in compost, contrasting sharply with only 17% up-regulated transcripts in both the differentiating casing-soil mycelium and the fruiting body (*SI Appendix, Fig. S6A and Table S9*). Four genes (two CE5 acetyl xylan esterases and two GH12 cellulases) showed the highest up-regulation compared with agar-grown mycelium (*SI Appendix, Table S9*). A rhamnogalacturonyl hydrolase (GH105) was induced 836-fold. Notably, growth on compost was accompanied by the up-regulation of all 16 genes encoding cellulose-binding motif 1-containing proteins. Although significantly up-regulated, the pectin digestion machinery was comparatively less prominent than that for cellulose and xylan (*SI Appendix, Fig. S6A and Table S9*). The transcript profile of the mycelium growing on compost is therefore compatible with a prevailing substrate preference for xylan and cellulose, a medium activity on pectin and a slight activity on mannan. This digestion pattern is well-matched to the known composition of grasses and straw (20, 21) and sequential changes in carbohydrates during composting and mushroom growth (22).

Lignin-converting oxidoreductase genes. To gain access to cellulose, wood-decaying white-rots use fungal class II lignolytic peroxidases (PODs) to degrade lignin (13). We searched sequenced Agaricomycotina genomes for 27 gene families encoding oxidoreductases and CAZymes that have been implicated in wood decay (*SI Appendix, Fig. S10*). *A. bisporus* has a distinctive pattern that is not seen in white- and brown-rotters, *C. cinerea* and *L. bicolor*. Comparative analysis of the distribution of genes encoding lignolytic

PODs [lignin peroxidase, manganese peroxidase (MnP), and versatile peroxidase] revealed that *A. bisporus* retains a limited POD machinery to degrade lignin (*SI Appendix, Table S11*). Of the genes encoding lignolytic PODs, which have been shown to be important for lignin degradation in wood decayers (13, 26, 27), only two MnP genes were found in the *A. bisporus* genome. In compost, transcript levels of one of these MnPs (MNPL, JGI ID#221245) were significantly up-regulated relative to agar medium (*SI Appendix, Fig. S6B*). Thus, the repertoire of *A. bisporus* lignolytic PODs differs from *P. chrysosporium* and other sequenced white-rot fungi, which feature 6–26 POD genes (*SI Appendix, Fig. S11*) (13). The number and types of lignolytic PODs is similar to brown-rot lineages and *C. cinerea* having a single POD (13) (*SI Appendix, Table S11*). In contrast, the *A. bisporus* genome contains the largest set (24 members) of HTP genes, including aromatic peroxygenases (APOs) and classic chloroperoxidases (CPOs), a significant expansion relative to wood-decay fungi (*Fig. 3 and SI Appendix, Table S11*) (13). Typical APOs and CPOs are secreted, versatile enzymes with multiple catalytic activities with organic hydrocarbons and lignin-like aromatic compounds, including peroxidative oxidation, epoxidation, hydroxylation, and oxygen transfer reactions (27). Moreover, 16 of these HTP genes were significantly up-regulated in compost relative to defined medium, including an APO-induced 1,492-fold (*SI Appendix, Fig. S6B and Table S12*). Several transcripts coding for β -etherases, one of the *A. bisporus* expanding family (*SI Appendix, Table S7*), were also significantly up-regulated in compost relative to agar medium (*SI Appendix, Fig. S6B*). Possibly, the number and expression patterns of *A. bisporus* HTPs and β -etherases are related to the heterogeneous nature of humic-rich lignocellulosic materials, such as compost (22, 25). To estimate patterns of duplication and loss of genes encoding HTPs in the organismal phylogeny, we performed gene-tree/species-tree reconciliation analyses using Notung (28). This analysis suggests that the ancestor of the Agaricomycotina possessed six HTP gene copies, and that the number of HTP paralogs has been more or less steady throughout the evolution of most Agaricomycetes (*Fig. 3 and SI Appendix, Fig. S14*). However, an abrupt expansion of HTPs is reconstructed in the lineage leading from the common ancestor of *Agaricus*, *Coprinopsis*, and *Laccaria*, which is reconstructed as having seven HTP gene copies, and *Agaricus*, which has 24 HTP gene copies (*Fig. 3 and SI Appendix, Fig. S14*). Expansion of HTPs could have been an adaptation for decomposition of nonwoody plant matter and humic substances in soil, which is a common substrate for species of Agaricaceae.

Substrate complexity may also explain the presence of three compost-induced glyoxal oxidase-encoding genes. These copper radical oxidases are thought to play a role in production of extracellular H_2O_2 from simple aldehydes, such as glyoxal and methylglyoxal, and they are typically associated with class II PODs (*SI Appendix, Tables S11 and S12*). Catalytically distinct from the copper radical oxidases, at least 12 laccases *sensu stricto* were also confidently predicted (*SI Appendix, Table S11*). Three *A. bisporus* laccase-encoding genes were significantly up-regulated (>10-fold, $P < 0.05$) in compost relative to agar medium (*SI Appendix, Fig. S6B and Table S12*). Other potential H_2O_2 -generating extracellular enzymes include various glucose-methanol-choline oxidoreductases that include a likely aryl alcohol oxidase (JGI ID#185801) and a methanol oxidase (JGI ID#195553). The gene coding for the latter enzyme is highly up-regulated (*SI Appendix, Fig. S6B and Table S12*).

Intracellular metabolism of lignin metabolites and related compounds is poorly understood but cytochrome P450s (CYPs) are generally thought to play an important role. The *A. bisporus* genome contains a relatively low number of CYPs (109 genes) (*SI Appendix, Table S11*), but many were highly expressed and regulated. Specifically, six CYP64-encoding genes were up-regulated >10-fold in compost relative to defined medium (*SI Appendix, Table S12*).

Induction of protease genes on compost. Litter decomposers share the ability to use proteins as a sole source of carbon and nitrogen (26). The “casing soil” layer used in cultivation is extremely nutrient-poor, and induces *A. bisporus* hyphae to aggregate and form

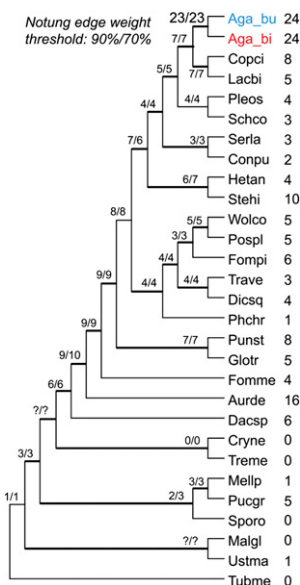


Fig. 3. The expansion of HTPs in *A. bisporus*. HTP copy numbers at internal nodes in the Agaricomycetes as reconstructed by gene-tree species-tree reconciliations in Notung (28) under two edge-weight threshold values. Numbers after species names denote the extant copy numbers of HTPs found in the genomes of these species.

a corded morphology where nutrient transport and proteinase activity is important. In line with this view, the two sequenced *A. bisporus* genomes reflect a remarkable metabolic capability for protein degradation and remobilization through amino acid and peptide transporters. Using the MEROPS protease nomenclature, which is based on intrinsic evolutionary and structural relationships (29), we identified 111 genes coding for proteases that possessed a secretion signal (SI Appendix, Table S13). We did not detect in *A. bisporus* any protease unique to this species. However, several transcripts coding for serine proteases (S8, S9, S10, and S12 families) were among the most highly up-regulated transcripts (>50-fold in comparison with agar-grown mycelium) in compost-grown mycelium (SI Appendix, Fig. S6C and Table S14). From the microarray data, a gene cluster coding for three serine proteases (located on chromosome IX) showed high expression on compost compared with other conditions. The most abundantly expressed of these was SPR1 (JGI ID# 194648), showing a 100-fold induction in mycelium colonizing compost, which is consistent with a major role in nutrient acquisition (11). *A. bisporus* serine proteinase has been shown to be synthesized specifically in response to humic-protein complex; transgenic analysis of the SPR1 promoter elements confirmed that the promoter is able to regulate mycelial serine proteinase production in response to specific nitrogen sources (11, 30). The abundance and substrate-induction of active proteinases may enable niche specialization in *A. bisporus*.

Metabolites released during litter (and compost) decay can efficiently be taken up by *A. bisporus* mycelium colonizing this substrate, as several genes coding for monosaccharide, cellodextrins, nicotinic acid, and amino acid transporters also were strikingly up-regulated on compost (cluster IV in SI Appendix, Fig. S12).

To identify the regulatory regions involved in adaptation to humic rich environments, transcriptomic data were compared with promoter sequences. Expression ratios were calculated and ranked from the microarray data comparing *A. bisporus* growing in humic (compost) vs. nonhumic (agar-medium) environments. A conserved sequence motif (TC[CA][TG]G[AT][GTA]A[AC]AATCTC) in the promoters of 23 of the top 33 compost-induced genes occurs at a much higher rate than in orthologs of *C. cinerea* and *L. bicolor* (SI Appendix, Fig. S12 and Table S15A), and exists in compost-induced genes of both *A. bisporus* genomes at a frequency significantly greater than random chance (SI Appendix, Table S15B). It is interesting to speculate (and to ultimately determine) whether this motif, which occurs more frequently in promoters of genes very highly expressed in humic-rich substrates, forms a common regulatory mechanism governing adaptation to the ecological niche.

Mushroom Development. The fruiting bodies of *A. bisporus* are the most commonly sold mushrooms in Europe and North America. However, some aspects of the sexual life cycle are poorly understood, although they are critical factors for breeding and other industrial applications. Fruiting body formation is a highly complex developmental process that has been studied in the model basidiomycetes *S. commune* and *C. cinerea* (31–33). After the substrate has been colonized and the application of specific environmental signals (reduced temperature and levels of CO₂ and 8-carbon volatiles), hyphae differentiate into the fruiting body where meiosis occurs (Fig. 1). The mating-type loci are the master regulators of fruiting-body development in fungi (31, 33). The sexually unifactorial *A. bisporus* contains only a single mating-type locus (34). The locus coding for homeodomain proteins typical for the *A* mating type in bifactorial species, such as *C. cinerea* and *S. commune*, and for the single mating-type locus in other unifactorial Agaricomycetes (35) was found on chromosome I (scaffold 1), supporting earlier studies (36). On the other hand, the genes encoding conserved pheromone and pheromone receptor genes are dispersed across the genome and the unifactorial *A. bisporus* has dispensed with the *B* mating-type specificity deployed by bifactorial species.

To identify genes associated with fruiting-body formation, we compared transcript profiles of undifferentiated mycelium grown

on agar medium, compost, or casing-soil, and of fruiting bodies (SI Appendix, Fig. S6, and Tables S8, S9, S12, S14, and S16). Of the 7,538 transcripts detected in fruiting bodies, 613 (8%) were significantly up-regulated ($P < 0.05$; false-discovery rate < 0.05) in fruiting bodies in comparison with undifferentiated mycelium grown on agar medium or compost. Among the 50 most highly induced genes in fruiting bodies, 40% are coding for orphan, lineage-specific genes. Genes coding for hydrophobins, lectins, tyrosinases, and transcriptional factors were among the most highly induced genes. Hydrophobins and tyrosinases are known to accumulate in mushroom caps during *A. bisporus* fruit-body development (37, 38). We compared genes induced during the fruiting-body formation in *A. bisporus*, *L. bicolor*, and *S. commune* to identify common developmental gene networks (SI Appendix, Table S16A). Only 35 and 22 homologous genes were significantly up-regulated ($P < 0.05$) in both *A. bisporus*/*L. bicolor* fruiting bodies or *A. bisporus*/*S. commune* fruiting bodies, respectively. Only 13 genes were significantly up-regulated ($P < 0.05$) in the fruiting bodies of these three species (e.g., aromatic-ring hydroxylase, GH16, FAD-linked oxidase, fatty acid desaturase), suggesting that genes induced during mushroom development are mainly clade-specific.

Recently, a set of transcription factors has been identified in *S. commune* that act downstream of the mating-type loci (3, 39). These transcription factor genes have orthologs in *A. bisporus* (SI Appendix, Table S16B). Based on transcript profiling, several of these transcription-factor genes may be involved in the regulation of mushroom formation in *A. bisporus*. The DNA binding protein *pcc1* gene, which leads to *A*-regulated sexual morphogenesis in *C. cinerea* (40), was highly expressed in *A. bisporus* mycelium grown on agar medium, compost, or casing, and in fruiting bodies. The expression profiles of the orthologs of *c2h2*, *fst3*, *fst4*, and *hom1* were similar in *S. commune* and *A. bisporus* (i.e., up-regulation of gene expression in mushrooms compared with mycelium), suggesting that these agarics share similar master developmental switches for fruiting-body formation, making these genes primary targets for a functional analysis. Studying the regulatory mechanisms underlying fructification in *A. bisporus* would allow the control of mushroom-pin formation, considered by mushroom growers as the most important step in managing the mushroom crop.

Our genomic and transcriptomic data suggest that *A. bisporus* has the decay machinery to decompose lignocellulosic material; yet to our knowledge, it has not been shown to decompose wood in nature. Although there are similarities in genome composition, *A. bisporus* fits neither brown-rot nor white-rot classifications. We hypothesized that a humicolous fungus adapted to growth in a humic-rich environment is atypical of classic wood-degrading fungi, and transcriptome expression data support this view. The wide repertoire of HTP, β -etherases, multicopper oxidase, and CYP450 oxidoreductases and their striking up-regulation in mycelium-colonizing compost suggest a broad mode of attack on decaying lignin and related metabolites, and an adaptation to challenges posed by complex composts. The large gene repertoire of compost-induced CAZymes and oxidoreductases, together with high protein degradation and nitrogen-scavenging abilities, are key features of *A. bisporus* adaptation to humic-rich ecosystems. This study reveals genetic and enzymatic mechanisms governing adaptation of *A. bisporus* to a humic-rich ecological niche created by primary degradation of plant material, demonstrating the critical role such fungi contribute to soil structure and carbon sequestration.

Materials and Methods

Genome Sequencing, Assembly, and Annotation. The homokaryotic *A. bisporus* var *bisporus* H97 and *A. bisporus* var. *burnettii* JB137-S8 strains were sequenced by whole-genome sequencing and were assembled into predicted 30.2-Mb and 32.6-Mb genomes, respectively (SI Appendix). The protein-coding genes were predicted with a combination of automated gene callers, ESTs produced from each *A. bisporus* strain, and filtering dubious genes with similarity to transposable elements (SI Appendix). In total, the

gene sets included 10,438 and 11,289 predicted genes for H97 and JB137-S8, respectively; these were the basis for multigene family analyses. The *A. bisporus* H97 genome sequence can be accessed at http://jgi.doe.gov/Abisporus_var_bisporus and the *A. bisporus* var. *burnettii* JB137-S8 genome sequence can be accessed at http://genome.jgi-psf.org/Agabi_varbur_1.

Microarray Analysis of Gene Expression. Gene expression was assessed in mycelium grown on defined medium, casing substrate and compost, and fruiting bodies, using specific custom 60-mer Agilent microarrays (as described in *SI Appendix*).

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