Molecular aspects of growth and fruiting of the edible mushroom *Agaricus bisporus*

Piet de Groot

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The research described in this thesis was part of a study to isolate marker genes and to develop a genetic map of the white button mushroom *Agaricus bisporus*, financially supported by Bromyc BV, a subsidiary of the Netherlands Mushroom Growers Cooperative and was performed at the Mushroom Experimental Station, Horst and the section Molecular Genetics of Industrial Microorganisms, Wageningen Agricultural University.

Cover: *in situ* hybridisation of *hypA* and *hypB* in an *A. bisporus* fruit body at stage 2

ISBN 90-9012349-0

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EEN WETENSCHAPPELIJKE PROEVE
OP HET GEBIED VAN DE NATUURWETENSCHAPPEN,
WISKUNDE EN INFORMATICA

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE KATHOLIEKE UNIVERSITEIT NIJMEGEN,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 4 FEBRUARI 1999
DES NAMIDDAGS OM 1.30 UUR PRECIES

DOOR

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GEBOREN OP 12 APRIL 1968
TE OLDEHOLTPADE

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Abstract

Molecular aspects of growth and fruiting of the edible mushroom *Agaricus bisporus*

The research described in this thesis was part of a project aimed at the isolation of marker genes and the development of a genetic map of the white button mushroom *Agaricus bisporus*. The ultimate purpose of the project was to make the white button mushroom more amenable to strain improvement by classical genetics. The thesis concentrates on molecular aspects of growth and fruiting of *A. bisporus*. In order to study the genetic processes that are involved in fruiting of *A. bisporus*, we focused on the cloning and characterisation of genes that are specifically expressed during fruit body development and maturation. Two fruit body specific genes identified by differential screening techniques appeared to encode small hydrophobic cell wall proteins belonging to the fungal family of surface-active hydrophobins. One of those proteins, HYPA, is part of a protective layer surrounding the mushroom caps. It showed, like other fungal hydrophobins, a self-assembling behaviour which was shown by *in vitro* studies using Atomic Force Microscopy. Additionally, to study genetic aspects of compost utilisation by *A. bisporus*, we aimed at the cloning of genes involved in the release of sugars from the plant polysaccharides present in compost. Cloning and characterisation of one of such genes, the *xlnA* gene encoding an endo-1,4-ß-xylanase, gave us a better insight in the regulation of enzymes that can degrade lignocellulosic compounds of compost. Expression studies with *xlnA* and *cel3*, encoding a cellobiohydrolase, showed that both genes are strongly induced on compost and the regulation of their transcription seems to be an elegant example of adaptation of a fungus to its natural niche. Furthermore, experiments were focused on the isolation and characterisation of housekeeping genes. Cloning of the housekeeping genes *pgkA*, *pkiA* and *aldA* enabled us to compare the structure of *A. bisporus* genes with homologous genes of related organisms and to monitor their expression in different parts of mature mushrooms. The cloning of a large number of genes combined with chromosome separation techniques forms a solid base for the development of a gene-based linkage map and will make *A. bisporus* more amenable for breeding purposes.

Additional index words: Agaricus bisporus, edible mushroom, Horst® U1, fruit body development, hydrophobin, hypA, gene duplication, ATP-synthase δ-subunit, septin, hypB, atomic force microscopy, self-assembly, housekeeping gene, pgkA, pkiA, aldA, compost utilisation, xlnA, endo1,4-β-xylanase, chromosome separation, CHEF, linkage map.

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General introduction

A modified version of this chapter was published in *Mycological Research* (1998) as "Biochemical and molecular aspects of growth and fruiting of the edible mushroom *Agaricus bisporus*" by Piet W. J. De Groot, Jaap Visser, Leo J. L. D. Van Griensven & Peter J. Schaap

General introduction

Cultivation of mushrooms represents a technology in which low-cost lignocellulosic agricultural by-products are converted into a high value human food crop. Fruit bodies of several basidiomycete fungi are presently cultivated and used as a vegetable crop, but by far the most popular species is the common white button mushroom *Agaricus bisporus*. Cultivation of this mushroom for human consumption started in France already early in the seventeenth century (Spencer, 1985). In the last decades mushroom production has expanded all over the world and production rates increased from 300,000 tonnes in 1970 to over two million tonnes of fresh weight mushrooms in 1997 (source: FAOSTAT Database). Production rates were not increased only by scale expansion of the mushroom industry but also by higher crop yields and increased efficiency achieved by optimisation of the cultivation process. A significant improvement in efficiency was made a few years ago with the introduction of full-grown compost, a compost already fully colonised with *A. bisporus* mycelium prior to deposition in the growing chambers. This shortened the duration of the vegetative growth stage in the growing chambers drastically and led to an increase in the number of cultivation cycles that can be run in a fixed amount of time.

The cultivation of *A. bisporus* for the production of mushrooms

Cultivation of the edible fungus *A. bisporus* for the production of mushrooms is a complex process. High mushroom yields are obtained on a specifically fermented and non-sterile substrate. The biochemistry and physiology of the processes involved in utilisation of this compost and in fruit body formation are, however, poorly understood. Nevertheless, optimising modifications of the cultivation procedure have rendered commercial production of mushrooms profitable and has led to the development of a large industry. Here, an overview of mushroom cultivation is given with emphasis on aspects that directly seem to affect substrate utilisation, compost colonisation, fruit body development and, hence, mushroom yields. Detailed descriptions of commercial cultivation procedures can be found elsewhere (Wood, 1984; Van Gils, 1988; Baars, 1996).

Preparation of compost

An efficient substrate for commercial cultivation of mushrooms is a moist mixture of wheat straw, horse manure, chicken manure and gypsum that has been subjected to an extended fermentation procedure (Gerrits, 1974). The basic nutritional requirements of *A. bisporus in vitro* are known and in principle are easily met by the resultant compost (Wood & Fermor, 1985). The purpose of the composting procedure is to prepare a medium that will favour growth of the mushroom in preference to other micro-organisms (Fermor *et al.*, 1985). The procedure involves 2 - 3 weeks of uncontrolled self-heating followed by a pasteurisation step and 8 - 9 days conditioning at 45°C (Gerrits, 1988). The raw materials are humified by a diverse range of micro-organisms. By manipulating the succession of these micro-organisms during the various stages of composting, the physical state (moisture content, bulk density) and chemical composition (available nutrients, pH) change and the compost becomes "selective" for growth of the mushroom mycelium. It is, however, largely unknown how this selectivity can be described in biological or chemical terms (Straatsma *et al.*, 1989; Smith *et al.*, 1995).

Growth of the compost microflora proceeds initially at the expense of soluble carbon and nitrogen compounds and after these have been depleted growth takes place using degradation products of insoluble fractions such as cellulose, hemicellulose and protein (Wood, 1984). During composting about two-thirds of the initial plant cell wall polysaccharides are consumed (Iiyama *et al.*, 1994). However, little or no degradation of lignin is observed during composting (Wood & Leatham, 1983), but alterations in the chemical structure of lignin, due to condensation and oxidation reactions, do occur (Iiyama *et al.*, 1994).

An electron microscopy study of wheat straw degradation during composting showed a considerable increase in numbers of bacterial cells until the pasteurisation stage. Due to the pasteurisation process, other bacterial populations, actinomycetes and thermophilic fungi take their place (Atkey & Wood, 1983). During the conditioning phase, volatile ammonia levels are lowered by emission and bioconversion to levels that do not inhibit growth of the mushroom mycelium. Ammonia disappearance from the compost was linked with the presence of the thermophilic fungus *Scytalidium thermophilum* (Ross & Harris, 1983). Thermophilic fungi were therefore suggested to play a role in compost selectivity by fixation of ammonia and nitrates in biomass (Fermor *et al.*, 1985).

After completion of the composting process, the compost mainly consists of the plant-derived components lignin, cellulose, hemicellulose and protein and microbial biomass

(Fermor & Wood, 1979). The lignin fraction encrusts cellulose and is chemically bound to hemicellulose (Cullen & Kersten, 1996) making the compost a poor substrate for micro-organisms other than *A. bisporus*.

Biochemical and molecular aspects of compost utilisation

After inoculating the compost with millet or rye grains overgrown with *A. bisporus* mycelium (a procedure called spawning), *A. bisporus* is allowed to colonise the compost for two weeks. During colonisation, an array of enzymes secreted by *A. bisporus* mycelium has been detected using various axenic and non-axenic culture conditions (Wood & Goodenough, 1977; Wood, 1984; Yagüe *et al.*, 1997; De Groot *et al.*, 1998). Furthermore, a broad range of lignocellulose degrading activities was recovered from spent mushroom compost including cellulose degrading activities such as endoglucanase, cellobiohydrolase and ß-glucosidase activities, xylan degrading activities such as acetylesterase and arabinofuranosidase activities and peroxidase activity (Gerrits *et al.*, 1967; Ball & Jackson, 1995). Although it is not clear in all cases whether these activities are exclusively produced by *A. bisporus* or by other micro-organisms still present in the compost, tests *in vitro* indicate that the fungus can degrade most of the plant-derived fractions of the compost. For a number of the detected activities, the responsible enzymes were characterised and/or the encoding genes cloned (Table 1.1).

Cellulose degradation

The major constituent of the lignocellulose complex present in compost is cellulose, a linear polymer of ß-1,4 linked D-glucose residues. When *A. bisporus* is grown on crystalline cellulose as the sole carbon source it induces a complete cellulose degrading system including endocellulase, cellobiohydrolase and ß-glucosidase activities (Gerrits *et al.*, 1967; Manning & Wood, 1983; Yagüe *et al.*, 1997).

Screening of a cDNA expression library, constructed of RNA isolated from cellulose-grown mycelium, with an antibody raised against an *A. bisporus* protein fraction with endocellulase activity, resulted in the isolation of four cDNAs designated *cel1* to *cel4* (Table 1.1). Of these, *cel2* shows similarity to cellobiohydrolase I proteins belonging to glycosyl hydrolase family 7, *cel3* shows similarity to cellobiohydrolase II proteins belonging to glycosyl hydrolase family 6 and *cel4* shows similarity to ß-mannanase proteins belonging to glycosyl hydrolase family 5. For the *cel1* gene (Raguz *et al.*, 1992), no homology was found with catalytic domains of members of cellulose degrading enzyme families. However, like CEL2, CEL3 and CEL4, the N-terminal part of CEL1 shows

similarity to fungal cellulose binding domains (CBD) which in many cellulases facilitate binding of cellulose (Gilkes *et al.*, 1991). Because the 'anti-endocellulase' antiserum was unable to recognise CEL1 protein from which the CBD was removed (Armesilla *et al.*, 1994), the antiserum used was probably more selective for proteins with a CBD than for cellulases per se.

Xylan degradation

Xylan, the most abundant hemicellulosic component in straw-based mushroom compost (Coughlan & Hazlewood, 1993), is a complex polymer consisting of ß-1,4-linked xylosyl residues which can be acetylated or can have covalently linked arabinosyl and glucuronic acid side-groups. Complete enzymatic conversion of xylan into monomeric sugars requires the concerted action of several enzymes including endo-1,4-ß-xylanase, ß-xylosidase, α-L-arabinofuranosidase, α-glucuronidase and a number of esterases. Wood & Goodenough (1977) detected in compost xylanase activity associated with *A. bisporus* which indicated the significance of *A. bisporus* xylanases for the release of sugars as substrates for growth. Nevertheless, in *Agaricus* and other basidiomycetes, information on the characterisation of these enzymes and the corresponding genes is still scarce and was until recently limited to the purification of an endo-1,4-ß-xylanase of *Schizophyllum commune* (Oku *et al.*, 1993).

Lignin degradation

Degradation of lignin, a lignocellulosic component formed by non-enzymatic polymerisation of phenoxy radicals, has been studied extensively in the basidiomycetous white-rot fungus *Phanerochaete chrysosporium* (Broda *et al.*, 1996). From *P. chrysosporium*, two types of peroxidases, lignin and manganese peroxidases, have been characterised that are known to catalyse the initial depolymerisation of lignin. Hydrogen peroxide producing enzymes such as glyoxal oxidase, and laccase (copper-containing polyphenol oxidase) are also considered important in the process of lignin bio-degradation (Cullen & Kersten, 1996). Laccase may also act as a scavenger system by promoting polymerisation of toxic compounds produced by lignin degrading enzymes (Thurston, 1994). During vegetative growth, the protein is constitutively expressed at high levels (Wood, 1980) and has been suggested as being a good indicator of *A. bisporus* mycelial biomass in compost (Matcham *et al.*, 1985). Laccase protein from *A. bisporus* was purified (Perry *et al.*, 1993a) and two laccase encoding genes were cloned (Perry *et al.*, 1993b). Comparison of these laccase genes with other fungal laccases and functionally related

Table 1.1. Temporal expression and mode of action of lignocellulolytic enzymes of *Agaricus bisporus*

Biopolymer	Enzymatic activity	Available data	Reference
Cellulose	Endocellulase	Activity in cellulose-grown culture and compost; in phase with fruit body enlargement	Manning & Wood, 1983
	Cellobiohydrolase	Activity in cellulose-grown culture; *cel2* (CBHI) and *cel3* (CBHII) genes, both induced on cellulose	Yagüe *et al.*, 1997 Chow *et al.*, 1994
	ß-glucosidase	Activity in cellulose-grown culture	Yagüe *et al.*, 1997
Mannan	Mannanase	*cel4* gene, induced on cellulose	Yagüe *et al.*, 1997
Xylan	Endoxylanase	34 kDa enzyme partially purified from compost; *xlnA* gene, induced on compost	De Groot *et al.*, 1998
	ß-xylosidase	Activity in compost	De Groot *et al.*, 1998
Lignin	Manganese peroxidase	Activity in compost, in phase with lignin degradation	Bonnen *et al.*, 1994
	Laccase	65 kDa enzyme purified; activity in compost in phase with lignin degradation; *lcc1* and *lcc2* genes	Perry *et al.*, 1993a Perry *et al.*, 1993b

ascorbate oxidases from plants showed that, whilst regions around the amino acids that are involved in copper binding are absolutely conserved, the overall sequence similarities are low.

In composts colonised with *A. bisporus*, using both axenic and non-axenic conditions, manganese peroxidase activity is present whereas lignin peroxidase activity was not detected (Bonnen *et al.*, 1994). The manganese peroxidase activity appeared to be developmentally regulated and is strongly correlated with the also developmentally regulated laccase activity. Activity patterns of both enzymes parallel that observed for degradation of radio-labelled and synthetic lignin by *A. bisporus*, suggesting that both enzymes are involved in lignin degradation by *A. bisporus* (Bonnen *et al.*, 1994).

Protein degradation

By supplementing compost with protein-rich materials prior to inoculation with *A. bisporus*, mushroom yields can be 25 - 50% increased (Schisler & Sinden, 1962; Fermor *et al.*, 1985). Associated with supplementation are a risk of excessive temperatures developing in the compost and the occurrence of weed moulds. Widely used supplements are soybean meal and cotton-seed meal.

Studies on protein utilisation by basidiomycetous fungi showed that *A. bisporus* shares with *Coprinus cinereus* and *Volvariella volvacea* the ability to grow on protein as sole source of carbon, nitrogen and sulphur (Kalisz *et al.*, 1986). Interestingly, in these three basidiomycetes protein utilisation and extracellular proteinase activity are not repressed by supplementing protein-containing media with glucose, ammonium or sulphate (Kalisz *et al.*, 1987). It therefore appears that proteinase expression is free of catabolite repression and this is in contrast to what has been observed in *Aspergillus* spp. and in *Neurospora crassa* (Cohen, 1980), which are in this respect the best studied fungi.

When grown on a microbial biomass or protein as sole nitrogen and carbon sources, acid, neutral and alkaline proteinase activities have been identified in the culture filtrate of *A. bisporus* (Fermor & Wood, 1981). One of the proteinases produced was an extracellular prolyl endopeptidase. A similar, intracellular, protein with only a slight difference in isoelectric point, was purified from fruit bodies (Sattar *et al.*, 1990). Intracellular proteinases may be functional during maturation or post-harvest sporophore development. From fresh mature fruit bodies of *A. bisporus* an enzyme active against bradykinin was purified (Kizuki & Moriya, 1982). Enzyme inhibition experiments indicated that this enzyme is probably a cysteine proteinase. From the stipes of post-harvest *A. bisporus*

mushrooms Burton *et al.* (1993) purified a serine proteinase and the corresponding gene was cloned (Kingsnorth *et al.*, 1997). Proteinase activity is strongly increased in post-harvest mushrooms possibly for recycling amino acids for the nutritionally starved mushroom and/or to activate other degrading enzymes.

The microbial biomass

The rate of colonisation of the compost by *A. bisporus* is stimulated by the microbial biomass present in compost at spawning. The most abundant fungus present after composting, *Scytalidium thermophilum*, was found to have a growth promoting effect on mycelial growth of *A. bisporus* upon addition to sterilised compost (Straatsma *et al.*, 1989; 1994b). The same stimulating effect, but now measured as a significant increase in mushroom yields, was found when this thermophilic fungus was inoculated into compost that was pasteurised at some point in the composting process just before *S. thermophilum* normally starts to accumulate (Straatsma *et al.*, 1994a).

Large variations were found in the compost in the composition of the bacterial population depending on the distance to *A. bisporus* hyphae. The strongest stimulators of growth of the mushroom mycelium were found in the area close to the surface of hyphae, the hyphosphere. Growth of the hyphosphere micro-organisms on the other hand was stimulated by culture filtrates of *A. bisporus* mycelium and it was suggested that this advantageous interaction enables the mycelium to colonise compost rapidly after spawning (Stanek, 1974). A number of micro-organisms including bacteria, actinomycetes and thermophilic fungi were isolated from compost and *A. bisporus* was able to grow on the dead biomass of several of these killed micro-organisms as its sole source of carbon, nitrogen and phosphorus (Fermor & Wood, 1981; Fermor & Grant, 1985). The main bacteriolytic enzyme activity in culture filtrates of mycelium grown on killed *Bacillus subtilis*, a bacterium often found in mushroom composts, was found to be ß-*N*-acetylmuramidase (Grant *et al.*, 1984, Lincoln *et al.*, 1997). Another cell wall degrading enzyme present in this culture filtrate was ß-*N*-acetylglucosaminidase and in cultures grown on killed *S. thermophilum* mycelium ß-*N*-acetylgalactosaminidase and laminarinase (endo-1,3-ß-D-glucanase) were detected. Additionally, acid, neutral and alkaline proteases, DNase, RNase and lipase activities were found in culture filtrates (Fermor & Wood, 1981; Fermor & Grant, 1985).

Mineralisation of microbial biomass, radiolabelled with ^{14}C during composting, showed that *A. bisporus* was able to utilise the microbial biomass efficiently (Fermor *et al.*, 1991). The contribution of the microbial biomass to the mushroom biomass is around 10%, which

suggests that under commercial cultivation conditions *A. bisporus* derives the bulk of its carbon nutrition from the plant polymers within straw. Although not the most important carbon source, the microbial biomass in the compost may be a concentrated source of nitrogen and/or other nutrients, especially during the early stages of spawning and mushroom development (Sparling *et al.*, 1982).

Fruit body development

Fruiting of *A. bisporus* depends on a complex set of variables including atmospheric factors such as carbon dioxide concentration, temperature, humidity and pH and on the nutritional status of the substrate (Flegg & Wood, 1985). To induce synchronous fruiting during commercial cultivation of *A. bisporus*, the full-grown compost is covered by a (non-sterile) casing layer consisting of peat and lime. After colonisation of the casing layer, fruiting is stimulated by lowering the ambient temperature and carbon dioxide concentration in the growing rooms.

Using a method known as the 'halbschalentest', Eger (1961) showed that the casing layer must contain a viable microbiota for normal fruiting to occur. The interaction between *A. bisporus* and the micro-organisms present in the casing layer was studied by electron microscopy. Masaphy *et al.* (1987) found that rodlike bacteria from the casing soil attached firmly to hyphae at the time of fruit body initiation and similarly, attachment of the casing layer micro-organisms *Pseudomonas putida* and *Pseudomonas tolaasii* to sterile hyphae was observed by Miller *et al.* (1995). Upon addition to sterilised casing, isolates of *P. putida* induced formation of fruit body initials (Hayes *et al.*, 1969; Reddy & Patrick, 1990) and the same effect was observed on malt agar (Hume & Hayes, 1972). With commercial strains, aseptic fruiting on malt agar was also attempted and, at a low frequency, formation of hyphal aggregates or cords and primordia was observed. However, these fruit bodies were arrested at the primordial stage of development (Wood, 1976).

On the other hand, the requirement of bacteria for fruit body initiation and further development is not absolute and may be strain dependent. Among the *A. bisporus* isolates that have been collected from the wild, some strains fruit readily under axenic conditions (Kerrigan, 1995). These strains and developmental mutants that are able to produce numerous primordia under axenic conditions and become blocked at the pin stage, prove to be important research tools to study factors that are involved in fruit body initiation (Elliott & Wood, 1978; Hammond & Burton, 1996).

The role of the micro-organisms in the inducing nature of the casing layer was also studied by replacing the non-sterile casing layer by activated charcoal, a chemical

absorbent, or by a sterilised casing layer. Whereas fruiting was inhibited or delayed on a sterile casing layer, normal fruiting appeared on activated charcoal. This suggests that the micro-organisms in the casing layer remove one or more inhibitory compounds of fruiting produced by the mushroom mycelium rather than that they produce fruiting inducing substances (Eger, 1961; Long & Jacobs, 1974; Wood, 1984).

Morphogenetic, biochemical and molecular aspects of fruit body development

Morphogenetic aspects of fruit body development

The different types of cells, tissues and organs of fruit bodies of basidiomycetes are the result of a differentiation process preluding the production of basidiospores for sexual reproduction. The development of such an *A. bisporus* multicellular structure begins with hyphae consolidating into a mycelial cord, the first well-organised but undifferentiated fungal tissue, by adhesive mucilaginous substances (Umar & Van Griensven, 1998). Under cultivation conditions multiple primordia, consisting of frequently branching and actively growing hyphae, develop within or in close relation to the mycelial cords. Some of these primordia grow within two days to a length of about 6 mm and then their development either ceases or continues through a so-called histo-organogenetic stage (Umar & Van Griensven, 1997). At this stage vertically oriented hyphae which form the future stipe, and a dense mass of radial oriented hyphae which form the future cap, can already be distinguished microscopically. At later stages, other cell types and tissues (Figure 1.1) can be distinguished (Craig *et al.*, 1977; Craig *et al.*, 1979; Umar & Van Griensven, 1997).

By comparison of the hyphal organisation in various regions of expanded stipes different types of cells were distinguished (Craig *et al.*, 1979). The base of the stipe is formed by a dense mass of long thin hyphae. The core region of the middle and upper parts of the stipe consist of similar hyphae, but these are loosely-packed and vertically oriented. Larger, closely-packed vertically oriented hyphae containing vacuolated cells are seen in the major part of the stipe whereas the surface of the stipe is covered by a thin layer of closely packed long and slender hyphae. Stipe cells are multinucleate with a mean number of 5 to 9 nuclei per cell in all regions. During the final stages of fruit body development the upper part of the stipe elongates and in the basidiomycete fungus *Coprinus cinereus* this is accompanied by high levels of chitin synthase activity. In expanding stipe cells of *A. bisporus*, synthesis

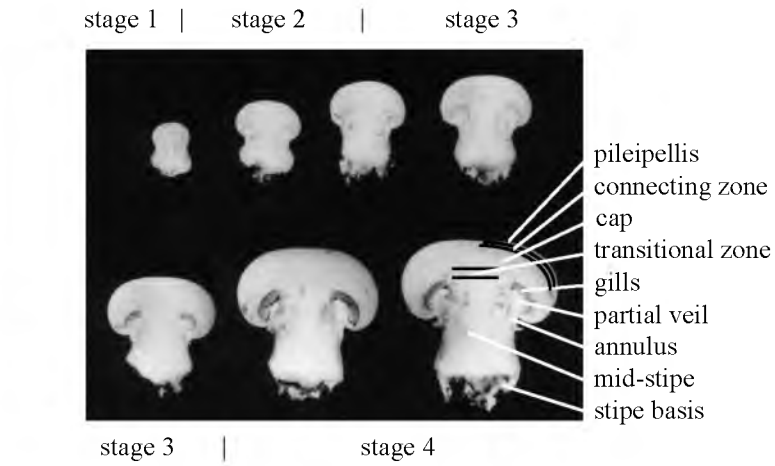

Figure 1.1. Progressive morphology of fruit bodies. Morphologically different tissues of a mature mushroom are indicated. Stages of development are according to De Groot *et al.* (1997).

of chitin, a major cell wall component, was allocated to the cell walls and also occurred at the ingrowths in between dividing cells, the septa.

Microscopic analysis of *A. bisporus* gill tissue revealed the highly organised structure of these cells (Craig *et al.*, 1977). The centre of the gills constitutes of elongate tramal cells that run from the cap to the edge of the gill. From the tramal cells extensively branched subhymenial cells arise and perpendicularly to the tramal cells a hymenial layer (basidia) covering the whole gill surface is formed. The number of nuclei in the basidia is reduced to two nuclei per cell, and after nuclear fusion and meiosis basidiospores develop on the apex of the basidial cells.

Hyphal filaments of both the vegetative mycelium and fruit bodies are interrupted by septa or cross-walls (Flegg & Wood, 1985). The septal structure is of a complex type known as the dolipore septum and is unique to the Basidiomycetes. In subhymenial cells it is surrounded by an outer cap most likely consisting of ribosomal aggregates (Craig *et al.*, 1977). The physiological function of this structure is unknown, but it would certainly prevent migration of larger organelles such as nuclei (Flegg & Wood, 1985).

The biochemistry of fruit body development

In terms of chemical composition, the main difference that has been observed between *A. bisporus* mycelium and fruit bodies is the accumulation, up to 25 - 35% dry weight, of mannitol in fruit bodies (Hammond & Nichols, 1976). Labelling studies showed that the turnover rate of mannitol in fruit bodies is low (Hammond & Nichols, 1977). It has been suggested that mannitol accumulation may be a mechanism to move water into the fruit body in order to create the hydrostatic pressure necessary for inflation of the fruit body hyphae (Hammond, 1985). Mannitol is synthesised in *A. bisporus* from fructose, a reaction mediated by mannitol dehydrogenase, using NADPH as a cofactor (Edmundowicz & Wriston, 1963; Ruffner *et al.*, 1978). Increased glucose-6-phosphate dehydrogenase levels at flush emergence, allowing a high activity of the pentose phosphate pathway and NADPH production, coupled with high levels of glycogen and trehalose would provide the substrates for mannitol production at the time of flush initiation (Hammond, 1985). The gene encoding glucose-6-phosphate dehydrogenase has been cloned and shows complex regulation at the level of transcription during fruit body development (Schaap, unpublished results).

In a mathematical model which describes the flushing pattern of fruit body production, the stimulus for fruit body initiation was suggested to be an increase in a hypothetical substrate in the mycelium above a threshold level (Chanter, 1979). The model also accounts for the decrease in yield normally observed in consecutive flushes as a result of substrate depletion. Good candidates for being such a substrate are trehalose and glycogen which are considered important carbohydrate resources for the developing sporophore. Glycogen and trehalose concentrations in sporophores and supporting mycelium fluctuate and these fluctuations correlate well with the flushing periods (Hammond & Nichols, 1979) and this is also true for the activity of trehalase and glycogen phosphorylase, the enzymes releasing sugars from these compounds (Wells *et al.*, 1987).

Obviously, maintenance of an energy supply to sustain growth of sporophores requires release of compost compounds by the action of cellulase and other hydrolytic enzymes. Endocellulase activity fluctuates in parallel with the flushing cycles and is directly correlated with crop yield (Claydon *et al.*, 1988; Smith *et al.*, 1989). It therefore seems that the rapid increase in biomass during fruit body enlargement will act as a (carbon) sink and will drive mechanisms that temporarily increase the carbohydrate uptake by the mycelium.

In contrast to cellulase activity, the activity of the extracellular enzyme laccase in compost oscillates out of phase with fruit body enlargement (Wood & Goodenough, 1977; Smith *et al.*, 1989). The reason for this out of phase regulation during fruiting is not certain.

Possibly, the enzyme produces fruiting inhibitory substances or, since laccase is a very abundant protein exported by the mycelium, demand for nitrogen supplies during fruiting requires re-assimilation of excreted nitrogen-containing compounds (Flegg & Wood, 1985).

Molecular aspects of fruit body development of basidiomycetes

Fruit body development in basidiomycetes is normally initiated in heterokaryotic mycelium upon induction by environmental stimuli. In heterothallic species such as *Schizophyllum commune* and *Coprinus cinereus*, fruiting competence is under control of mating type genes and analysis of the mating type loci has been the subject of many studies (Griffin, 1994). In a number of basidiomycetes, however, fruit body development also occurs in homokaryotic strains (Elliott, 1985a). The structures of homokaryotic fruits may vary from slightly differentiated tissue to fully differentiated fruit bodies and this suggests that especially in the latter case the expression of genes controlling fruit body development are not strictly controlled by the mating type loci.

Gene expression studies in various fungi including the basidiomycetes *S. commune* (Mulder & Wessels, 1986) and *C. cinereus* (Yashar & Pukkila, 1985) showed that sporulation is accompanied by a temporarily enhanced and specific expression of a large number of genes (Griffin, 1994). For the ascomycetes *Saccharomyces cerevisiae* (Griffin, 1994) and *Aspergillus nidulans* (Timberlake, 1990) cloning of such genes and analysis of developmental mutants have led to schemes explaining the regulation of the differentiation process during sporulation. In these schemes, developmental growth is preceded by a pre-induction phase during which developmental competence is acquired. After induction, an interactive cascade of genes regulates reproductive development. Using differential screening techniques, cDNAs from genes showing elevated expression during fruit body development have been cloned from the basidiomycetes *Agrocybe aegerita* (Salvado & Labarère, 1991), *C. cinereus* (Yashar & Pukkila, 1985) and *S. commune* (Mulder & Wessels, 1986).

Three of the *S. commune* genes that are abundantly expressed in dikaryotic fruit bodies, *Sc1*, *Sc4* and *Sc6*, and a gene highly expressed in both mono- and dikaryotic mycelium, *Sc3*, show significant sequence similarity (Schuren & Wessels, 1990; Wessels, 1997). These genes all encode small moderately hydrophobic proteins and have a conserved pattern of eight cysteine residues and a putative signal sequence for secretion. From the Euascomycetes *A. nidulans* (Stringer *et al.*, 1991; Stringer & Timberlake, 1995) and *Neurospora crassa* (Bell-Pedersen *et al.*, 1992; Lauter *et al.*, 1992) genes encoding

analogous proteins with the same cysteine pattern were cloned and gene disruptants formed conidia lacking an external wall layer. The *S. commune* Sc3p protein self-assembles in the walls of emergent hyphae and these Sc3p aggregates were shown to be resistant to washing with 2% sodium dodecyl sulphate (SDS) solutions at 100°C but can be solubilised into monomers with trifluoroacetic acid (Wösten *et al.*, 1994). Purification strategies based on this property resulted in the isolation of similar proteins from fungi of different taxonomic classes. These proteins, presently known as hydrophobins, represent a major class of fungal secreted proteins that self-assemble into amphipathic films at hydrophobic-hydrophilic interfaces. By interfacial self-assembly at the outer surface, hydrophobins determine the hydrophobicity of the surface of aerial hyphae (Wessels, 1997).

Regulation of the *S. commune* hydrophobin genes was studied by following the expression in strains blocked in basidiocarp development. All hydrophobin genes were suppressed in a *thin* (*thn*) mutant that neither forms aerial hyphae nor shows signs of fruit body development (Wessels *et al.*, 1991). In a *fruit body formation* (*fbf*) mutant, formation of aerial hyphae appears normal but fruit body development is blocked. The formation of aerial hyphae in the *fbf* strain is accompanied by expression of *Sc3* gene but expression of the fruit body specific *Sc1*, *Sc4* and *Sc6* genes is suppressed (Springer & Wessels, 1989; Wessels *et al.*, 1995). This suggests that, as proposed for asexual sporulation in *A. nidulans* and sexual development in *S. cerevisiae*, sexual reproduction in *S. commune* is a two phase process. Fruiting competence is gained during a pre-inductive phase regulated by the mating type factors and the *thn* gene. After induction, fruit body development regulated by the *fbf* gene occurs, and is associated with a temporary high expression of a large number of genes.

The genetics of *A. bisporus*

Breeding of *A. bisporus* is complicated because of its unusual secondarily homothallic life-cycle which differs at crucial points from the life-cycle of a 'text-book' basidiomycetous fungus (Raper *et al.*, 1972; Elliott, 1985b). Instead of forming basidia with four monokaryotic spores, the majority of the basidia produce two spores each containing two nuclei of opposite mating-type. These spores thus retain heterozygosity for most of the parental markers and after germination a fertile multinuclear heterokaryotic mycelium is formed. Such heterokaryotic offspring cannot be used directly in (out)crossing experiments. Approximately 2% of the *A. bisporus* basidia produce three or four spores (Summerbell *et al.*, 1989) and most of these will form homokaryotic mycelia upon germination. In typical

basidiomycetes, fertile heterokaryotic mycelium is usually binucleate and the binucleate nature is maintained by the formation of clamp connections (Elliott, 1985b). Clamp connections could therefore be diagnostic for heterokaryotic mycelium but, unfortunately, *A. bisporus* lacks clamp connections and, until recently, successful matings could only be recognised by differences in growth rate of the vegetative mycelium and laborious fruiting trials. Other features of the atypical basidiomycete *A. bisporus* are the multinuclear character of its mycelium and the absence of asexual spores, which make it difficult to generate mutants.

Together, these features made *A. bisporus* not very favourable among scientists and caused the application of recombinant DNA technology to be underdeveloped in mushroom research. This is reflected by the fact that the first reports of *A. bisporus* genes being cloned did not appear until 1992 (Harmsen *et al.*, 1992, Raguz *et al.*, 1992) and that a transformation system is not yet available, although integration of homologous DNA was reported in a mutant strain (Van de Rhee *et al.*, 1996a; 1996b).

Due to the relative difficulty of breeding of *A. bisporus*, most of the strains that are used nowadays for commercial cultivation are identical to or derived from only two "hybrid" strains, Horst®U1 and Horst®U3, which were developed by Fritsche (1983). The lack of genetic variation between the commercial mushroom strains forms a severe threat to the mushroom industry. The industry is sensitive to crop losses caused by bacterial and fungal infections and losses caused by 'spontaneous' degeneration processes leading to the formation of ill-shaped or malformed mushrooms (Sonnenberg *et al.*, 1995). To enlarge the genetic diversity and simultaneously improve the agronomic performance of the mushroom strains efficiently, a collection of wild *A. bisporus* strains must be systematically screened for industrially important traits using molecular techniques.

Towards a gene-based linkage map of *A. bisporus*

Efficient breeding can be achieved with a linkage map in which industrially important genetic traits are linked to well-defined genetic loci and a collection of strains with a large genetic diversity. Kerrigan *et al.* (1993) constructed a small linkage map of *A. bisporus*. For this linkage map a set of 52 haploid offspring and restriction fragment length polymorphisms (RFLP) of anonymous DNA fragments and randomly amplified polymorphic DNA (RAPD) markers were used. The value of this linkage map was demonstrated shortly thereafter with the localisation of the mating type locus (Xu *et al.*, 1993) and the locus determining the basidial spore number (Imbernon *et al.*, 1996) on the

largest linkage group. However, many of the markers of the RFLP/RAPD-based linkage map were located in highly repetitive loci. Furthermore, polymorphisms observed between parental homokaryons were often based on the presence or absence of amplified DNA fragments rather than on fragment length polymorphisms. Consequently, mapping results obtained with these unidentified markers were not always consistent (Sonnenberg *et al.*, 1996) and altogether this made the use of a linkage map based mainly on unidentified DNA fragments an unfavourable starting point for future breeding strategies.

Therefore a project was started which was aimed at creating a gene-based linkage map of the widely used *Agaricus bisporus* strain Horst®U1 (Schaap *et al.*, 1995b). The project involved the isolation and characterisation of genes and their use as well-defined genetic markers in linkage analyses. Chromosomal positions of markers can be identified by Southern analysis of size separated chromosomes. Using an optimised method of contour-clamped homogeneous electric field (CHEF) electrophoresis, 9 chromosomes of the *A. bisporus* genome were separated (Chapter 8, Sonnenberg *et al.*, 1996). Because of differences in length between homologous chromosomes of strains H39 and H97, the homokaryotic constituents of Horst®U1, 11 of the 13 chromosomes of *A. bisporus* could be resolved in this way. Markers for which a RFLP or a sequence mutation is found between strains H39 and H97, can be used for identification of homokaryotic single spore isolates among meiotic offspring using RFLP or allele specific amplified fragment (ASAF) techniques. In this way, markers can be assigned to linkage groups and the genetic distance between markers of the same linkage group can be determined. A combination of CHEF-analysis and linkage analysis would therefore form a sound basis for the development of a gene-based linkage map for the cultivated mushroom *A. bisporus*, provided that there are sufficient marker genes available.

The isolation of marker genes

One of the strategies that has been followed to generate a large number of well-defined gene markers was sequencing of random cDNA clones. As cDNA clones specify open reading frames of functional genes, the products of many of these expressed sequence tags (EST) could be identified easily by similarity comparison using database analysis. Among the identified ESTs were *s15A* (Schaap *et al.*, 1995a), *rs13A* and *l41A* all encoding ribosomal proteins, *htbA* and *hhfA* encoding core histone proteins, *tefA* encoding translation elongation factor 1α (Sonnenberg *et al.*, 1996), *aldA* encoding aldehyde dehydrogenase (Chapter 6) and *pruA* encoding pyrroline-5-carboxylate (P5C) dehydrogenase (Schaap *et al.*, 1997), an enzyme involved in the conversion of proline to glutamate. Besides

identification of ESTs, other cloning strategies were aimed at the isolation of genes whose products are involved in specific parts of the mushroom life-cycle.

Developmentally regulated genes of A. bisporus

One of our main objectives in the process of generating marker genes was the isolation of genes which are specifically involved in the development of fruit bodies. During commercial cultivation of *A. bisporus*, fruit body initiation and growth is controlled by empirically optimised manipulations of the environment. The molecular basis of this process is poorly understood. In our view, isolation and identification of genes which are expressed specifically during fruit body development is a first but important step towards elucidation of molecular mechanisms involved in fruit body development. Since not many developmentally regulated genes from the class of basidiomycetous fungi have been cloned and identified, isolation of fruit body specific genes from *A. bisporus* was executed by exploiting a cDNA library that was constructed of RNA isolated from fruit body primordia (De Groot *et al.*, 1995, 1996, 1997). In order to identify the functions of the gene products they encode, the DNA sequences of cloned fruit body specific genes will be subjected to database analyses. Additionally, expression studies will clarify whether (some of) the genes are co-regulated which would be indicative for a general control of transcription of these genes.

Compost utilisation genes

A second group of genes we were interested in were those encoding enzymes involved in degradation of the lignocellulosic fraction of compost. Elucidation of the regulation of these 'compost utilisation' genes will contribute to gain more insight in processes that are important for mushroom cultivation. Also, a better understanding of the enzyme activities involved might enable mushroom breeders to focus breeding strategies on mushroom strains which have high activities of those enzymes.

Knowledge on the degradation of xylan, one of the major compost components, by *A. bisporus* and basidiomycetes in general, is poor. In contrast to the field of basidiomycete fungi, cloning of multiple xylanase encoding genes has been reported for a number of ascomycetes and deuteromycetes including a number of industrially used *Aspergillus* spp. (Ito *et al.*, 1992a, 1992b; De Graaff *et al.*, 1994; MacCabe *et al.*, 1996). Cloning of *A. bisporus* xylanases was therefore attempted with heterologous hybridisation techniques using various *Aspergillus* genes as probes.

Housekeeping genes

A number of *A. bisporus* genes encoding key enzymes of carbon and nitrogen metabolism have been cloned. These so-called housekeeping genes are highly conserved throughout nature which enabled direct cloning using the isofunctional genes from other fungal sources and heterologous hybridisation techniques or polymerase chain reaction (PCR) approaches and degenerate primers.

For housekeeping genes involved in carbon metabolism, high overall levels of homology are observed between enzymes of different species and cloning of *A. bisporus* genes was performed with heterologous hybridisation techniques using the isofunctional genes of *Aspergillus niger* as probes. This resulted in the isolation of the genes encoding pyruvate kinase and phosphoglycerate kinase, both enzymes of the glycolytic pathway (Chapter 6) and in cloning of the gene encoding glucose-6-phosphate dehydrogenase, an enzyme active in the pentose phosphate pathway (Schaap *et al.*, 1995b). These housekeeping genes can be used to screen for metabolic differences between various parts of the fruit body.

The enzymes glutamine synthetase (*glnA*, Kersten *et al.*, 1997), $NADP^+$-glutamate dehydrogenase (*gdhA*, Schaap *et al.*, 1996) and NAD^+-glutamate dehydrogenase (*gdhB*, Schaap, unpublished results) are all involved in nitrogen metabolism and at least glutamine synthetase is directly involved in ammonium assimilation. Isolation of their corresponding genes thus enabled a study of the regulation of this important aspect of the nitrogen metabolism. Using highly conserved regions between the enzymes of different species, reverse genetic techniques were exploited for the cloning of these genes. Comparison of steady state transcript levels with enzyme activity measurements revealed that the levels of enzyme activity are mainly controlled by regulation of transcription of the corresponding genes. Glutamine synthetase activity is, however, also subject to posttranscriptional regulation (Kersten *et al.*, 1997). Northern analyses of *gdhA* and *glnA* showed that transcription of these genes is repressed by the presence of ammonium in the medium (Kersten *et al.*, 1997; Schaap *et al.*, 1996). For *gdhA*, the low mRNA levels found in the presence of ammonium were in agreement with $NADP^+$ glutamate dehydrogenase activity measurements (Baars *et al.*, 1995). The results are also in agreement with $NADP^+$ glutamate dehydrogenase enzyme activities found in the basidiomycetes *Schizophyllum commune* (Dennen & Niederpruem, 1964) and *Coprinus cinereus* (Stewart & Moore, 1974) but are contradictory to what has been found in ascomycetes (Pateman *et al.*, 1969) and suggest that $NADP^+$-GDH is not primarily involved in ammonium assimilation in these basidiomycetes (Schaap *et al.*, 1996).

Pyrroline-5-carboxylate (P5C) dehydrogenase is encoded by the fortuitously cloned *pruA* gene. This enzyme is involved in the second step of the conversion of proline to glutamate. Schaap *et al.* (1997) studied *pruA* expression in cultures grown on glutamate or proline as the sole nitrogen source and detected constant mRNA levels before and after addition of ammonium to proline or glutamate utilising cultures. These results suggest that ammonium and the amino acids proline and glutamate are equally preferred nitrogen sources for *A. bisporus* and this is consistent with earlier observations that *A. bisporus* continues to degrade protein and secrete ammonium even if ammonium and glucose are present in the culture medium (Kalisz *et al.*, 1987).

The architecture of *A. bisporus* genes

Characterisation of the structure of *A. bisporus* genes showed that all genes reported thus far and for which the genomic organisation was resolved are heavily spiked with small intervening sequences. The introns almost exclusively begin with the di-nucleotide GT and end with the di-nucleotide AG thereby following the consensus for spliceosomal introns. The introns are usually between 48-60 bp in length although larger introns have been found occasionally, e.g. in *gdhA* (Schaap *et al.*, 1996), *cel1* (Raguz *et al.*, 1992) and the *gpd* genes (Harmsen *et al.*, 1992). Comparison of the intron positions in the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase (Harmsen *et al.*, 1992) and $NADP^+$-dependent glutamate dehydrogenase (Schaap *et al.*, 1996) encoding genes with intron positions in other basidiomycetes and other fungi showed their use as phylogenetic indicators. However, more detailed information on the evolutionary relation of *Agaricus* to other fungi was obtained by comparing *gpd* amino acid sequences (Harmsen *et al.*, 1992), sequence comparison (Chapela *et al.*, 1994) and RFLP analysis (Bunyard *et al.*, 1996) of ribosomal DNA and analysis of mitochondrial DNA (Robinson *et al.*, 1997).

In the *A. bisporus* genes that have been reported thus far, gene duplication is frequent (Harmsen *et al.*, 1992; De Groot *et al.*, 1996; Van de Rhee *et al.*, 1996b; Smith *et al.*, 1998). In all cases the duplicated copies are immediately adjacent and in the same orientation, but only for the HYPA/HYPC and the laccase encoding genes, transcriptional activity of both copies was detected using RT-PCR techniques (De Groot *et al.*, 1996; Smith *et al.*, 1998).

Analysis of the codon usage of the reported *A. bisporus* genes, revealed that *A. bisporus* genes have a preference for C or T in the third base position. The six fold degenerate codons of arginine and leucine have a preference for C at the first base position while serine

codons preferably start with TC (see Chapter 6 for more details). For future research this information may be helpful when designing degenerative oligonucleotides.

Thus, the isolation of gene markers not only supplied tools for genome mapping purposes but also enabled us to investigate the architecture of *A. bisporus* genes and allowed us to study processes involved in different aspects of the mushroom metabolism using Northern analyses.

Aim and Outline

This thesis presents a molecular analysis of certain processes involved in growth and fruiting of the edible mushroom *A. bisporus*. A crucial step towards the construction of a gene-based linkage map for *A. bisporus* is the identification of a large number of genes. The isolation of genes that are only expressed during mushroom development will provide valuable research tools to study the genetics involved in this process. Furthermore, the isolation of genes coding for proteins involved in compost utilisation and enzymes from common biochemical routes enable us to study the regulation of those genes and to compare their structure with isofunctional genes from other organisms. Therefore, the aim of this study is to clone and characterise genes involved in various aspects of the life-cycle of *A. bisporus* using different approaches.

Chapters 2, 3 and 4 describe the isolation of genes that are specifically expressed during fruit body development using differential screening techniques. Cloning and characterisation of a highly expressed fruit body specific gene (*hypA*) encoding a hydrophobin, is reported in Chapter 2. Chapter 3 describes the isolation of additional fruit body specific genes and in Chapter 4 a second, differently regulated, fruit body specific hydrophobin encoding gene is characterised. In Chapter 5, the self-assembling nature of the HYPA hydrophobin was studied using Atomic Force Microscopy. Cloning of housekeeping genes involved in carbon metabolism using heterologous hybridisation techniques is reported in Chapter 6. The structures of the genes are analysed and the genes are also used to monitor metabolic routes in fruit bodies by studying their expression levels in various parts of mature mushrooms. In Chapter 7, cloning and characterisation of a compost specific endoxylanase encoding gene is described. Chapter 8 reports the isolation and identification of random cDNAs and their use as expressed sequence tags for the construction of a gene-based linkage map for *Agaricus bisporus*. Finally, the results presented in this thesis are summarised and discussed.

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2

The *Agaricus bisporus hypA* gene encodes a hydrophobin and specifically accumulates in peel tissue of mushroom caps during fruit body development

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Journal of Molecular Biology **257,** 1008-1018 (1996)

The *Agaricus bisporus hypA* gene encodes a hydrophobin and specifically accumulates in peel tissue of mushroom caps during fruit body development

Differential screening of a cDNA library was used to clone genes that are specifically expressed during mushroom development in the basidiomycete *Agaricus bisporus*. One of the isolated genes encodes a polypeptide of 112 amino acids and belongs to the fungal gene family encoding hydrophobins. This gene, *hypA*, has the characteristic pattern of eight cysteine residues at conserved positions and a hydrophobicity pattern that is very similar to class I hydrophobins. Elucidation of the genomic structure of *hypA* led to the identification of a second copy, *hypC*, located downstream of *hypA*. Although at a much lower level, *hypC* is like *hypA* specifically expressed in fruit bodies. The *hypA* mRNA level is transiently increased ten days after fruit body induction and expression appears to be associated with rapid expansion of the mushroom caps. In mushroom caps very high concentrations of *hypA* messengers were found in the (outer) peel tissue where they accumulate to more than 60% of the total mRNA mass. The corresponding protein with a molecular mass of 8 - 9 kDa was purified from this peel tissue and was identified by N-terminal sequencing. Our results suggest that HYPA forms a protective hydrophobic layer instrumental in cap formation.

Introduction

During fruiting of the edible mushroom *Agaricus bisporus* fruit bodies appear in ten to twelve day flushes and this process can be precisely controlled by empirically optimised manipulations of the environment (Van Gils, 1988). During the flushing cycles four stages of morphological appearance of fruit bodies are distinguished. In a twelve day flush, tiny pins appear synchronously five days after fruit body induction (stage 1). After ten days mushroom caps begin to expand. Shortly thereafter spores are visible in sectioned mushrooms and they now have the typical button like morphology (stage 2). In stage 3 and 4 the fruit body further expands and at stage 4 gills become visible from the outside (Hammond & Nichols, 1975; Figure 2.1(a)). The molecular basis of the processes underlying fructification, growth and differentiation, and maintenance of *A. bisporus* and other basidiomycetes is still largely unknown. Furthermore, our knowledge on the genetics of *A. bisporus* is still rather limited due to the aberrant secondarily homothallic life cycle in which the majority of the spores are fertile and contain two nuclei of opposite mating type (Raper *et al.*, 1972). Studies to get more insight in fructification and fruit body development in *A. bisporus* have, due to these reasons, been focused mainly on the biochemical and morphological differences between vegetative mycelium and fruit bodies.

The aim of this study was to isolate genes involved in fructification and fruit body development. We constructed a cDNA library of RNA isolated from primordia and young developing mushrooms and screened this library for highly expressed fruit body specific genes. One of the two fruit body specific genes that were isolated, *hypA*, was identified as a member of the fungal gene family encoding hydrophobic cell wall proteins.

Hydrophobins were discovered in three fungal classes. Of the basidiomycete *S. commune* three hydrophobin genes were cloned. Two of these genes, *Sc1* and *Sc4*, were very abundantly expressed in fruiting dikaryons, whereas *Sc3* was transcribed independent of fruiting (Schuren & Wessels, 1990). Sc3p and Sc4p proteins could be purified from cell wall fractions of emergent structures (Wessels *et al.*, 1991) and purified Sc3p was observed to self-assemble into a hot SDS-insoluble monolayer at hydrophilic/hydrophobic interfaces (Wösten *et al.*, 1993, 1994). In the class of ascomycetic fungi the *eas/ccg-2* gene of *Neurospora crassa* and the *Aspergillus nidulans rodA* and structurally different *dewA* genes encode components of the outer spore wall thereby contributing to spore wall hydrophobicity (Stringer *et al.*, 1991; Bell-Pedersen *et al.*, 1992; Lauter *et al.*, 1992; Stringer & Timberlake, 1995). Hydrophobins were also detected in two phytopathogenic (Stringer & Timberlake, 1993; Talbot *et al.*, 1993) and two entomopathogenic (St. Leger *et al.*, 1992; Bidochka *et al.*, 1995) species. In these deuteromycetes, hydrophobins seem to be involved in the elaboration of infection structures.

In this study we have elucidated the genomic structure of *hypA*. We show here that the *hypA* transcript accumulates in peel tissue of mushroom caps and we have purified the corresponding protein. In conjunction with the self-assembling behaviour of hydrophobins (Wösten *et al.*, 1994), these results suggest that HYPA assembles into a sheath surrounding the mushroom cap thereby protecting the mushroom against bacterial infection and other environmental influences.

Materials and Methods

Strains and culture conditions

Agaricus bisporus strain Horst®U1 and its homokaryotic constituents H39 and H97 were grown as mycelial mats in DT80 medium (Sonnenberg *et al.*, 1988), supplemented with 3% glucose or 2% xylan as carbon source. After 14 days of growth at 24°C, the mycelium was harvested by filtration over nylon gauze, immediately frozen in liquid nitrogen, and stored at -70°C. In order to obtain *Agaricus bisporus* Horst®U1 fruit bodies of different growth stages, this strain was grown at the Mushroom Experimental Station on compost covered with a casing soil. Primordia and mushrooms were harvested for seven days (day six to twelve) after fruit body induction in the first flush (Van Gils, 1988) corresponding with stages 1 to 4 of the 7 developmental stages defined by Hammond & Nichols (1975). After immediate freezing into liquid nitrogen, fruit bodies were stored at -70°C.

Some stage 4 fruit bodies (mushrooms) were separated into pileus, stipe, gill and peel tissue before they were frozen.

Escherichia coli XL1-blue MRF' and XL1-blue cells (Stratagene) were used for initial plating of the packaged cDNA constructs and *in vivo* excision of cloned cDNA inserts. *E.coli* LE392 (Promega) was used for phage amplification and λ DNA isolation. *E. coli* DH5α (Bethesda Research Laboratories) was used for plasmid transformation and propagation.

Recombinant DNA techniques and enzymes

Plasmid pUC19 (Yanisch-Perron *et al.*, 1985) was used as cloning vector for genomic DNA fragments. For the cloning of PCR products the pGEM-T vector system of Promega was used.

Standard DNA manipulations were carried out essentially as described by Sambrook *et al.* (1989). Restriction enzymes and other enzymes used for DNA manipulations were purchased from BRL and used according to the supplier's instructions.

Construction of the pin stage cDNA library

A cDNA expression library was constructed from poly(A) enriched RNA from pin stage mushrooms that were picked eight days after fruit body induction. Total RNA was isolated by extraction of RNA using guanidium thiocyanate followed by centrifugation through a cesium chloride cushion (Sambrook *et al.*, 1989). Poly(A) enriched RNA was purified from total RNA using an oligo(dT)-cellulose column (Promega). The cDNA expression library was constructed using the ZAP-cDNA® Synthesis Kit (Stratagene) following the manufacturer's instructions, except that packaging was performed using a packaging extract from Promega. About 2 x 10^6 transformants per µg of double stranded cDNA were obtained.

Selection of fruit body induced clones

A two step screening procedure was used to select fruit body induced clones. First we screened for clones that were highly expressed in pin stage mushrooms. A part of the cDNA library was randomly excised and 100 of the resulting colonies were transferred to a Hybond-N (Amersham) transfer membrane and lysed. The membrane was then hybridised with a cDNA probe that was prepared by labelling total first strand pin stage cDNA with [α-^{32}P] dATP using the method of Feinberg & Vogelstein (1983). Fruit body induced clones among the selected highly expressed cDNA clones were detected by comparing the steady-state mRNA levels in fruit bodies and vegetative growing mycelium of Horst®U1 of each of the individual clones by Northern analysis.

Total RNA isolation and Northern analysis

Total RNA for Northern analysis was isolated from fruit body and mycelium samples using TRIzol™ Reagent (BRL). The concentration of the RNA samples was determined spectrophotometrically and equal amounts of RNA were denatured in 10 x SSC and 6.15 M formaldehyde and spotted on Hybond-N membrane, or denatured using glyoxal and DMSO by standard techniques, separated on a 1.6% agarose gel and transferred to Hybond-N membrane. Messenger RNA lengths were determined by co-electrophoresis of RNA molecular mass markers (BRL) that were stained separately after transfer with methylene blue (Sambrook *et al.*, 1989). For Northern analysis of the individual cDNA clones, complete inserts were labelled and used as probes. Hybridisation was performed at 65°C in standard hybridisation buffer (SHB; 6 x SSC, 5 x Denhardt's solution, 0.5% SDS and 100 µg ml^{-1} denatured herring sperm DNA) and stringently washed (65°C,

0.1 x SSC, 0.1% SDS). When appropriate Northern blots were scanned using an Ultroscan XL laser densitometer (LKB).

Isolation of total genomic DNA, Southern blot analysis, cloning and sequencing of *hypA* and *hypC*

Total DNA from strains H39 and H97 was isolated according to the method of De Graaff *et al.* (1988), digested with various restriction enzymes, separated on 0.8% agarose gels and transferred onto Hybond-N membranes (Amersham). CHEF electrophoresis of intact chromosomal DNA of H39 and H97 was performed as described by Sonnenberg *et al.* (1991) using a Biorad CHEF DrII system. The chromosomes were transferred by capillary blotting to a Hybond-N membrane. The membranes were hybridised overnight at 65°C in SHB using a *hypA* cDNA clone as probe. The membranes were stringently washed at 65°C and exposed.

Genomic sequences of the *hypA* and *hypC* genes were obtained by screening a λEMBL4 genomic library of *A. bisporus* strain H39 by standard methods using the same *hypA* cDNA probe. Cloned hybridising fragments, cloned PCR products and cDNA clones were sequenced by the method of Sanger *et al.* (1977) using a T7 sequencing Kit (Pharmacia) and standard and sequence specific oligonucleotides.

Nucleotide and protein sequence comparisons

The sequence data from this article have been deposited with the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X89242 and X90818 for *hypA* cDNA and *hypA*/*hypC* genomic sequences respectively.

The nucleotide sequences were compared using the University of Wisconsin Genetics Computer Group (GCG) program BESTFIT (Devereux *et al.*, 1984). The predicted *hypA* and *hypC* amino acid sequences were compared with translated sequences from GenBank and EMBL using the program BLASTX (Gish & States, 1993).

Amino acid alignments were made using the GCG programs PRETTY and PILEUP. Hydrophobicity plots were generated with the program DNA Strider Version 1.1 (Marck, 1988) based on the Kyte-Doolittle algorithm with a window size of seven (Kyte & Doolittle, 1982).

Comparison of *hypA* and *hypC* mRNA levels by competitive PCR and amplification of *hypC* cDNA products

Comparison of *hypA* and *hypC* transcript levels by competitive PCR was accomplished using as template double stranded cDNA also used for the cDNA library and cDNA obtained from xylan-grown mycelium. Reactions were performed with a common *hypA*/*hypC* primer in antisense direction and a sense *hypA* specific primer positioned over intron 1 and/or a sense *hypC* specific primer positioned over intron 2 (Figure 2.5). For cloning of *hypC* specific cDNA products, the common primer was also used in a PCR reaction with a second *hypC* specific primer (primer 4). Amplification was achieved using 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C and 1 min extension at 72°C, followed by a final cycle of 5 min at 72°C. Products of competitive PCR were separated on a 1.5% agarose gel and analysed by Southern blotting using a *hypC* specific probe.

Purification and sequencing of the HYPA protein

HYPA was purified from a hot SDS-insoluble fraction of peel tissue of stage 4 mushrooms according to De Vries *et al.* (1993). After trifluoroacetic acid (TFA) extraction, TFA was removed by evaporation under a stream of air. The TFA extracted proteins were dissolved in 0.1 M Tris pH 7.0 and analysed by SDS-PAGE using 15% polyacrylamide gels (Laemmli, 1970). Protein molecular mass markers were obtained from Serva. For N-terminal sequencing, the thus purified protein was dialysed against water and lyophilised. Amino acid sequences were determined using a gas phase sequenator equipped with a PTH analyser as described by Amons (1987).

Results

Isolation of fruit body induced genes

For rapid isolation of cDNA clones encoding genes that were either highly induced or specifically expressed in fruit bodies of *A. bisporus* we have constructed a cDNA library of pin stage mushrooms. This cDNA library was subsequently screened in a two step selection procedure as described in Materials and Methods.

The first step resulted in the selection of eleven clones that are abundantly present in the cDNA library (results not shown) and these clones were subsequently screened for their fruit body linkage (Figure 2.1). As expected, all clones showed high transcript levels at pin stage. Surprisingly, only one clone, pDG71, was also abundantly expressed in vegetative mycelium. The other ten clones appeared to have very low expression levels in vegetative mycelium. Based on the relative signal strength observed in these three stages of development, the fruit body induced clones could be divided into two groups. Six clones (group A) appeared to have equal transcript levels in pin stage and stage 4 sporophores (Figure 2.1(b)) and these clones were shown to contain highly homologous cDNA sequences by DNA hybridisation (data not shown) and did not cross-hybridise with the four remaining B type clones.

Comparison of the protein bank databases with the deduced amino acid sequence of a typical group A clone, pDG04, showed that this clone encodes a putative polypeptide that shows homology with a class of developmental genes encoding fungal cell wall proteins having hydrophobic properties. We therefore named the gene *hypA*.

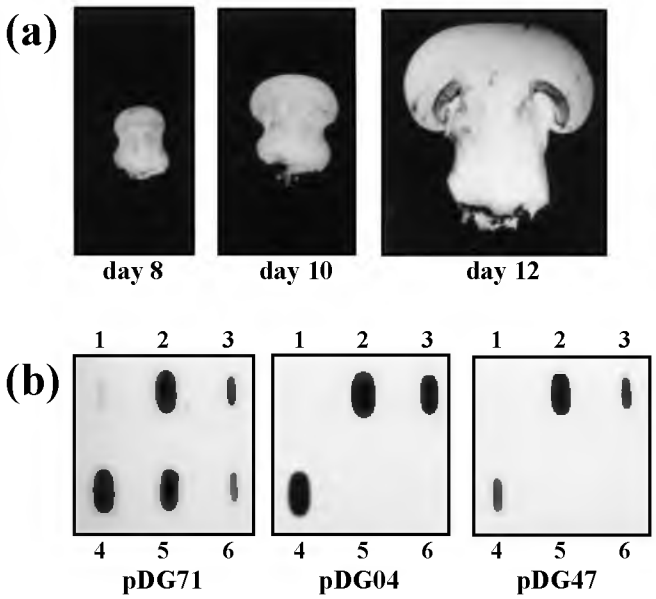

Figure 2.1. Progressive morphology of fruit bodies and developmental regulation of genes highly expressed at pin stage. (a) Typical appearance of sectioned fruit bodies eight, ten and twelve days after fruit body induction in a twelve day flushing cycle. Day eight fruit bodies (pin stage) are in between stage 1 and 2, day ten fruit bodies are almost at stage 2, while day twelve mushrooms have reached stage 4. (b) Slot blots probed with the constitutively expressed clone pDG71, an A type clone (pDG04) and a B type clone (pDG47), respectively. (slot 1) Control sample without RNA; (slots 2 and 3) 5 µg and 0.05 µg pin stage RNA; (slot 4) 5 µg stage 4 mushrooms RNA; (slots 5 and 6) 5 µg and 0.05 µg glucose-grown mycelial RNA.

Chromosomal location of the *hypA* locus

The chromosomal location of the *hypA* gene was determined by Southern analysis of Clamped Homogeneous Electric Field (CHEF) resolved chromosomes of strains H39 and H97, the homokaryotic constituents of the commercial strain Horst®U1. The *hypA* gene was assigned to either chromosome III or IV of both strains (Figure 2.2(a)). Since these two chromosomes were not separable by CHEF-analysis, definite assignment of *hypA* to either of these chromosomes has to await the isolation of more genetic markers to establish linkage.

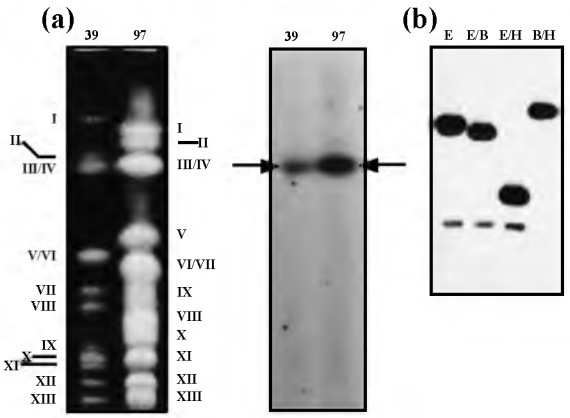

Figure 2.2. Genomic organisation of *hypA*. (a) Assignment of *hypA* to chromosome III or IV. *A. bisporus* chromosomes were isolated from the homokaryotic strains H39 and H97, fractionated by CHEF gel electrophoresis, stained with ethidium bromide (left panel) and hybridised with *hypA* (right panel). Chromosomes are identified by Roman numerals and numbered according to Sonnenberg *et al.* (1991). (b) Hybridisation of a *hypA* λ-clone with *hypA* cDNA. Fragments were separated on a 0.8% agarose gel. The following digestions were performed: (E) *Eco*RI; (E/B) *Eco*RI/*Bam*HI; (E/H) *Eco*RI/*Hin*dIII; (B/H) *Bam*HI/*Hin*dIII.

Cloning and primary structure of *hypA* genomic sequences

Two *hypA* λ-clones were isolated by standard methods from a genomic library of strain H39 using the insert of type A plasmid pDG04 as a probe. Both λ-clones and genomic DNA of strains H39 and H97 were subjected to Southern analysis. The two λ-clones showed identical hybridisation patterns when the insert of pDG04 was used as a probe. These restriction patterns were also found for both strains when the Southern blot with digested genomic DNA was hybridised with the same probe.

Two fragments of λ_1, a strongly hybridising 1.8 kb *Eco*RI-*Hin*dIII fragment and a weaker hybridising 1.4 kb *Eco*RI fragment were isolated and cloned (Figure 2.2(b)). Surprisingly, the complete coding region of *hypA* was found on the 1.8 kb *Eco*RI-*Hin*dIII fragment whereas hybridisation patterns indicated that *hypA* homologous sequences were positioned on both cloned fragments. The 1.4 kb *Eco*RI fragment, however, was found to comprise sequences showing a high degree of homology to *hypA* and regulatory sequences. These related sequences presumably encode another hydrophobic cell wall protein which was tentatively called *hypC* (for copy).

To determine the orientations and the relative positions of *hypA* and *hypC* we additionally cloned and sequenced a 4.1 kb *Bgl*II genomic fragment encompassing both *hypA* and *hypC* sequences. Sequence analysis of this clone proved that the 1.8 kb *Eco*RI-*Hin*dIII and the 1.4 kb *Eco*RI fragments are immediately adjacent and share an *Eco*RI site. The *hypA* and the putative *hypC* gene have the same orientation and are separated by a 2.4 kb intergenic region (Figure 2.3).

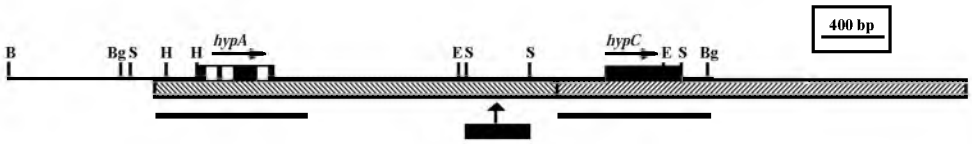

Figure 2.3. Molecular map of the *hypA* and *hypC* region. The coding regions of *hypA* and *hypC* are represented by black boxes. Introns in the coding region of the *hypA* transcript are shown by open boxes. Arrows indicate orientation of transcription. Duplicated parts in the sequenced 4142 bp *Bgl*II fragment are shown by thick lines. Striped boxes represent the duplicated 2.8 - 2.9 kb fragment. The black box underneath the map indicates the 447 bp *Sal*I fragment that was used as a probe to determine the extend of the duplication. The following restriction sites are indicated: (B) *Bam*HI; (Bg) *Bgl*II; (E) *Eco*RI; (H) *Hin*dIII; (S) *Sal*I.

Translation of *hypA* cDNA and genomic sequences starting at the first ATG in the reading frame led to the deduction of the amino acid sequence shown in Figure 2.4(a). The polypeptide consists of 112 amino acids with a molecular mass of 11,190. The coding region is interrupted by three small introns of 53, 56 and 57 bp respectively. The putative amino acid sequence and intron positions of the duplicated *hypC* sequence were deduced by comparison with *hypA* cDNA and genomic sequences. This open reading frame codes for a slightly larger polypeptide of 115 amino acids with a molecular mass of 11,471.

To determine the exact level of homology between *hypA* and *hypC* sequences and surroundings, we aligned the relevant parts of the sequenced *Bgl*II fragment (Figure 2.5). The homology between the *hypA* and *hypC* sequences starts 311 bp upstream of the *hypC* start codon and probably extends beyond the *Bgl*II site located downstream of the putative *hypC* gene. When the *hypA* coding sequences were compared with the putative *hypC* coding sequences we found 84% homology at the nucleotide level. Deviations appeared to be random since there was no bias observed towards third basepair mutations. The high level of homology further extends to the promoter regions, the introns and the 3' noncoding sequence of *hypA* and *hypC*.

(a)

```
                                                                *       **
A. bisporus  hypA   ..MISRVLVAALVALPALVTATPAPGKPK..........ASSQCDVGEIH..CCDTQQTPDHT
A. bisporus  hypC   ..MFSRVLVAALVALPVLVSASPTPGGYP.......DSTTVVSQCNVGELH..CCNTQQTPDHT
S. commune   Sc1    ....MRFSLAILALPVLAAATAVPRGG..............ASKCNSGPVQ..CCNTLVDTKDK
S. commune   Sc3    MFARLPVVFLYAFVAFGALVAALPGGHPGTTYPPSTTTIAAGGTCTTGSLS..CCNQVQSASSS
S. commune   Sc4    .....MRFSLALLALPALAAAAPVPGGGK..........GAGQACNSGPVQ..CCNETTTVANA
N. crassa    eas    ...MQFTSVFTILAIAMTAAAAPAEVVPR.......ATTIGPNTCSIDDYKPYCCQSMSGPAGS
Consensus                                    G------------------C--G-----CCN--------

                          *             *      **                *
hypA   SAAA..SGLLGVPIN.LGAFLGFDCTPISVLGV.GGNNCAAQPV.CCT.G.NQFTA....LINALDCSPVNVNL
hypC   NAAG...GLLGAAAN.VGALLGFDCTPISVIGI.GGNNCAAQPV.CCE.A.NEFTG....LINALSCSPINVNL
Sc1    HQTNIVGALLGLDLGSLTGLAGVNCSPVSVIGV.GGNSCSTQTV.CCE.G.TQFNG....LVN.VGCTPINVGL
Sc3    PVTALL.GLLGIVLSDLNVLVGISCSPLTVIGV.GGSGCSAQTV.CCE.N.TQFNG....LIN.IGCTPINIL.
Sc4    QKQGLLGGLLGVVVGPITGLVGLNCSPISVVGVLTGNSCTAQTV.CCD.HVTQ.NG....LVN.VGCTPISL..
eas    ..PGLLN.LIPVDLS..ASL.G..C....VVGVIGSG.CGA.SVKCCKDDVTN.TGNSFLIINAANCVA.....
       -------GLLG--------L-G--C-P-SV-GV-GGN-C-AQ-V-CC-----QF-G----LIN---C-PIN---
```

(b)

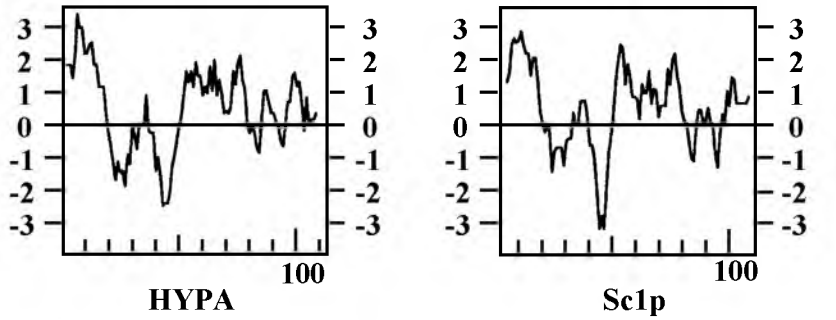

Figure 2.4. Comparison of the translated polypeptide sequences encoded by *hypA* and *hypC* with other class I hydrophobins. (a) Alignment of the inferred *hypA* and *hypC* amino acid sequences to those of *S. commune Sc1*, *Sc3* and *Sc4* (Schuren & Wessels, 1990) and *N. crassa eas* (Bell-Pedersen *et al.*, 1992; Lauter *et al.*, 1992). The consensus sequence is composed by residues shared by at least four of the (predicted) mature proteins. The determined N-terminal amino acids of HYPA, Sc3p and Sc4p are underlined. The eight conserved cysteines are marked by asterisks. (b) Hydrophobicity plots of deduced amino acid sequences of HYPA and Sc1p. Points above the x-axis indicate hydrophobic regions.

Since the results suggested that the *hypC* gene and surroundings were the result of a recent duplication event involving a 2.8 - 2.9 kb DNA fragment, this was further investigated by Southern analysis. A 447 bp *Sal*I fragment, which is located downstream of *hypA* and adjacent to the duplicated sequence (Figure 2.3), was used as a probe on genomic DNA of strains H39 en H97 which was digested with *Bgl*II and *Sal*I. Hybridisation patterns showed that besides expected 447 bp *Sal*I and 4.1 kb *Bgl*II fragments also additional weaker hybridising fragments downstream of *hypC* hybridised to the 447 bp *Sal*I probe (results not shown). This indicated that the duplicated sequence encompasses the complete 2.8 - 2.9 kb fragment.

The deduced amino acid sequences of *hypA* and *hypC* were compared with the deduced amino acid sequences of analogous proteins of fungal origin. The amino acid alignment showed that the HYPA protein is of the same size as the already described hydrophobins. All proteins have an aliphatic N-terminal amino acid sequence and contain eight cysteine residues at conserved positions of which four occur in pairs. The overall homology between HYPA and the other hydrophobins is low. The highest level of homology (42%) was found with Sc1p, a fruit body specific hydrophobin from *Schizophyllum commune*

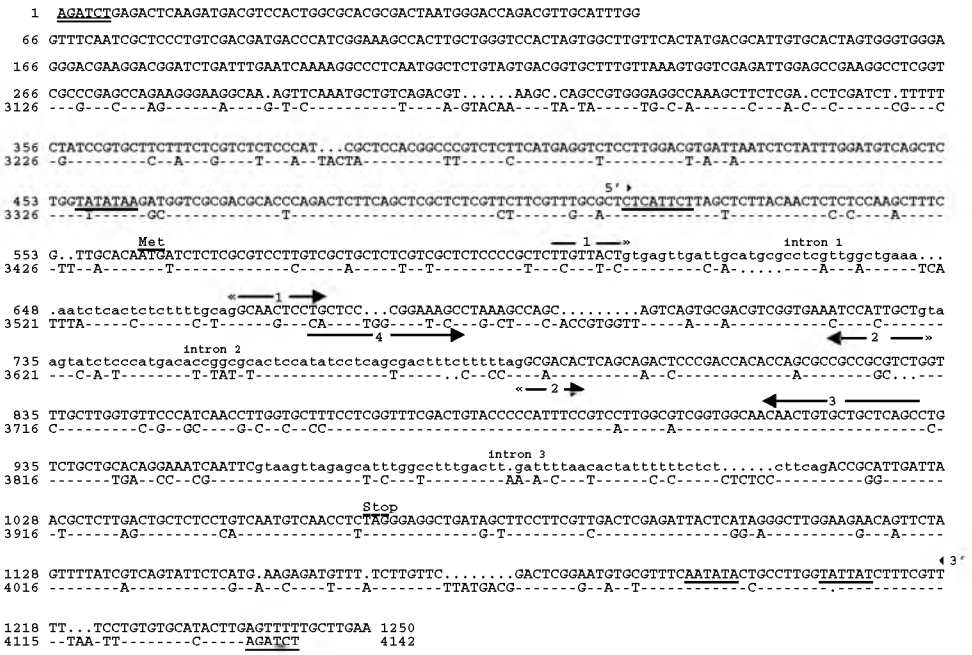

Figure 2.5. Alignment of homologous sequences within the 4.1 kb *Bgl*II fragment. Numbering starts at the first nt of the 5' *Bgl*II recognition site. *Bgl*II restriction sites are double underlined. The complete *hypA* and flanking sequences are shown (upper row). The duplicated region starts around nt 3126 and is aligned with the first 1250 nt starting at nt 266. Identical nucleotides are indicated by dashes in the duplicated sequence (lower row). Gaps are indicated by dots. Translation start codon and stop codon of *hypA* are indicated. Intervening sequences in *hypA* are shown in lowercase letters. A putative TATA-box, putative polyadenylation recognition sites and a putative Inr core minimal promoter sequence (Adams & Timberlake, 1990) are underlined. The 5' ultimate base of the longest *hypA* cDNAs is marked by 5' ▸ and the 3' ultimate base by ◂ 3'. Primers used in PCR experiments are indicated by numbered arrows.

(

Figure 2.4(a)). Of the lower fungi, the *ccg-2/eas* protein of *Neurospora crassa* showed the highest level of homology (30%). However, the hydrophobicity pattern of HYPA was very similar to the hydrophobicity patterns of these class I hydrophobins (Stringer & Timberlake 1995). This is exemplified by the almost identical hydrophobicity patterns of HYPA and Sc1p (Figure 2.4(b)).

Regulation of *hypA* expression

The transcriptional regulation of the *hypA* gene was studied more extensively by Northern analysis using 5 µg of total RNA isolated from whole fruit bodies picked during the first flush six to twelve days after fruit body induction and from pileus, stipe, peel and a mixture of gill and basidia tissue from stage 4 mushrooms. In addition, RNA isolated from vegetative mycelium grown on xylan was used as a control. After hybridisation of the Northern blots with a *hypA* cDNA clone, the amount of rRNA in each lane was examined by rehybridisation with a part of the *A. bisporus* 28S rDNA repeat (Schaap *et al.*, 1996) (Figure 2.6(a) and 2.6(b)). After densitometric scanning of the obtained signals from both hybridisations, the *hypA* mRNA levels were estimated relative to the *hypA* mRNA level found in whole stage 4 mushrooms.

As in glucose-grown mycelium (Figure 2.1(b)), no detectable amount of *hypA* mRNA was observed in xylan-grown mycelium (Figure 2.6), whereas *hypA* transcripts of about 600 nt were very abundant from day 6 (stage 1) to day 12 (stage 4) during sporophore development. However, a substantial transient increase of *hypA* mRNA was observed at day 10 (Figure 2.6(c)). Northern analysis on different parts of stage 4 mushrooms showed that *hypA* mRNA accumulated in very large amounts in the peel of the mushroom caps (Figure 2.6(d)) whereas very low *hypA* mRNA levels were observed in stipe tissue.

Competitive PCR between *hypA* and *hypC*

Since the 5' end of the duplication extends to 295 bp before the translation start site of *hypA*, we wanted to know if the second copy was an active gene, and if so how the mRNA levels of the two genes were related. Therefore the cDNA library was screened for *hypC* cDNAs with a *hypC* genomic probe. However only phages derived from *hypA* transcripts, which are 84% homologous to putative *hypC* transcripts, were found. This suggested that either *hypC* is a pseudo gene or that *hypC* is transcribed at much lower levels than *hypA* due to loss of essential promoter elements in the duplication. The high level of homology

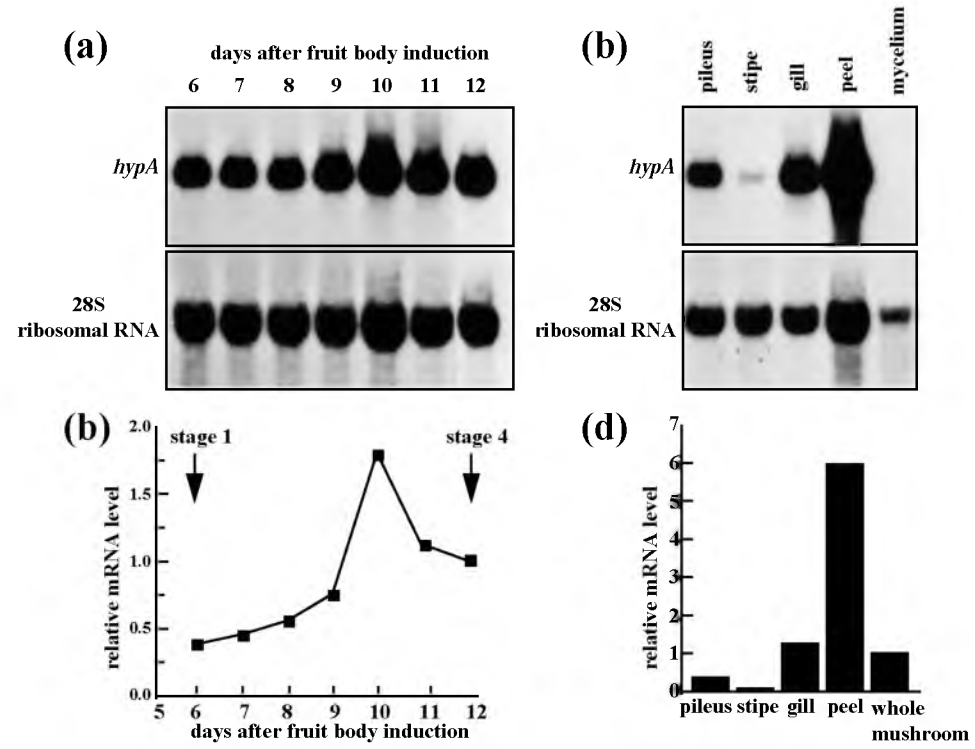

Figure 2.6. Transcript levels of *hypA* in developing fruit bodies. (a) Northern blots showing the levels of *hypA* mRNA and 28S ribosomal RNA in mushrooms harvested at day 6 (stage 1) to day 12 (stage 4) during fruit body development and (b) in different parts of stage 4 mushrooms and in xylan-grown mycelium (indicated above the lanes). (c) Graphs of relative *hypA* mRNA level during mushroom development and (d) in different parts of stage 4 mushrooms. Transcript levels were normalised against 28S ribosomal RNA and the mRNA level obtained for stage 4 whole mushrooms was set to 1.0. The *hypA* hybridisation signal of the xylan-grown mycelium sample was below the detection limit.

between *hypA* and *hypC* also made it difficult to study *hypC* expression by Northern analysis. Therefore, we compared the *hypA* and *hypC* transcript levels by competitive PCR using three oligonucleotides: A sense *hypA* specific primer annealing over intron 1 (primer 1), a sense *hypC* specific primer annealing over intron 2 (primer 2) and a common *hypA*/*hypC* primer (primer 3) annealing in antisense direction (Figure 2.5). In this way we could exclude unwanted PCR products by priming on residual genomic DNA. Double stranded cDNA synthesised from poly(A) enriched RNA isolated from pin stage mushrooms

or mycelium grown on xylan was used as a template in the PCR reaction. Amplification of pin stage cDNA with primers 1 and 3 resulted in a *hypA* specific product of 215 bp, while a *hypC* specific product of 151 bp was obtained if primers 2 and 3 were used. However, when all three primers were added in equal amounts in a competitive PCR reaction, the 215 bp *hypA* product was the major amplified product whereas a very low amount of the 151 bp *hypC* product was formed. When xylan-grown mycelial cDNA was added as template and the same conditions were used, *hypA* and *hypC* transcripts were hardly visible on an ethidium bromide stained agarose gel. The amplified fragments were visualised by hybridisation with a *hypC* specific cDNA probe (Figure 2.7).

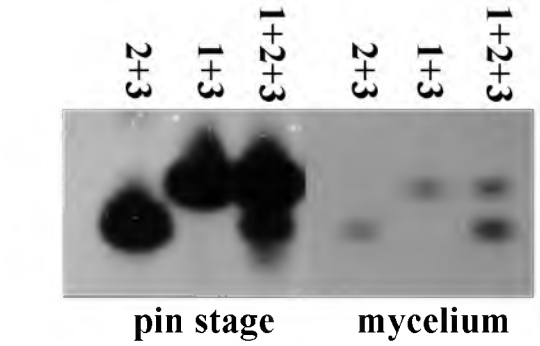

Figure 2.7. Analysis of *hypA* and *hypC* relative transcript levels by competitive PCR. Amplification was performed using 1 ng total pin stage cDNA or 1 ng total xylan-grown mycelial cDNA. A *hypA* specific primer (primer 1) and/or *hypC* specific primer (primer 2) were used together with a *hypA*/*hypC* common primer (primer 3). PCR products were separated on a 1.5% agarose gel and hybridised with the 151 bp *hypC* PCR product. Primer combinations are shown above the lanes.

To finally prove that *hypC* is an active gene, a second *hypC* specific fragment was amplified using a different *hypC* specific primer located between intron 1 and 2 (Figure 2.5, primer 4), resulting in a PCR product of 209 bp. Both *hypC* products were cloned and sequenced and analysis confirmed that they were amplified from *hypC* cDNA sequences. In this way we could also prove that the second intron of *hypC* is spliced at the same position as the second intron of *hypA*.

Purification of the HYPA protein

Hydrophobin aggregates from *S. commune* are insoluble in hot SDS, but can be solubilised in trifluoroacetic acid (TFA) (Wessels *et al.*, 1991). These properties were used to purify the *Agaricus bisporus* hydrophobic protein encoded by *hypA* from peel tissue of the mushroom cap. After TFA extraction of the hot SDS insoluble fraction we obtained a single protein band on SDS-PAGE with a molecular mass of 8 - 9 kDa (Figure 2.8). This protein was sequenced from its N-terminus resulting in the sequence ^{24}g-K-P-K-A-S-S-Q-?-d-v-g-E-I^{37} (smaller case letters indicate amino acid residues that were determined with some uncertainty). This amino acid sequence perfectly matched the deduced amino acid sequence of the *hypA* gene after cleaving an N-terminal signal peptide of 23 amino acids. The mature HYPA peptide should then have a molecular mass of 8,933.

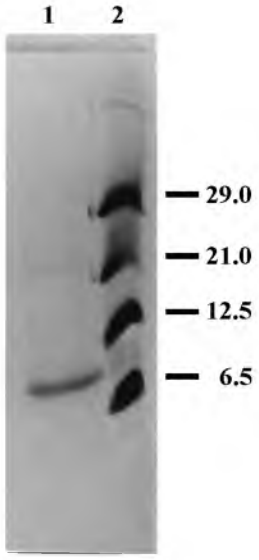

Figure 2.8. Analysis of TFA extracted protein from peel tissue of fruit body caps by SDS-PAGE. Lane 1, TFA extracted protein from peel tissue of fruit body caps; lane 2, protein molecular mass markers (Serva). Relative molecular mass of marker proteins are indicated in kDa.

Discussion

We have developed a screening procedure for the detection of *A. bisporus* genes that are either specifically expressed in fruit bodies or specifically expressed at the pin stage of development. One of these genes was, based on the characteristics of the protein it encodes, identified as a hydrophobin encoding gene.

Highest transcript levels of this gene, *hypA*, were found late in the development of mushrooms indicating a role for this gene in expansion and/or maintenance of the fruit body rather than development. By analysis of the genomic structure of *hypA* and its flanking regions, a second copy of *hypA* was found in the same orientation located 2.4 kb downstream of *hypA*. This gene, *hypC*, appeared to be the result of a 'recent' duplication event since the high level of homology was not only found in the coding sequences but also extended to the introns and 5' and 3' flanking regions. In a competitive PCR experiment, substantially more *hypC* messengers were detected in pin stage mushrooms than in mycelium, showing that *hypC* is an active gene and developmentally regulated.

Comparison of the size of the *hypA* transcript as detected by Northern analysis and the length of the inserts of the isolated cDNA clones indicated that the clones contained full length cDNAs. The longest five of the six sequenced cDNA clones all started with the sequence ^{517}CTCATTCT^{524} (Figure 2.5). This sequence exactly matches with the core minimal promoter Inr element found in a number of developmentally regulated *A. nidulans* genes and which in those cases overlaps the transcription initiation site (Adams & Timberlake, 1990). Approximately 95 bp upstream of the *hypA* and *hypC* translation start sites putative TATA signals (Nussinov, 1990) were found and both translation start sites are preceded by an A at position (-3) which is in accordance with the consensus translation start sequence for highly expressed genes (Kozak, 1989). The 5' donor and 3' splice acceptor sites of *hypA* and *hypC* conform to the consensus sequences for fungal splice junctions (Unkles, 1992). Putative polyadenylation signals (Humphrey & Proudfoot, 1988) were also found in both sequences 5' adjacent to the polyadenylation site.

Competitive PCR between *hypA* and *hypC* cDNAs showed that only a very small fraction of the hydrophobin transcripts that appear during fruiting of the mushroom are derived from *hypC*. Furthermore, differences between the *hypA* and *hypC* coding regions are not biased towards third base pair changes and appear to occur randomly. These findings suggest that *hypC* expression may not be relevant for normal fruit body development. The duplication starts about 310 bp upstream of the *hypC* translation start indicating that the low activity of the *hypC* gene could be caused by loss of (an) essential promoter element(s) located further

upstream. The promoter of the circadian clock-controlled *ccg-2/eas* hydrophobin of *N. crassa* contains *cis*-acting elements located at least 625 bp upstream of the transcription unit required for normal expression (Bell-Pedersen *et al.*, 1992). Lauter *et al.* (1992) showed that an insertion between 1.0 and 1.5 kb upstream of the *eas* transcription initiation site greatly reduced the activity of the *eas* gene. Like *hypC*, the *eas* gene of this mutant strain was still developmentally regulated.

Comparison of the translated sequence of the *hypA* gene with protein bank databases showed highest homology for HYPA with the putative Sc1p protein of the hydrophobin family of the basidiomycete *S. commune*. The *Sc1* and *Sc4* genes are only expressed at high levels in fruiting dikaryons whereas Sc3p accumulates in specific cell wall structures of dikaryons and monokaryons and is involved in the formation of aerial hyphae (Schuren & Wessels, 1990). A hot SDS insoluble protein with an apparent molecular mass of 16 kDa could be extracted with TFA from aerial hyphae of *A. bisporus* (De Vries *et al.*, 1993). In this stage of the life cycle *hypA* is not expressed and this, together with the difference in apparent molecular mass with HYPA, indicates that more hydrophobins are present in different tissues and/or stages of the life cycle of *A. bisporus*.

HypA transcript levels were elevated in mushrooms picked at day ten of the flushing cycle and a very high concentration of *hypA* messengers was found particularly in the peel of mushroom caps. Since six of the originally screened pin stage (day eight) cDNA clones appeared to be *hypA* clones, we assumed that about 6% of the messengers at this stage encodes HYPA. The same percentage of *hypA* cDNA containing phages was found in a more extensive plaque screening for *hypC* cDNA containing phages. Similar high mRNA levels were found for *Sc1* and *Sc4* in the fruiting dikaryon of *S. commune* (Mulder & Wessels, 1986). Six percent *hypA* messengers at day eight would mean that approximately 20% of the messenger population of a day ten mushroom codes for HYPA and that *hypA* transcripts accumulate to more than 60% of the total mRNA population in stage 4 peel tissue cells. Furthermore, a band of about 600 nt was clearly visible in an ethidium bromide stained gel of total RNA of peel tissue. This suggests that HYPA is the major protein of the peel of the mushroom cap. The accumulation of *hypA* transcripts in the peel is in accordance with the increase of *hypA* transcripts observed at day ten. At this timepoint the mushroom cap starts to enlarge almost spherically while the stipe elongates (Figure 2.1(a)).

A high hydrophobin concentration in the peel of the mushroom cap is also in agreement with a proposed role for hydrophobins in the formation of a hydrophobic coating surrounding the (cap of the) fruit body. From previous work it is known that hydrophobin proteins self-assemble into an amphipathic layer at hydrophobic/hydrophilic phase

transitions (Wösten *et al.*, 1993). The transition from the hydrophilic exterior of the emergent fruit body to hydrophobic air is therefore thought to trigger self-assembly of hydrophobins on developing fruit bodies, thereby generating a hydrophobic sheath around the mushroom cap. Assembled hydrophobin proteins were found to form a hydrophobic coating surrounding spores of some ascomycetic fungi, as was exemplified in an *A. nidulans* mutant strain that was deficient for *rodA* encoding a hydrophobin-like rodlet protein. This strain lacked a rodlet layer resulting in easily wettable spores (Stringer *et al.*, 1991).

Northern analysis showed that the hydrophobin mRNA transcripts were concentrated in the peel of the mushroom caps and following the procedure described for the purification of hydrophobins from *S. commune*, a hot SDS resistant protein with a molecular mass of 8 - 9 kDa was obtained from peel tissue (Figure 2.8). N-terminal sequencing of this protein band proved that the purified protein was HYPA.

Acknowledgements

The authors thank J. Baars and A. van de Munckhof of the Mushroom Experimental Station for supplying the CHEF blot and for culturing the *A. bisporus* strains. We thank Y. Müller of MGIM for technical assistance.

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3

Isolation of developmentally regulated genes from the edible mushroom *Agaricus bisporus*

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A modified version of this chapter was published in *Microbiology* **143,** 1993-2001 (1997)

Isolation of developmentally regulated genes from the edible mushroom *Agaricus bisporus*

From a cDNA library, constructed from mushroom primordia, nine cDNAs were isolated which are either induced or specifically expressed during fruit body development and maturation of the basidiomycete *Agaricus bisporus*. These cDNAs varied in size from 372 to 1019 bp and hybridised to transcripts of 400 to 1600 nt. Four of the cDNAs are only expressed in the generative phase of the life cycle while the other five cDNAs are strongly induced but have low steady-state mRNA levels in vegetatively grown mycelium of the hybrid strain Horst®U1. Among the genes that showed a low level of expression in vegetatively grown mycelium, the peptide translation of one clone, *sepA*, showed significant homology with a family of cell division control proteins which are involved in septa formation. Highest expression levels of this septin related gene were found in the rapidly dividing cells in the upper part of the stipes of stage 4 mushrooms, suggesting that its product is also involved in septa formation. Two other clones appeared to encode the δ-subunit of the mitochondrial ATP-synthase complex and a cytochrome P450 protein, however, the relation between the developmentally regulated expression patterns and the possible roles during fruit body development of these two genes is at present not well understood. All cDNAs, except the presumed cytochrome P450 specifying cDNA (*cypA*), hybridised with single copy genes scattered over the *Agaricus* genome. For the *cypA* gene the presence of several additional copies was shown by heterologous hybridisations. Based on changes in expression levels of the fruit body induced genes during development coinciding with alterations in morphological appearance of mushrooms, four stages of development were distinguished during growth and maturation of *A. bisporus* fruit bodies.

Introduction

The basidiomycetous fungus *Agaricus bisporus* is cultivated all over the world in large quantities for human consumption. During the cultivation process, fruit bodies appear in flushes on a casing layer covering compost precultured with vegetatively growing *Agaricus* mycelium. After colonisation of the casing layer, fruit body formation is initiated by changing the temperature and carbon dioxide concentration of the environment.

In the past, fruit body development of *A. bisporus* has been studied by measuring activities of a number of enzymes involved in carbon metabolism. Mannitol dehydrogenase and glucose-6-phosphate dehydrogenase were found to have highest activities at the onset of fruit body formation of the successive flushes (Hammond & Nichols, 1976; Hammond, 1981) while trehalase and glycogen phosphorylase peak at mid-flush (Wells *et al.*, 1987). Although this knowledge led to a better understanding of some aspects of carbon metabolism during fruit body formation, the molecular biology underlying fruit body

initiation and maturation remained unclear. In order to study the genetics of *Agaricus* fruit body formation we have started with the isolation and characterisation of a number of genes that are specifically expressed during this process of morphogenesis. For the isolation of developmentally expressed genes, two methods are usually applied. Differential screening techniques have been used successfully to isolate developmentally regulated genes from the basidiomycetes *Coprinus cinereus* (Yashar & Pukkila, 1985), *Schizophyllum commune* (Mulder & Wessels, 1986) and *Agrocybe aegerita* (Salvado & Labarère, 1991). Of these genes, no sequence information or data concerning the function of the deduced proteins is available except for the *Sc1* to *Sc4* genes of *S. commune* (Schuren & Wessels, 1990) that belong to a fungal gene family encoding small hydrophobic cell wall proteins. Recently, using a similar differential screening method, we have cloned a hydrophobin encoding gene (*hypA*) from *A. bisporus* (De Groot *et al.*, 1996). This gene was one of only two individual cDNAs of developmentally expressed genes that were isolated by this approach. The high expression levels of these genes, accumulating to approximately 6% (*hypA*) and 4% (pDG47) of the total messenger RNA pool in pin stage mushrooms frustrated detection of other developmentally regulated genes.

Alternatively, when considerable sequence information of differentially expressed genes from related organisms is present, approaches using heterologous hybridisation or PCR techniques can be used to isolate differentially expressed genes, as was demonstrated for the ascomycete *Aspergillus fumigatus* (Thau *et al.*, 1994) and the basidiomycete *Lentinus edodes* (Kajiwara *et al.*, 1992). However, as was shown by the direct cloning of the *ras* genes of the basidiomycetes *L. edodes* (Hori *et al.*, 1991) and *Coprinus cinereus* (Ishibashi & Shishido, 1993), in this way also genes were cloned that are not differentially expressed, illustrating differences in gene regulation during development of apparently closely related organisms.

For further study of the genetics of fruit body formation we wanted to investigate the structure, function and expression of more genes involved in fruit body formation. Due to the unusual high expression levels of the two previously cloned genes we inferred that repetition of conventional differential screening methods would not result in cDNAs other than those already cloned. Therefore cDNA clones, present in a cDNA library constructed from small developing mushrooms, were individually screened for elevated mRNA levels in the generative phase of the life cycle. In this way, nine new cDNAs corresponding to unlinked genes were isolated which are strongly induced during the generative phase of the life cycle. Three of those cDNAs could be identified by sequence analysis combined with a database search.

Materials and Methods

Strains and culture conditions and recombinant DNA techniques

Agaricus bisporus strain Horst®U1 and its homokaryotic constituents H39 and H97 were grown as mycelial mats in DT80 medium (Sonnenberg *et al.*, 1988) supplemented with 3% (w/v) glucose. After 14 days of growth at 24°C, the mycelium was harvested by filtration over nylon gauze, immediately frozen in liquid nitrogen, and stored at -70°C. Horst®U1 was also grown on cellophane sheets in Petri dishes containing commercially available and sterilised compost solidified with 1.5% (w/v) agar. Colonies were collected after ten days of growth at 24°C and frozen in liquid nitrogen.

A. bisporus Horst®U1 fruit bodies were obtained using a ten or twelve day flushing cycle (Van Gils, 1988). Primordia and mushrooms were harvested from the first appearance of fruit bodies (five to six days after fruit body initiation) to harvest stage (ten to twelve days after fruit body initiation). For analysis of *sepA* expression in different parts of mature mushrooms, caps and stipes were separated and further fractionated. From caps we isolated the pileipellis (peel), cap tissue (plectenchyma), a connecting zone (between the pileipellis and the plectenchyma), partial veil tissue, and gill tissue (lamellae). The interconnected tissues of the partial veil and the pileipellis, both consisting of white hyphae (Umar & Van Griensven, 1997), are difficult to distinguish, making isolation of only partial veil tissue technically difficult. The partial veil sample therefore contained both partial veil and pileipellis tissue. From the stipes, we isolated the annulus as a separate fraction and the stipes were then sectioned horizontally into 3 mm slices. Of these, three sections were analysed: the transitional zone, comprising the connection with cap tissue, a mid-stipe section and the stipe basis that connects the mushroom with its mycelial cord. After immediate freezing into liquid nitrogen, fruit body samples were stored at -70°C.

Standard DNA manipulations were carried out essentially as described in Sambrook *et al.* (1989). Restriction enzymes and other enzymes used for DNA manipulations were purchased from Bethesda Research Laboratories and used according to the supplier's instructions. *Escherichia coli* SolR cells (Stratagene) were used for *in vivo* excision of cloned cDNA inserts. The cDNA clones were sequenced by the method of Sanger *et al.* (1977) using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) and an ALF automated sequencer (Pharmacia Biotech).

Total RNA isolation and Northern analysis

Total RNA for Northern analyses was isolated from fruit bodies and mycelium using TRIzol™ Reagent (BRL). The concentration of the RNA samples was determined spectrophotometrically and equal amounts of RNA were denatured in 10 x SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) and 6.15 M formaldehyde and spotted on Hybond-N membrane (Amersham), or denatured using glyoxal and dimethyl sulphoxide by standard techniques, separated on a 1.6% (w/v) agarose gel and transferred to Hybond-N membrane. Messenger RNA lengths were determined by co-electrophoresis of RNA molecular weight markers (BRL) that were stained separately with methylene blue after transfer of the RNA to Hybond-N membranes (Sambrook *et al.*, 1989). Hybridisation of RNA blots was executed at 42°C in standard hybridisation buffer (SHB is 6 x SSC, 5 x Denhardt's solution, 0.5% SDS and 100 µg ml^{-1} denatured herring sperm DNA) to which 10% (w/v) dextran sulphate and 50% (v/v) formamide was added. Washing was performed at 65°C to a final stringency of 0.1 x SSC, 0.1% SDS. To provide for a loading control, Northern blots were probed with an *A. bisporus* 28S ribosomal DNA fragment (Schaap *et al.*, 1996). Signal intensities were compared by scanning the autoradiograms with an Ultroscan XL laser densitometer (LKB).

Selection of fruit body induced cDNAs

For the detection of fruit body induced clones present in a primordial cDNA expression library (De Groot *et al.*, 1996), total RNA was isolated from mushrooms grown under the same conditions (twelve day flushing cycle) that were used to construct this cDNA library. Northern slot blots were prepared containing RNA of stage 1 (pin stage) mushrooms (5 and 0.05 µg), mature spore-bearing mushrooms (5 µg) and mycelium vegetatively grown on glucose (5 and 0.05 µg). The blots were hybridised with 100 individual cDNA clones obtained by random excision of part of the cDNA library. The complete inserts of the clones were individually amplified using polymerase chain reaction and pBluescript specific primers 5' and 3' adjacent to the insert sequences. The obtained products were labelled with [α-^{32}P] dATP by random priming (Feinberg & Vogelstein, 1983).

Clones, which showed a 100-fold or more increase in signal strength in fruit body RNA compared to glucose-grown mycelium RNA, were rehybridised to Northern blots containing size fractionated RNA isolated from mature fruit bodies and vegetative mycelium grown on sterilised compost, to confirm induction of the corresponding genes in the fruit bodies and to determine the length of the corresponding mRNA.

Isolation of total genomic DNA and chromosome assignment

Total DNA from strain H39 and H97 was isolated according to the method of De Graaff *et al.* (1988), digested with various restriction enzymes, separated on 0.8% agarose gels and transferred onto Hybond-N membranes. CHEF electrophoresis of intact chromosomal DNA of H39 and H97 was performed as described by Sonnenberg *et al.* (1996) using a Biorad CHEF DrII system. The chromosomes were transferred by capillary blotting to a Hybond-N membrane. Membranes were hybridised overnight in SHB at 65°C and stringently washed (0.1 x SSC, 0.1% SDS at 65°C). Heterologous hybridisations were performed at 56°C with a final washing in 1 x SSC, 0.1% SDS at 56°C. The amplified inserts of the cDNA clones were used as probes. For clones that could not be assigned to a single chromosome of one of the homokaryotic constituents of Horst®U1 using CHEF gel electrophoresis followed by Southern analysis, definite assignment was achieved by linkage analysis. Segregation of these clones and previously cloned marker genes was studied in a set of 86 homokaryotic offspring (Sonnenberg *et al.*, 1996).

Nucleotide and protein sequence comparisons

The sequence data for the putative *atpD*, *sepA*, and *cypA* cDNAs have been deposited with the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z82019, Z82020 and Z82021. Database searches and other sequence manipulations were performed with the University of Wisconsin Genetics Computer Group (GCG) sequence analysis software package. Homology values were calculated from optimal alignments between two sequences using the GCG program BESTFIT (Devereux *et al.*, 1984).

Results

Isolation of fruit body linked genes

In a previous study, differential screening techniques have led to the isolation of two *A. bisporus* genes that are specifically expressed in fruit bodies. The isolation of these genes, *hypA* and pDG47, was based on their abundant presence in a pin stage cDNA expression library (De Groot *et al.*, 1996). In order to isolate more developmental genes whose transcriptional activities are induced during fruit body formation we screened 100 cDNA clones, provisionally designated pDG101 - 200, from the same library individually for elevated mRNA levels during fruit body formation. For this, we compared the steady-state transcript levels of each clone in primordia, in spore-bearing mature mushrooms and in vegetative mycelium grown on glucose by Northern slot blot analysis. One cDNA clone showed reduced expression levels in fruit bodies compared to mycelium, 41 cDNA clones did not show significant differences in RNA expression levels between fruit bodies and mycelium, while 33 cDNA clones showed expression levels elevated up to a 100-fold in fruit bodies compared to mycelium. Twenty-five cDNA clones showed a 100-fold or more induction in the generative phase of the life cycle and these were further analysed. These 25 clones were screened by Southern analysis (not shown) for the occurrence of the two previously isolated fruit body specific genes. Consistent with the high expression levels of those genes, nine clones hybridised with *hypA* and six with pDG47.

Identification of *atpD*, *sepA* and *cypA*

The ten newly isolated cDNA clones were then subjected to sequence analysis. Two clones, pDG122 and pDG195, both specifying gene(s) with negligible expression levels in glucose-grown mycelium, were found to contain parts of the same gene. Clone pDG195 contained an insert of 433 bp ending with a poly(A) tail and this sequence appeared to be identical to the first 433 bp of clone pDG122. In the latter clone this sequence was followed by an additional insert sequence of 800 bp. Since Northern analysis of size fractionated RNA showed that clone pDG195 hybridised to a single transcript of only 550 nt (Table 3.1), pDG122 most probably is the result of a combination of two unrelated cDNAs. Therefore, of these two clones, only pDG195 was used for further study.

Of the remaining nine clones only clones pDG175 and pDG183 appeared to be full length (Table 3.1). A database search of clone pDG183, with an insert of 648 bp, identified

Table 3.1. Characteristics of developmentally regulated genes of *A. bisporus*.

Clone	Size of cDNA (bp)	Size of mRNA (nt)	mRNA level in veg. grown mycelium*	Chromosome†	Putative gene product
hypA	535	550	-	III	Cell-wall-associated hydrophobic protein
pDG47	481	500	-	XII	Unknown
pDG144	783	900	-	VII	Unknown
pDG174	532	1050	-	VII	Unknown
pDG175	372	400	-	VIII	Unknown
pDG195	433	550	-	VI	Unknown
pDG102	718	1600	+	II	Septin
pDG125	932	1600	+	XI	Cytochrome P450
pDG172	1019	1350, 1500	+	III	Unknown
pDG183	648	650	+	II	ATP synthase complex δ-subunit
pDG192	717	850	+	V	Unknown

* -, mRNA level below detection limit; +, low mRNA level detected in vegetatively grown mycelium.
† Chromosomal localisations were done by CHEF gel electrophoresis followed by Southern analysis. cDNAs hybridising to chromosomes III or IV were re-analysed by linkage analysis.

this cDNA as encoding the δ-subunit of the mitochondrial ATP-synthase complex (ATPD). The deduced *A. bisporus* ATPD amino acid sequence was found to have 63%, 51% and 36% identity with ATPD subunits of the ascomycetous fungus *Neurospora crassa*, the yeast *Saccharomyces cerevisiae* and bovine, respectively (Figure 3.1(a)). From the primary translation products of the *S. cerevisiae* and the bovine genes a 22 amino acids mitochondrial targeting peptide is cleaved off and also for *N. crassa* ATPD a mitochondrial targeting peptide was predicted. The *A. bisporus atpD* open reading frame encodes a putative protein of 162 amino acids that contains the conserved RXY↓(S/A) motif for cleavage of mitochondrial targeting peptides (Gavel & Von Heijne, 1990) and therefore a mitochondrial targeting sequence of 25 amino acids, rich in basic (arginines) and polar (serines) residues, is suggested.

For two partial cDNAs, pDG102 and pDG125, also significant homology with database sequences was found. The conceptual translation of the largest open reading frame of clone pDG102, with an insert of 718 bp, showed 46% identity with the carboxy terminal half of *Schizosaccharomyces pombe* SPN3, 42% identity with *S. cerevisiae* CDC11, 40% identity with the *Drosophila* PNUT protein and 39% identity with murine DIFF6 (Figure 3.1(b)). These proteins are mitotic cell division control proteins belonging to the septin family, and might be involved in cytokinesis. Clone pDG102, now renamed to *sepA*, hybridised with a

(a) ATPD

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                                        **                  **   ****      *   * *** **  **  * *   ***  * * * * *** * **
Ab          MSSLRLLASAARRATTHVAYTRRGY AE.ISDK... .....LKLSL ALPHKAIFSS QDVVQVNIPA ESGDMGILSS HVPSIEPLRP GVVEVVEDSG
Nc        MNSLRIARAALRVRPTAVRAPLQRRGY AEAVADK... .....IKLSL SLPHQAIYKS QDVVQVNIPA VSGEMGVLAN HVPSIEQLKP GLVEVIEESG
Sc            MLRSIIGKSASRSLNFVAKRSY AEAAAAS... ...SGLKLQF ALPHETLYSG SEVTQVNLPA KSGRIGVLAN HVPTVEQLLP GVVEVMEGSN
Bt            MLPSALLRRPGLGRLVRQVRLY AEAAAAQAPA AGPGQMSFTF ASPTQVFFNS ANVRQVDVPT QTGAFGILAA HVPTLQVLRP GLVVVHAEDG

    * *  * *** **** *   *       **  * * ** **  *  * ** *    *        * ***  ** *  **  *
Ab  SQK.WFVSGG FATVHPNNRL TINVVEAAPL EDFSIEAIRA NLQEANKVAA GSGSEADKME AQIEAEVYEA LQHALAK  162
Nc  SNKQYFLSGG FAVVQPGSKL SINAVEGYAL EDFSAEAVRA QIAEAQKIVS GGGSQQDIAE AQVELEVLES LQAVLK  165
Sc  SKK.FFISGG FATVQPDSQL CVTAIEAFPL ESFSQENIKN LLAEAKKNVS SSDAR.EAAE AAIQVEVLEN LQSVLK  160
Bt  TTSKYFVSSG SVTVNADSSV QLLAEEAVTL DMLDLGAAKA NLEKAQSELL GAADEATRAE IQIRIEANEA LVKALE  168
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(b) Septin

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     *  **    *  **   * ****** *   *  *    **  * **** ** * ** ****   ** *    * * ***  *    *** ***** **
Ab  LTEDDEETVQ DNTELRALLP FAVVGSEEEV EIDGE..PVR ARVYPWGLVE VDNPRHCDFV RLRGAILGSH LGDLKMLTED VLYETYRTEK LS........
Sp  IEEDEEAIIN LSQQLRATIP FAIVSSDRLI EMNGQ..TVR GRAYPWGVVE VDNPRHSDFL ALRSALFATH IEDLHNITSN QLYETYRTEK LS........
Sc  EDEISDEDYE TNMYLRTLLP FAIIGSNEVY EMGGDVGTIR GRKYPWGILD VEDSSISDFV ILRNALLISH LHDLKNYTHE ILYERYRTEA LSGESVAAES
Dm  LEDAAEE.AK TTQNLRSRVP FAVVGANTII EQDGK..KVR GRRYPWGLVE VENLTHCDFI ALRNMVIRTH LQDLKDVTNN VHYENYRCRK LSELGLVDGK
Mm  DSDEDEEFKK QNEEMKENIP FAVVGSCEVV RDGTR..PVR GRRYSWGTVE VENPHHCDFL NLRRMLVQTH LQDLKEVTHD LLYEGYRARC LQSLARPGAR

                *                                    *  *  **    *         *       **
Ab  .......... .KSILDDSQD SEMLPEDLAN QSVLLKEQQL VKEQERLREY ELRAKHEIGL MKQQLLAKEE ALRNLEARTG SSPY     164
Sp  .......... .TSQL..... ..LLDSTVGL DGKNLSQHDQ VLREDRLRAI ELSVQKEIEE KRRQLLAREE ALRALEEKLA ASTAAMANAS VSTLPSSVSS
Sc  IRPNLTKLNG SSSSSTTTRR NTNPFKQSNN INNDVLNPAS DMHGQSTGEN NETYMTREEQ IRLEEERLKA FEERVQQELL LKRQELLQRE KELREIEARL
Dm  ARLSNKNPLT QMEEEKREHE QKMKKMEAEM EQVFDMKVKE KMQKLRDSEL ELARRHE..E RKKALELQIR ELEEKRREFE REKKEWEDVN HVTLEELKRR
Mm  DRASRS.... .......... .KLSRQSATE IPLPMLPLAD TEKLIREKDE ELRRMQEMLE KMQAQMQQSQ AQGEQSDVL           365

Sp  TNHSQS                  412
Sc  EKEAKIKQEE              415
Dm  SLGANSSTDN VDGKKEKKK KGLF  539
```

(c) Cytochrome P450

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                                          *    *                          *   * *** *          *   **  *** *  * *** *
Ab  WSYINSIVAN SLWNSVKERM EAGTAKPCIA TAMLEDLLDD DSAESKEEET VRRGACANGF LGGADTTVSL VTSFFMAMAL YPDVQKKAQA ELDQVLGG..
Ph  LHKKFDALLT KMFDEHKATS YERKGKPDFL DCVMEN..RD NSEGERLSTT NIKALLLNLF TAGTDTSSSA IEWALAEMMK NPAILKKAQG EMDQVIGNNR
Gg  RDQLLQQKFT EHKEAFCGDT VRDLMDALLQ VRLNAENNSP LEPGLELTDD HLLMTVGDIF GAGVETTTTV LKWAVLYLLH YPEVQKKIQE EMDQKIGLAR
Oc  ERFYSFTQER VKEHCRSFEK GHIRDITDSL IKHYRVDRLD ENANVQVSDE KTVGIVLDLF GAGFDTVTTA ISWSLMYLVT KPRIQRKIQE ELDAVVGRA.
Om  KAKVYIQEQA EIRLKTLNIS EPQDFIEAFL VKMLEEK... DDPNTEFNNG NMVMTAWSLF AAGTETTSST LRQSFLMMIK YPHIQESVQK EIDEVIG.SR

    * *  ****  ***  *   *   *  *  **  * * *        ******* * *  * **  ****    *   * * ***       *              **
Ab  RLPEFSDRPS LPYVNALLKE SERWQPVPPL AIAHMSSNAD EYDGYYIPKG TYVIGNAWSI LHDPEFYKDP LVFNPDRFL. .KDGEIDPSV RDPNVASFGF
Ph  RLLE.SDIPN LPYLRAICKE TFRKHPSTPL NLPRISNEPC IVDGYYIPKN TRLSVNIWAI GRDPEVWENP LEFYPERFLS GRNSKIDPRG NDFELIPFGA
Gg  H.PHLSDRPL LPYLEATISE GLRIRPVSPL LIPHVSLADT SIGEYSIPKG ARVVINLWSV HHDEKEWDKP EEFNPGRFLD EQGQHIHSPS PSY..LPFGA
Oc  RRPRFSDRPQ LPYLEAVIME TFRHTSFLPF TIPHSTTRDT SLGGFYIPKG RCVFVNQWQN NHDPELWGDP EAFRPERFLT PSGAVDKALT EK..VLLFGL
Om  V.PTVDDRVK MPYTDAVIHE VQRYMDLSPT SVPHKVMRDT EFYNYHIPEG TMVLPLLSSV LVDPKLFKNP DEFDPENFLD ENG..VFKKN DGF..FAFGV

    * * * *       *       **  *          *
Ab  GRRICPGRF FPDASLYSTVT HVLTVFD... ....IKPNLD ENGKEIGIKP DMTDGLLSIP M            250
Ph  GRRICAGTR MGIVMVEYILG TLVHSFDWKL PSEVIELNME EAFGLALQKA VPLEAMVTPR LPIDVYA PLA  508
Gg  GIRVCLGEV LAKMELFLFLA WVLQRFTLEC PQDQPLPSLE GKFGVVLQVQ KF.RVKARLR EAWRGEM VR   508
Oc  GKRKCIGET IGRLEVFLFLA TLLQQVEFSV SPGTTV.DMT PIYGLTMKHA RCEHFQAKLR FEA          518
Om  GKRACPGEA LARVELFLFFT SVLQRFTFTG TKPPEEINIE PACSSFGRLP RSYDCYIKLR TEK          499
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Figure 3.1. Alignment of the conceptual translation products of three fruit body induced clones pDG102, pDG125 and pDG183 with putative homologous proteins. Residues which are identical to *A. bisporus* in at least two of the related proteins are marked by asterisks above the sequences. (a) Predicted amino acid sequence of ATPD translated from clone pDG183. The *A. bisporus* (*Ab*) ATPD protein was aligned with *Neurospora crassa* ATPD (*Nc*, Kruse & Sebald, 1984), *Saccharomyces cerevisiae* ATPD (*Sc*, Giraud & Velours, 1994) and *Bos taurus* (bovine) ATPD (*Bt*, Runswick *et al.*, 1990). (b) Predicted amino acid sequence of SEPA translated from clone pDG102. The partial *A. bisporus* (*Ab*) SEPA was aligned with *Schizosaccharomyces pombe* SPN3 (*Sp*, Gen Bank accession number U29889), *S. cerevisiae* CDC11 (*Sc*, Gen Bank accession number L16550), *Drosophila melanogaster* PNUT (*Dm*, Neufeld & Rubin, 1994) and *Mus musculus* (murine) DIFF6 (*Mm*, Nottenburg *et al.*, 1990). (c) Predicted amino acid sequence of CYPA translated from clone pDG125. The partial *A. bisporus* (*Ab*) CYPA was aligned with *Petunia hybrida* CYP75A3 (*Ph*, Holton *et al.*, 1993), *Gallus gallus* (chicken) CYP17 (*Gg*, Ono *et al.*, 1988), *O. cuniculus* (rabbit) CYP1A1 (*Oc*, Kagawa *et al.*, 1987) and *Onchorychus mykiss* (rainbow trout) CYP2M1 (*Om*, Gen Bank accession number U16657).

transcript of 1600 nt (Table 3.1) which could encode a protein of about 400-500 amino acids, a size which is in accordance with the size of the four septin proteins to which SEPA was aligned.

Clone pDG125 has an insert of 932 bp ending in a poly(A) tail. On the positive strand a large open reading frame of 750 bp was found of which the deduced amino acid sequence showed significant homology with the carboxy terminal half of cytochrome P450 proteins which are currently classified into 74 families (Nelson *et al.*, 1996). Highest homology was found with two flavonoid 3',5' hydroxylases CYP75A1 and CYP75A3 of *Petunia hybrida*, proteins that belong to cytochrome P450 family 75. The partial *A. bisporus* amino acid sequence showed 36% identity with these proteins. The homology found with members of other cytochrome P450 subfamilies was 32% with chicken CYP17, 31% with rabbit CYP1A1 and 28% with trout CYP2M1 (Figure 3.1(c)). Northern analysis showed that pDG125 hybridised to a transcript of 1600 nt (Table 3.1) that could encode a protein of about 500 amino acids and this is in accordance with the size of cytochrome P450 proteins. Therefore, pDG125 was tentatively renamed to *cypA*.

Classification into fruit body specific and fruit body induced genes

Besides determination of transcript size the Northern analyses of size-separated RNA were also used to re-establish the induction of expression of the nine clones in fruit bodies by comparing expression in mature fruit bodies with expression in mycelium grown on sterilised compost. The previously isolated fruit body specific *hypA* gene and clone pDG47, the constitutively expressed *tefA* gene (Sonnenberg *et al.*, 1996) and 28S rDNA (Schaap *et al.*, 1996) were used as controls. Four of the newly isolated cDNAs and the controls *hypA* and pDG47 showed strong signals upon hybridisation with fruit body RNA while their mRNA levels in RNA from mycelium grown on compost were the same as those obtained with glucose-grown mycelium, below the detection limit (Figure 3.2), even after prolonged exposure (not shown). These cDNAs were therefore considered as being obtained from genes that are only expressed in the generative phase of the life cycle and were classified as fruit body specific genes. For the other cDNAs weak hybridisation signals were observed with RNA from compost mycelium as was also observed with RNA from glucose-grown mycelium in the initial screening, but they showed a strong increase in signal strength upon hybridisation with RNA obtained from fruit bodies (Figure 3.2). These clones are further denoted as being obtained from fruit body induced genes. The *tefA* control showed comparable levels of expression in compost mycelium and fruit bodies. All cDNA clones, except pDG172, hybridised to transcripts of apparent single size. For pDG172, two

transcripts with a small size difference are visible (Figure 3.2). The results from the Northern analyses are summarised in Table 3.1. The previously isolated genes *hypA* and pDG47 are added to the Table for comparison.

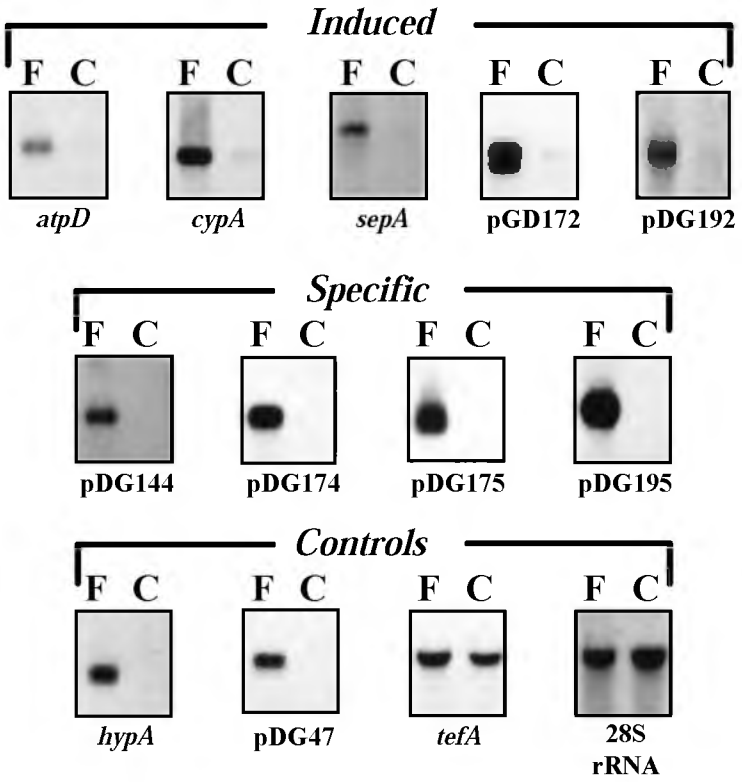

Figure 3.2. Transcript levels of cDNA clones in fruit bodies (F) and compost-grown mycelium (C). The inserts of all inferred fruit body specific and induced cDNA clones were individually used as probes. Control hybridisations were performed with *hypA*, pDG47 (De Groot *et al.*, 1996), *tefA* (Schaap *et al.*, 1997) and a 28S rDNA probe (Schaap *et al.*, 1996). Sizes of the messenger RNAs of all fruit body specific or induced genes are listed in Table 3.1.

The fruit body specific and induced genes are scattered over the genome

The genomic organisation of the fruit body linked cDNAs was studied using genomic DNA of the homokaryotic constituents of Horst®U1. Southern analyses of digested DNA fragments showed that all clones, except *cypA*, probably represent single copy genes. For *cypA* several strong and weak hybridising bands were observed using heterologous hybridisation conditions and this indicates that there are additional copies of the cytochrome P450 encoding *cyp* gene family in the *Agaricus* genome (results not shown).

The chromosomal localisation of all clones was analysed using clamped homogeneous field electrophoresis (CHEF) of total DNA from the parental strains H39 and H97 followed by Southern analysis. All clones, except *cypA*, hybridised to single chromosome bands representing homologous chromosomes of the two karyotypes (Sonnenberg *et al.*, 1996) and the corresponding genes are divergently located on the *Agaricus* genome (Figure 3.3 and Table 3.1). For *cypA*, using heterologous hybridisation conditions, a strong hybridisation signal was observed for chromosome XI while additional weaker signals were found for at least chromosomes VII, VIII, XIII and the unseparated chromosomes III/IV (Figure 3.3). This is consistent with the multiple signals found by Southern analysis of digested total DNA. It therefore appears that other cytochrome P450 encoding genes are located on different chromosomes in the *Agaricus* genome.

The *hypA* gene (De Groot *et al.*, 1996) and pDG172 were localised with CHEF analysis on either chromosome III or IV (Figure 3.3) and had to be assigned by linkage analysis. Using homokaryotic offspring, for both genes 100% linkage was found with two markers, the *pgkA* gene (Sonnenberg *et al.*, 1996) and anonymous marker p33N5 (Kerrigan *et al.*, 1993) both residing on chromosome III. No linkage was found with the marker genes *rpaB* and *pruA* both localised on chromosome IV (Sonnenberg *et al.*, 1996). Consequently, *hypA* and the gene corresponding to pDG172 are localised on chromosome III of both parental strains. None of the clones that are localised on same chromosomes cross-hybridised or shared significant sequence homology. All these clones hybridised with transcripts of different size and taken together this indicates that all cDNAs represent genes of different loci of the *A. bisporus* genome.

Developmental expression of the fruit body specific and induced genes

The mRNA levels of all the newly isolated fruit body linked cDNAs, the previously identified *hypA* gene and pDG47 were followed during development and maturation of Horst®U1 fruit bodies during a first flush using Northern slot blot analysis. For this, fruit

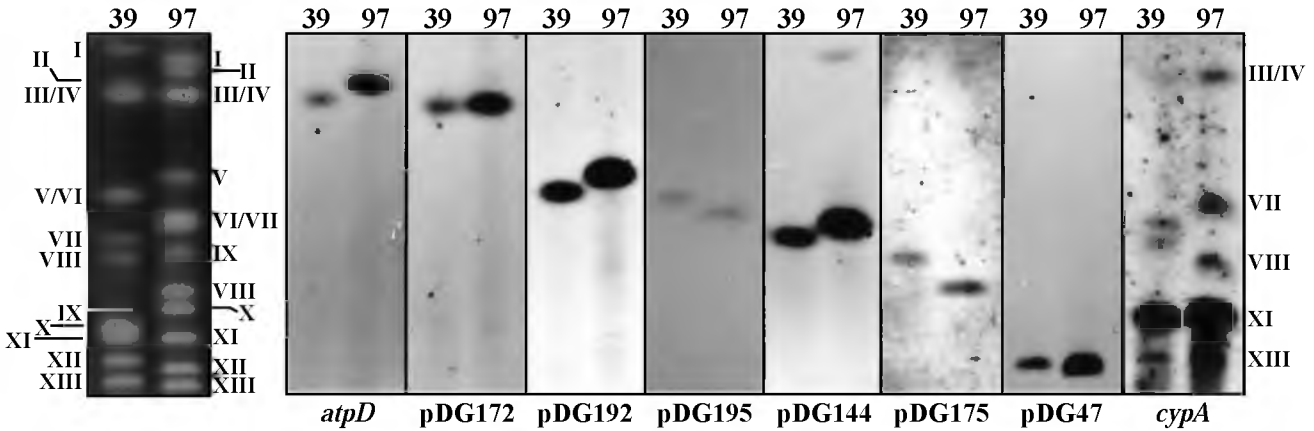

Figure 3.3. CHEF-analysis of *A. bisporus* strains H39 and H97. Chromosomes of the parental lines of Horst®U1 separated by CHEF gel electrophoresis after staining with Ethidium bromide (left panel) and after hybridisation with the different fruit body specific and induced clones. For all chromosomes to which fruit body specific or induced genes were assigned one representative hybridisation is shown. For *cypA*, a heterologous hybridisation showing the presence of additional *cypA* copies, is presented. Chromosomes are indicated by Roman numerals according to Sonnenberg *et al.* (1996).

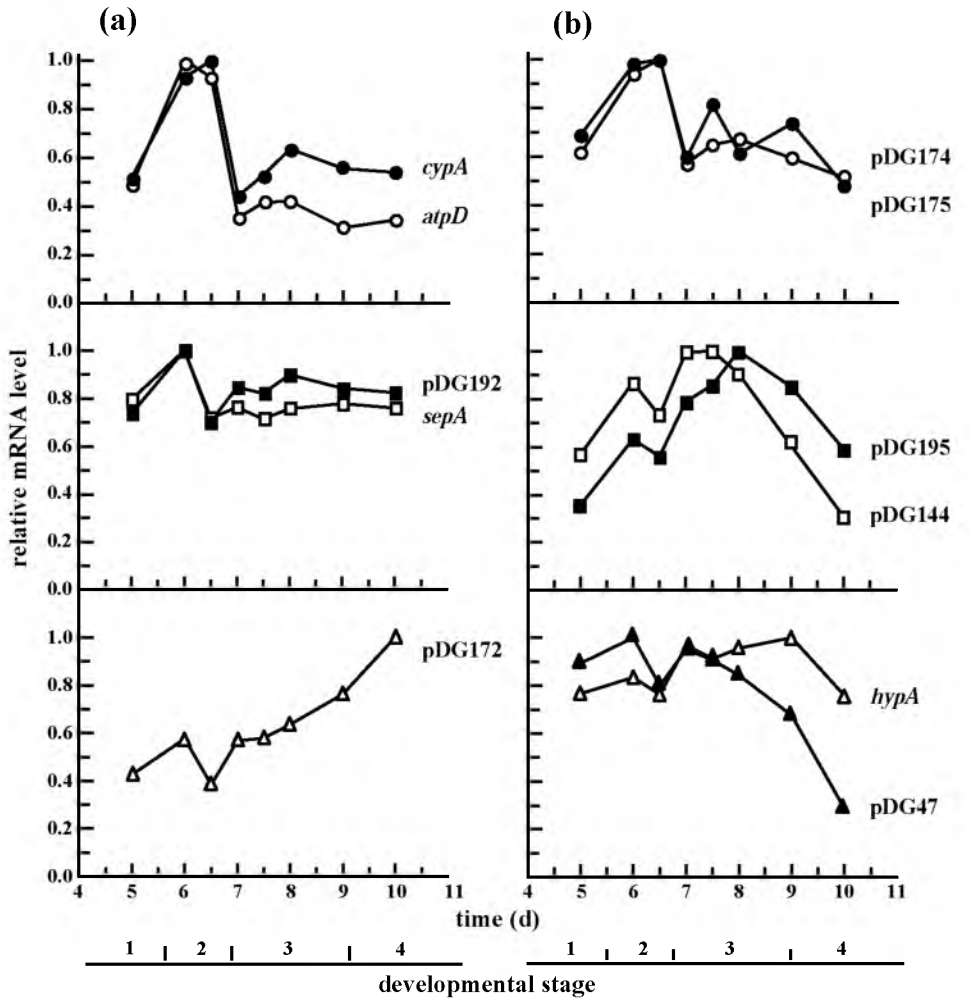

Figure 3.4. Graphs of the relative mRNA levels of the fruit body induced (a) and specific (b) genes in mushrooms harvested at day 5 to day 10 during fruit body development. Samples were taken daily at 9 a.m. and at day 6 and 7 additional samples (6.5 and 7.5) were isolated at 9 p.m. The inserts of the fruit body induced and specific cDNA clones and of *hypA* and pDG47, were used as probes. Transcript levels were normalised against 28S ribosomal RNA and for each gene the highest level recorded was arbitrary set to 1.0.

bodies were grown in a ten day flushing cycle using commercial cultivation conditions. Transcript levels were followed from the first visual appearance of primordia, five days after initiation of fruit body formation until spore-bearing gills are exposed and the veil is broken. Several types of expression patterns were observed (Figure 3.4). Two in fruit body induced genes (*atpD* and *cypA*) and two fruit body specific genes (pDG174 and pDG175), have highest mRNA levels six days after fruit body initiation. Two other fruit body specific genes, pDG144 and pDG195, show highest transcript levels from day 7 to day 9 whereas their mRNA levels in mature mushrooms (day 10) are clearly decreased. In these mature mushrooms fruit body specific gene pDG47 also showed its lowest expression levels. Two fruit body induced genes, pDG192 and *sepA*, and the *hypA* gene appeared to have a more or less constant expression level during these six days of mushroom development. Finally, the fruit body induced gene pDG172 was unique in showing its highest level of expression in mature fruit bodies.

For the *sepA* gene, whose product was suggested to be involved in septa formation during cell division, we also determined the spatial expression pattern in fractionated mature mushrooms. An accumulation of *sepA* messengers was found in the transitional

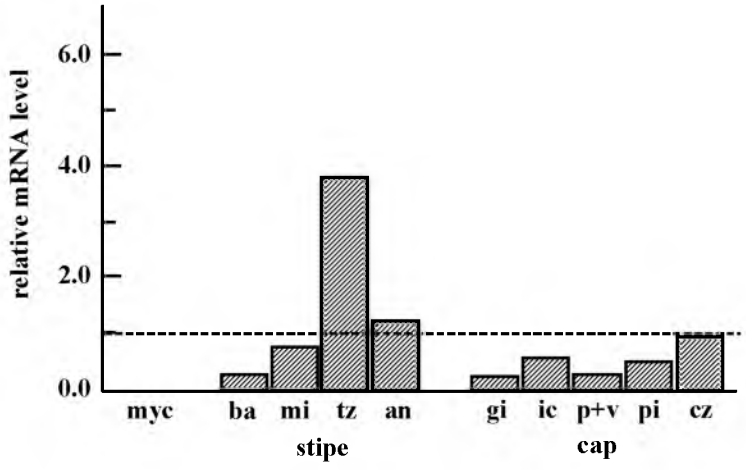

Figure 3.5. Spatial expression of *sepA* in different parts of mature mushrooms and in compost grown mycelium. Abbreviations are as follows: myc, mycelium; ba, stipe basis; mi, mid-stipe; tz, transitional zone; an, annulus; gi, gills; ic, inner cap; p+v, pileipellis + partial veil; pi, pileipellis; cz, connecting zone. Relative transcript levels were calculated by normalisation against 28S rRNA and the mRNA level obtained for whole stage 4 mushrooms was set to 1.0.

zone fraction (Figure 3.5). This fraction contains the upper stipe regions which have been found to be comprised of rapidly dividing cells (Craig *et al.*, 1979), providing additional evidence that SEPA is involved in septa formation.

Discussion

Differential screening techniques have been successfully applied for the cloning of developmentally regulated genes from several fungi among which a few basidiomycetes. However, with this approach we succeeded in isolating only two fruit body specific genes (De Groot *et al.*, 1996). In our new strategy we screened a 100 cDNA clones from a fruit body cDNA library individually for elevated expression of the corresponding genes in fruit bodies. Although only a small part of the cDNA population in the cDNA library was examined, nine new differentially regulated cDNAs were isolated originating from genes that are induced coordinately with fruit body formation.

Northern analyses showed that most of the cDNA clones isolated in this study are, although not detected by the previous differential screening, highly expressed in *A. bisporus* fruit bodies. This is underlined by the results from hybridisation of pDG144 to the cDNA library. This clone hybridised with approximately 1% of the clones of this library and this emphasises the unusual strong expression of the two fruit body specific clones that were found by differential screening.

Sterile compost supports good growth in the vegetative phase of the life cycle. Under normal cultivation conditions, other micro-organisms present in the compost and necessary for normal fruit body induction somehow accelerate the colonisation rate of the compost by *A. bisporus* but these micro-organisms appear to have no effect on the total mycelial biomass formed (Smith *et al.*, 1995). Nevertheless, we tried to avoid false positives, that have variations in expression levels as a consequence of metabolic differences resulting from the different growth conditions used, by selecting clones of which the corresponding mRNA level in primordia and/or mature mushrooms was elevated at least a 100-fold compared to the level in mycelium. Furthermore all selected fruit body induced clones showed similar expression levels in mycelium grown on compost and glucose and this excludes any carbon source effects in our screening results. We can however, not exclude that a part of the difference in expression levels observed for the fruit body induced clones is due to metabolic differences as a result of different growth conditions.

The cDNA library that is used in this study was composed of poly(A) RNA isolated from a mixture of primordia and small mushrooms which were picked at the same day and is thus

enriched in cDNAs from genes that show high mRNA levels late in the development of primordia and their transition into mature mushrooms. In order to isolate genes that are active at earlier stages of fruit body development, fertile mycelium that is starting to form mycelial aggregates, a process occurring prior to primordium formation, would be a better source of material for constructing such a cDNA library. In the basidiomycetes *S. commune* and *A. aegerita*, culturing under axenic conditions leads to normal fruit body formation and this enables the isolation of mycelium that undergoes developmental changes. *A. bisporus*, on the other hand, is very reluctant to fruit under axenic conditions and under those conditions mycelial aggregates have only been observed in a mutant, that however shows no sign of further differentiation or maturation. The mutant is also unable to produce normal fruit bodies on compost (Hammond & Burton, 1996). Normal *A. bisporus* fruit body development requires the presence of a particular kind of microflora in the compost during cultivation (Miller *et al.*, 1995). The isolation of genes involved in the initial steps of fruit body formation of *A. bisporus* may therefore require the isolation of RNA from aggregated mycelium from non-sterile compost shortly after fruiting is initiated.

Based on changes in morphological appearance that can be visualised by light microscopy (De Groot *et al.*, 1996), we can divide *A. bisporus* mushroom development into four stages. These stages differ from the arbitrary stages previously defined by Hammond & Nichols (1975). The transition from pin shaped (stage 1) mushrooms into stage 2 mushrooms is accompanied by a transient increase of expression of four genes at day six. At this timepoint, previously described as belonging to stage 1, cap tissue becomes visible as a more dense type of mycelium. One day later, non spore-bearing gills are already visible in cross-sections of these fruit bodies and the cap starts to enlarge rapidly (stage 3). During this stage, previously subdivided into stages 2 and 3, clones pDG47, pDG144 and pDG195 have their highest levels of expression. Ten days after fruit body initiation the now spore-bearing mushroom starts to unveil its gills and is considered mature (stage 4). This coincides with a decrease in expression of pDG144 and pDG195 and increased expression of pDG172 (Figure 3.4).

The three cDNA clones, *atpD*, *sepA* and *cypA*, for which significant homology with database sequences was found, all belong to the group of genes that are also expressed during the vegetative phase of the life cycle. Identification of genes specifically expressed during fruiting, on the other hand, seems to be more difficult and is hampered by the lack of morphological mutants and sequence information from genes involved in fruiting of other basidiomycetes. However, further studies on the regulation of the now cloned genes will lead to a better understanding of mushroom development.

Acknowledgements

The authors thank Dr J. Baars and Mrs. A. van de Munckhof of the Mushroom Experimental Station for performing the CHEF-electrophoresis and for culturing the *A. bisporus* strains. We also thank Ms. D. Basten and Mrs. Y. Müller of MGIM for technical assistance.

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4

Temporal and spatial expression of two hydrophobin encoding genes of the edible mushroom *Agaricus bisporus*

A modified version of this chapter is accepted for publication in *Microbiology* (1999) as "Different temporal and spatial expression of two hydrophobin encoding genes of the edible mushroom *Agaricus bisporus*" by Piet W. J. De Groot, Robert T. P. Roeven, Leo J. L. D. Van Griensven, Jaap Visser & Peter J. Schaap

Temporal and spatial expression of two hydrophobin encoding genes of the edible mushroom *Agaricus bisporus*

In a search for genes that are only expressed in fruit bodies of the basidiomycete *Agaricus bisporus* we previously isolated two cDNAs, *hypA* and *hypB* that encode hydrophobins. In this study, the structure of the *hypB* gene is resolved and it is shown that the two genes are differently expressed, indicating that the encoded hydrophobins serve different functions in *A. bisporus* mushrooms. The *hypB* gene encodes a polypeptide of 119 amino acids with eight cysteines arranged in a way exclusively found in hydrophobins but otherwise shows little sequence identity with HYPA. The temporal and spatial expression of the two hydrophobin encoding genes during fruit body development was compared using Northern analyses and *in situ* hybridisation techniques. Accumulation of *hypA* messengers was found in tissue fractions consisting of undifferentiated white hyphae. *In situ* hybridisations show that the highest *hypA* mRNA levels are not found in the outermost cell layers of the pileipellis but in the cell layers adjacent to that. This suggests that the cells of the zone connecting the pileipellis and the plectenchyma excrete high amounts of HYPA monomers that do not self-assemble before they have reached the surface of the fruit body caps. Highest expression of the *hypB* gene occurs early in development when the primordium differentiates into densely packed, randomly oriented cap hyphae and loosely packed, vertically oriented stipe hyphae. In mature mushrooms, a strong accumulation of *hypB* transcripts was found only in the transitional zone between cap and stipe tissue, demonstrating that transcription regulation of *hypB* is clearly distinct from *hypA*.

Introduction

The edible fungus *Agaricus bisporus* is commercially cultivated on a large scale for mushroom production. The differentiation process leading to these reproductive structures is influenced by available nutrients and abiotic factors such as temperature, humidity and the carbon dioxide concentration (Flegg & Wood, 1985). By manipulating environmental conditions during cultivation, the onset and further development of fruit body formation can be controlled to optimise mushroom yields. At present, however, it is unclear what molecular mechanisms program the vegetatively growing mycelium to start differentiation.

For a better understanding of this process, we have previously isolated cDNAs specifying genes that were either strongly induced or specifically expressed in fruit bodies (De Groot *et al.*, 1996, 1997, 1998). From various filamentous fungi, structural genes associated with

cellular differentiation processes have been isolated and amongst them are often genes encoding hydrophobins (reviewed by Wessels, 1997). Hydrophobins are small cell wall proteins of about 110 amino acids that have eight cysteine residues at characteristic positions, a signal sequence for secretion and a specific hydrophobicity pattern. By interfacial self-assembly, hydrophobins form amphipathic films that provide aerial structures with a hydrophobic surface. Hydrophobin aggregates are normally not dissolved in solutions of 2% sodium dodecyl sulphate (SDS) but can be dissociated into monomers using trifluoroacetic acid (TFA) or formic acid (Wessels, 1997).

One of the fruit body specific genes isolated from *A. bisporus*, *hypA*, was previously shown to encode a hydrophobin. Accumulation of *hypA* messengers was found in the peel (pileipellis) of mushroom caps, constituting more than 60% of the total mRNA population in that tissue (De Groot *et al.*, 1996). Treatment of the peel tissue with SDS followed by dissociation of the hydrophobin aggregates by TFA extraction resulted in isolation of the corresponding HYPA protein. HYPA was therefore proposed to be the major constituent of the protective hydrophobic layer surrounding mushroom caps (De Groot *et al.*, 1996). Protein extracts of HYPA adhere strongly to hydrophobic surfaces thereby forming a hot-SDS resistant monolayer (Lugones *et al.*, 1996; Gunning *et al.*, 1998) as has also been shown for the well-studied Sc3p hydrophobin from the basidiomycete *Schizophyllum commune* (Wösten *et al.*, 1994b).

In addition to *hypA*, other hydrophobin encoding genes were discovered in *A. bisporus*. The genomic region immediately downstream of *hypA* comprises the *hypC* gene, a fruit body specific gene for which only low levels of expression were detected. The *hypC* gene is overall 84% identical at the nucleotide level to the *hypA* gene and is probably the result of a duplication event. Recently, a hydrophobin (ABH3) specifically secreted by vegetatively growing hyphae of *A. bisporus* was isolated (Lugones, et al., 1998).

In an effort to elucidate the functions of more fruit body specific genes of *A. bisporus*, we analysed the sequence of a number of the corresponding cDNAs. In this study, we show that pDG47 specifies a protein that also has all the characteristics that are typical of fungal hydrophobins but is distinct from HYPA. This hydrophobin was designated HYPB. Detailed analyses of temporal and spatial expression of both hydrophobins are presented. We show that *hypA* and *hypB* are regulated differently and probably serve disparate functions in developing mushrooms.

Materials and Methods

A. bisporus strains and culture conditions

Cultivated *Agaricus bisporus* strain Horst®U1 and one of its homokaryotic constituents, H39, were grown on cellophane sheets in Petri dishes containing DT80 medium (Sonnenberg *et al.*, 1988) that was supplemented with either 3% (w/v) glucose or 7.5% (w/v) powdered and sterilised compost (Van Gils, 1988), and solidified with 1.5% (w/v) agar. Colonies were collected after ten days of growth at 24°C and frozen in liquid nitrogen.

A. bisporus Horst®U1 fruit bodies were obtained using a ten day flushing cycle (Van Gils, 1988). Primordia and mushrooms were harvested from the first appearance of fruit body initials (five days after fruit body initiation) until spore-bearing mature mushrooms have developed (ten days after fruit body initiation). Some mature mushrooms were separated into different fruit body parts before freezing into liquid nitrogen.

E. coli strains, recombinant DNA techniques and enzymes

Escherichia coli strain LE392 (Promega) was used for phage amplification and λ DNA isolation. *E. coli* DH5α (Bethesda Research Laboratories) was used for plasmid transformation and propagation. Standard DNA manipulations were carried out essentially as described in Sambrook *et al.* (1989). Restriction enzymes and other enzymes used for DNA manipulations were purchased from Bethesda Research Laboratories and used according to the supplier's instructions. Plasmid pUC19 (Yanisch-Perron *et al.*, 1985) was used as cloning vector for genomic DNA fragments. Cloned hybridising fragments and cDNA clones were sequenced by the method of Sanger *et al.* (1977) using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) and an ALF automated sequencer (Pharmacia Biotech).

Isolation of total genomic DNA, Southern blot analysis and cloning of the genomic sequence of *hypB*

For Southern analysis, total DNA of strain H39 was isolated according to the method of De Graaff *et al.* (1988), digested with various restriction enzymes, separated on 0.8% (w/v) agarose gels and transferred onto Hybond-N membranes (Amersham). The membranes were hybridised overnight at 65°C by standard methods with a *hypB* cDNA probe which was prepared by labelling with [α-^{32}P] dATP by random priming (Feinberg & Vogelstein, 1983). Cloning and selection of *hypB* cDNA plasmid pDG47 has been described by De Groot *et al.* (1996). Genomic sequences of the *hypB* gene were obtained by screening a λEMBL4 genomic library of *A. bisporus* strain H39 by standard methods using the *hypB* cDNA probe. Seven λ-clones were isolated of which four comprised the complete *hypB* gene and surroundings. Relevant parts of one of these λ-clones were subcloned and sequenced.

Nucleotide and protein sequence comparisons

The sequence data for the *hypB* genomic and cDNA sequences and the putative succinate dehydrogenase encoding *sudA* gene located adjacent to *hypB*, have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers Y15940, Y15941 and Y15942 respectively. Database searches were performed with the University of Wisconsin Genetics Computer Group (GCG) sequence analysis software package. Homology calculations and amino acid alignments were made using the GCG programs BESTFIT, PRETTY and PILEUP (Devereux *et al.*, 1984).

Hydrophobicity plots were generated with the program DNA Strider Version 1.1 (Marck, 1988) based on the Kyte-Doolittle algorithm with a window size of seven (Kyte & Doolittle, 1982).

Total RNA isolation, Northern analysis and primer extension

Total RNA for Northern analysis was isolated from fruit body and mycelium samples using TRIzol™ reagent (BRL). The concentration of the RNA samples was determined spectrophotometrically and equal amounts of RNA were denatured in 10 x SSC (SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate pH 7.0) and 6.15 M formaldehyde and spotted on Hybond-N membrane, or denatured by glyoxal using standard techniques, separated on a 1.6% (w/v) agarose gel and transferred to Hybond-N membrane. Hybridisation of RNA blots was executed at 42°C in standard hybridisation buffer (6 x SSC, 5 x Denhardt's solution, 0.5% SDS and 100 µg denatured herring sperm DNA ml^{-1}), to which 10% (w/v) dextran sulphate and 50% (v/v) formamide was added, using *hypA* and *hypB* cDNAs as probes. Washing was performed at 65°C to a final stringency of 0.1 x SSC, 0.1% SDS. To provide a loading control, Northern blots were rehybridised with an *A. bisporus* 28S ribosomal DNA fragment (Schaap *et al.*, 1996). Signal intensities were quantified by scanning the autoradiograms with an Ultroscan XL laser densitometer (LKB). Transcription start points of *hypB* were determined by primer extension techniques (Calzone *et al.*, 1987) using RNA, treated with DNase (Promega), from pin stage mushrooms and a primer positioned over the inferred translation startcodon.

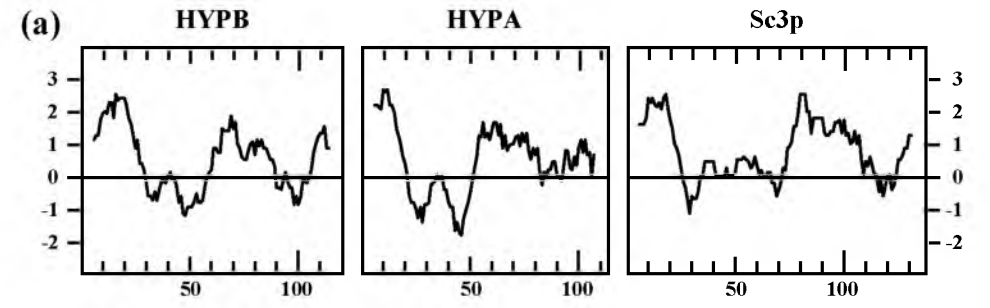
In situ *Northern analysis*

The protocol used for in situ hybridisation was a modification of the method used by Bochenek and Hirsch (1990). Fruit body tissue was fixed in 4% paraformaldehyde, 0.25% glutaraldehyde in 0.1 M potassium phosphate buffer pH 7.4 and sections were embedded in paraffin. Subsequently, 4 µm thick sections were made on a paraffin microtome. Proteinase K treatment was carried out at 37°C and acetylation was performed for ten minutes in 0.25% (v/v) acetic anhydride, 0.1 M triethanolamine pH 8.0. Prehybridisation buffer contained 50% (v/v) deionised formamide, 300 mM NaCl, 1% (w/v) Boehringer Mannheim blocking reagent, 0.15 mg ml^{-1} yeast tRNA, 1 mM EDTA and 10 mM Tris pH 8.0. For hybridisation (45°C), we used prehybridisation buffer to which 5% (w/v) dextran sulphate and 50 ng probe per slide was added. Single strand probes were generated from full length *hypA* and *hypB* cDNA plasmids using T7 and T3 RNA-polymerase. Plasmids were digested on one side of the insert to create a run-off reaction. The coding strands of *hypA* and *hypB* were used as negative controls. After hybridisation, slides were washed in 2xSSC, rinsed in NTE (500 mM NaCl, 0, 1 mM EDTA, 10 mM Tris pH 8.0 and incubated with 5 µg/ml RNAseA at room temperature. Blocking was performed for 4 hours with 1% (w/v) Boehringer blocking reagent in maleate buffer (150 mM NaCl, 100 mM maleic acid pH 7.5) and overnight with 10% (w/v) BSA. Detection of hybrids was carried out with anti-dioxygenin-alkaline-phosphatase conjugate (Boehringer) and NBT according to the manufacturer's instructions. Sections were viewed by light microscopy.

Results

Identification of a new fruit body specific hydrophobin

In a previous study, we screened a primordial cDNA library for genes that were strongly expressed during fruit body development but not in vegetatively grown mycelium (De Groot *et al.*, 1996, 1997). One of the isolated cDNA clones, pDG47, hybridised only in fruit bodies with a messenger of ±500 nt (De Groot *et al.*, 1997). Sequence analysis of this cDNA revealed only one large open reading frame (ORF) which would encode a polypeptide of 119 amino acids with a calculated molecular mass of 11,940. The peptide sequence contains eight cysteine residues located in a way typical of fungal hydrophobins. Furthermore, its small size and the presence of an aliphatic sequence at the N-terminus, which was predicted to comprise a signal sequence for secretion (Nielsen *et al.*, 1997), also indicated that pDG47 encodes a hydrophobin and we designated this gene *hypB*. Apart from the conserved spatial arrangement of the eight cysteine residues, hydrophobins generally have low levels of sequence identity. However, they display similar patterns of hydrophobicity. Based on small differences in these patterns, a division into class I and class II hydrophobins was made (Wessels, 1994). The hydrophobicity pattern of the HYPB protein is very similar to that of HYPA, Sc3p and the other class I hydrophobins (Figure 4.1(a)).



(b)

```
                                                                                *
A. bisporus   HYPB      MVSTFITVAKTLLVALLFVNINIVVG.....................TATTGKHCS..TGPIE
A. bisporus   HYPA       ...MISRVLVAALVALPALVTATPAP.....................GKPKASSQCD..VGEIH
A. bisporus   ABH3      MFARISTIITTLFFAMLAAATAVPR.....................TDPPPATGSQCTAVGGDVN
S. commune    Sc3p      MFARLPVVFLYAFVAFGALVAALPG..GHPGTTTPPVTTTVTVTTPPSTTTIAAGGTCT..TGSLS
P. tinctorius HydPt-1   MKFAAVVVLAAAAAAAVSAETNAQRMARGLPPKAPIRRHGTPADTEKRSHPSSTGGGQCN..TGPIQ
C. cinereus   CoH1       ...MQFKFLSTVALATLAVAAPAP.....................TDPTPIPPSQCN..TGPIQ
Consensus                                                                 C----G---

          **                          *           *     **            *
HYPB      CCKQVMDS.KSPQATELLTKNGLGLGVLAGVKGLVGANCSPITAIGIGSGSQCSGQTVCCQNNNFNG.VVAIGCTPINANV
HYPA      CCDTQQTP..DHTSAAAS...GLLGVPIN.LGAFLGFDCTPISVLGVG.GNNCAAQPVCCTGNQFTALINALDCSPVNVNL
ABH3      CCNSVQDA.SNPIVGLLA...GLLGIVLGPIQGLVGLTCNPISVIGG..GNSCSSQTVCCTGNNFSGGLLVIGCSPINIDL
SC3p      CCNQVQSA.SSSPVTALL...GLLGIVLSDLNVLVGISCSPLTVIGVG.GSGCSAQTVCCENTQFNG.LINIGCTPINIL.
HydPt-1   CCNTVATSGSQSGVDELL...TLLGLSVP.VGTQVGASCSPISAVGTGSGAQCSGQTVCCEQNEWNG.LVNIGCMPINLNA
CoH1      CCNTVTQA.SNPVAGLLL...GLLGIVLQDLNVLVGLTCSPISIIGLP.GNSCNAQPVCCQNNNFNG.LIAIGCTPININL
          CC--V----S------L----GLLG---------VG--C-PI---G---G--C--Q-VCC--N-F-G----IGC-PIN---
```

Figure 4.1. Comparison of the amino acid sequence of HYPB with other basidiomycete class I hydrophobins. (a) Hydrophobicity plots of HYPB, HYPA and Sc3p. Points above the x-axis indicate hydrophobic regions. (b) Alignment of the HYPB amino acid sequence to those of HYPA/ABH1 (De Groot *et al.*, 1996; Lugones *et al.*, 1996), ABH3 (Lugones *et al.*, 1998), *S. commune* Sc3p (De Vocht *et al.*, 1998), *P. tinctorius* HydPt-1 (Tagu *et al.*, 1996) and *C. cinereus* CoH1 (EMBL accession no. Y10627). The consensus sequence is composed of residues shared by at least five of the proteins. The N-terminal amino acids of the mature HYPA and Sc3p proteins and the putative N-terminal end of mature HYPB are underlined. The eight conserved cysteines which were used as fixed coordinates are marked by asterisks.

By comparison with protein database sequences we found that the polypeptide translated from *hypB* has significant homology with only some of the other class I hydrophobins. Highest levels of sequence identity with HYPB were found for hydrophobins of three other basidiomycetes; Sc3p of *S. commune*, HydPt-1 of the ectomycorrhizal fungus *Pisolithus tinctorius* and CoH1 of *Coprinus cinereus*. When the cysteine residues are used as fixed coordinates in aligning, the remaining amino acids can be aligned in a way that they show 37% - 39% identity (Figure 4.1(b)). Excluding the eight cysteine residues, the previously isolated *A. bisporus* hydrophobins HYPA, HYPC and ABH3 show only 25% - 31% identity with HYPB.

A genomic library of the homokaryotic strain H39 was then screened. Southern analysis of isolated λ-clones and genomic DNA resulted in the restriction map which is presented in Figure 4.2(a). Comparison of the genomic sequence with the sequence of the cDNA clone showed that the *hypB* ORF is interrupted by two intervening sequences of 79 and 71 bp with normal splice junctions (Figure 4.2(b)). Translation probably starts with the first ATG of the ORF at position 1. The sequence surrounding this start codon conforms to the consensus sequence for translation initiation of highly expressed genes (Kozak, 1989). The transcriptional start of the *hypB* gene was determined by primer extension analysis using RNA isolated from pin stage mushrooms as template. The major transcription start point in this tissue is located at position -97 relative to the inferred start codon and a minor start point was found further upstream at position -296. Fifty three bp downstream of the stop codon the transcript ends in a poly(A) tail, and this site is preceded by an AT-rich sequence, which is often found in eukaryotic genes (Humphrey & Proudfoot, 1988). In the *hypB* promoter a TATA-box (Bucher, 1990), starting 33 bp upstream of the major transcription initiation site, is found.

For a number of the *A. bisporus* genes that have been cloned and sequenced, gene duplications were found (Harmsen *et al.*, 1992; De Groot *et al.*, 1996; Van de Rhee *et al.*, 1996; Smith *et al.*, 1998) and in all cases the duplicated copies are immediately adjacent

and in the same orientation. This led us to analyse the regions surrounding *hypB* by hybridisation and sequence analysis. A second copy of the *hypB* gene was not found in the *A. bisporus* genome. However, 2.5 kb upstream of the *hypB* gene a large ORF was found (Figure 4.2(a)) and a cDNA corresponding to this ORF was isolated. By database analysis, this cDNA was recognised as coding for the iron-sulphur protein subunit of succinate dehydrogenase, showing 85% sequence identity with the iron-sulphur protein subunit of the oyster mushroom *Pleurotus ostreatus* (EMBL accession no. AB007361) and 74% identity with the same protein subunit of the basidiomycete yeast *Ustilago maydis* (Broomfield & Hargreaves, 1992). Northern analysis showed that the gene, designated *sudA*, is also

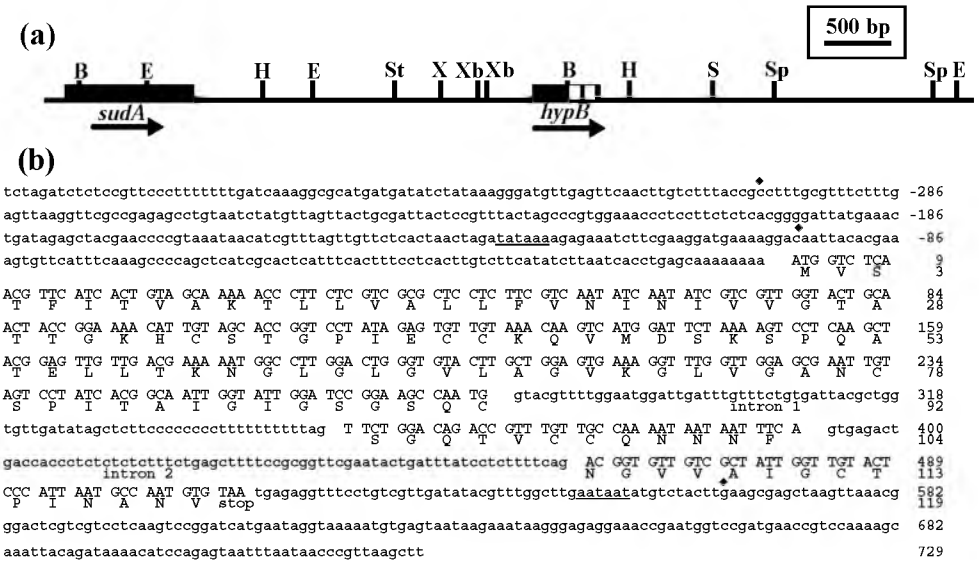


Figure 4.2. Genomic organisation of *hypB*. (a) Molecular map of the *hypB* locus. The *hypB* and *sudA* coding regions are represented by black boxes and introns in the coding region of *hypB* are represented by open boxes. Horizontal arrows indicate orientation of transcription of *hypB* and *sudA*. The following restriction sites are indicated: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sp, *Sph*I; St, *Sst*I; Xb, *Xba*I; X, *Xho*I. (b) Nucleotide sequence of the 1.1 kb *Xba*I - *Hin*dIII fragment comprising the *hypB* gene and its flanking regions. Coding regions of *hypB* are shown in uppercase letters, flanking regions and intervening sequences in lowercase letters. The deduced HYPB amino acid sequence is shown underneath the coding regions. Transcription start points and the polyadenylation site are indicated by diamonds. A putative TATAAA promoter element and the AT-rich sequence preceding the polyadenylation site are underlined.

expressed in vegetatively grown mycelium and thus is regulated independently from *hypB* (results not shown).

Temporal and spatial expression of *hypA* and *hypB*

The transcriptional regulation of the two fruit body specific hydrophobin genes was compared by determining the mRNA levels of *hypA* and *hypB* in total fruit body tissue during development and in various parts of mature mushrooms by Northern analyses. Developing fruit bodies were obtained from a ten day flushing cycle using commercial cultivation conditions. Fruit bodies were picked from the first visual appearance of primordia, five days after initiation of fruit body formation, until the mature mushrooms have their typical button-like appearance and expose their spore-bearing gills by breaking the veil (see Figure 4.3 for details). The normalised temporal expression of the two genes during fruit body development is presented in Figure 4.4(a). For *hypA*, a more or less constant mRNA level is observed during mushroom development and maturation. This pattern differs from *hypB*, for which highest mRNA levels are observed during the early stages of fruit body development and for which after day eight of development the mRNA level decreases threefold to the level observed in mature mushrooms.

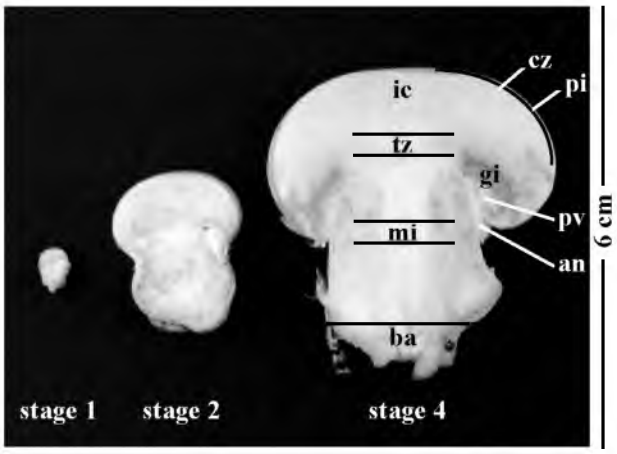


Figure 4.3. Progressive morphology of fruit bodies. Stages of development are according to De Groot *et al.* (1997). In the stage 4 mushroom the morphologically distinct tissues that are used for analysis of spatial expression patterns of *hypA* and *hypB* are indicated. Abbreviations are: ba, stipe

basis; mi, mid-stipe; tz, transitional zone; an, annulus; gi, gills; ic, inner cap; pv, partial veil; pi, pileipellis; cz, connecting zone.

For determination of the mRNA levels of *hypA* and *hypB* in various parts of mature fruit bodies, caps and stipes of mushrooms were separated and further fractionated (Figure 4.3). From caps we isolated the pileipellis (peel), inner cap tissue (plectenchyma), a zone between the pileipellis and the plectenchyma (the connecting zone), partial veil tissue, and gill tissue (lamellae). The interconnected tissues of the partial veil and the pileipellis, both consisting of white hyphae (Umar and Van Griensven, 1997), are difficult to separate, making isolation of only partial veil tissue technically difficult. The partial veil sample therefore contained both partial veil and pileipellis tissue. From the stipes, we isolated the annulus as a separate fraction and the stipes were then sectioned horizontally into 3 mm slices. Of these, three sections were analysed: the transitional zone (or upper part), comprising the physical connection of the stipe with the cap, a mid-stipe section and the stipe basis that connects the mushroom with its mycelial cord. Northern analyses were done

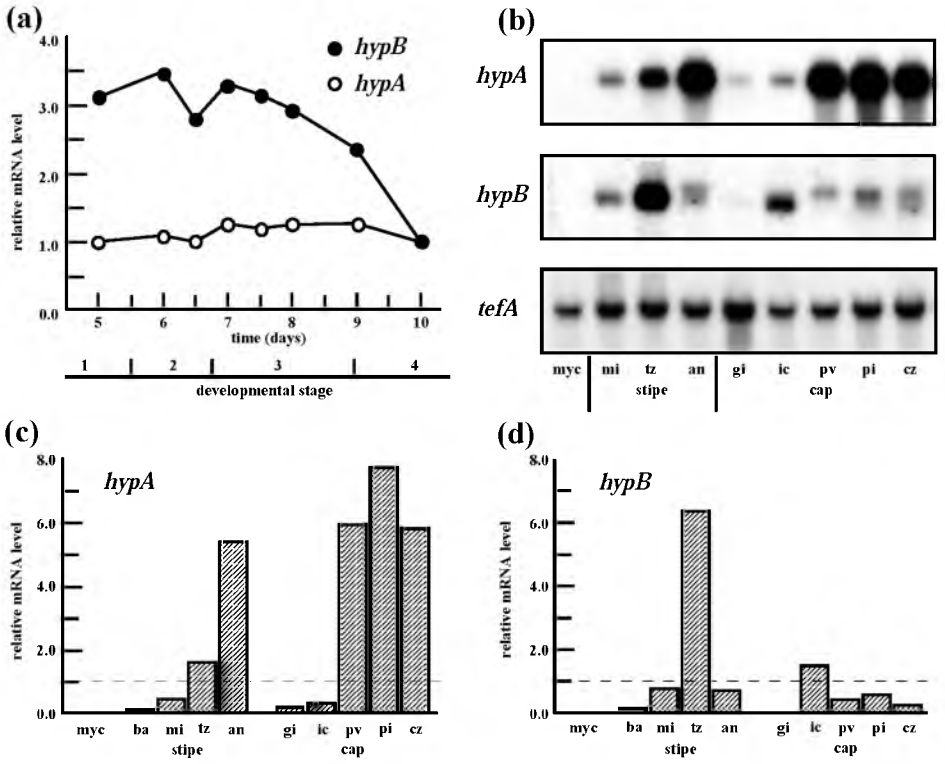


Figure 4.4. Northern analysis of the hydrophobin genes in developing fruit bodies. (a) Graphs of the relative mRNA levels of *hypA* and *hypB* in fruit bodies from day 5 (stage 1) to day 10 (stage 4). (b) Transcript levels of *hypA*, *hypB* and the *tefA* gene encoding translation elongation factor 1α in different parts isolated from stage 4 mushrooms and in compost grown mycelium. (c) and (d) Bar graphs of the relative mRNA levels of *hypA* and *hypB* in different parts of stage 4 mushrooms. The abbreviations of Figure 4.3 were used and myc denotes compost grown mycelium. The pv sample contains both partial veil and pileipellis. Relative transcript levels were calculated by normalisation against 28S rRNA and the mRNA level obtained for whole stage 4 mushrooms was set to 1.0.

with size fractionated RNA from the various fruit body parts except for RNA from the stipe basis which did not fractionate properly on agarose gels and was therefore analysed by slot blot techniques.

Figures 4.4(b) and 4.4(c) show that in mature fruit bodies *hypA* mRNA accumulates in the annulus, the partial veil, pileipellis tissue and in the zone connecting the pileipellis and the plectenchyma, which is in agreement with previous results (De Groot *et al.*, 1996). The *hypA* mRNA level in the transitional zone was more than threefold lower, whereas *hypA* is expressed at relatively low levels in inner cap tissue, in gills and in stipe tissue.

For *hypB* the distribution of mRNA in mature mushrooms is completely different (Figure 4.4(b)). A high concentration of *hypB* messengers is found in the transitional zone and an approximately fivefold lower *hypB* mRNA level is detected in inner cap tissue (Figure 4.4(d)). In gills, *hypB* mRNA was below the detection level. In all other fractions, *hypB* messenger levels were significantly lower than in the transitional zone and the inner cap tissue. Furthermore, within and between assayed fruit body parts, *hypB* messengers of different sizes are found. In accordance with the primer extension experiment, this again indicates that *hypB* transcription can start at different positions.

For both hydrophobin genes, no transcripts were found in compost grown mycelium. The distribution of *hypA* and *hypB* messengers RNA in mature mushrooms was compared with the expression pattern obtained with the *tefA* gene encoding translation elongation factor 1α. As was found previously (Schaap *et al.*, 1997), the *tefA* gene shows equal transcript levels in all assayed parts of the fruit body and in vegetative mycelium. This suggests that all assayed fruit body parts are metabolically active.

Spatial expression of *hypA* and *hypB* during development

The previous experiment using conventional Northern analysis techniques showed that in mature mushrooms highest expression of *hypB* is found in the transitional zone. It also shows that *hypB* transcription is more prominent during the early stages of development and declines thereafter. In order to study the spatial expression of the hydrophobin genes at the early stages of fruit body development we applied *in situ* hybridisation techniques on primordia. Figure 4.5(a) shows that, with this technique differentiation into cap and stipe tissue can already be observed in pin-shaped primordia that appear five days after initiation of fruit body formation. At this stage of development *hypA* messengers can be detected in the universal veil covering the whole primordium. However, highest *hypA* mRNA levels are found in the outer regions of future cap tissue. For *hypB*, highest mRNA levels are also found in the future cap, but the expression is concentrated in other, more inward, cell

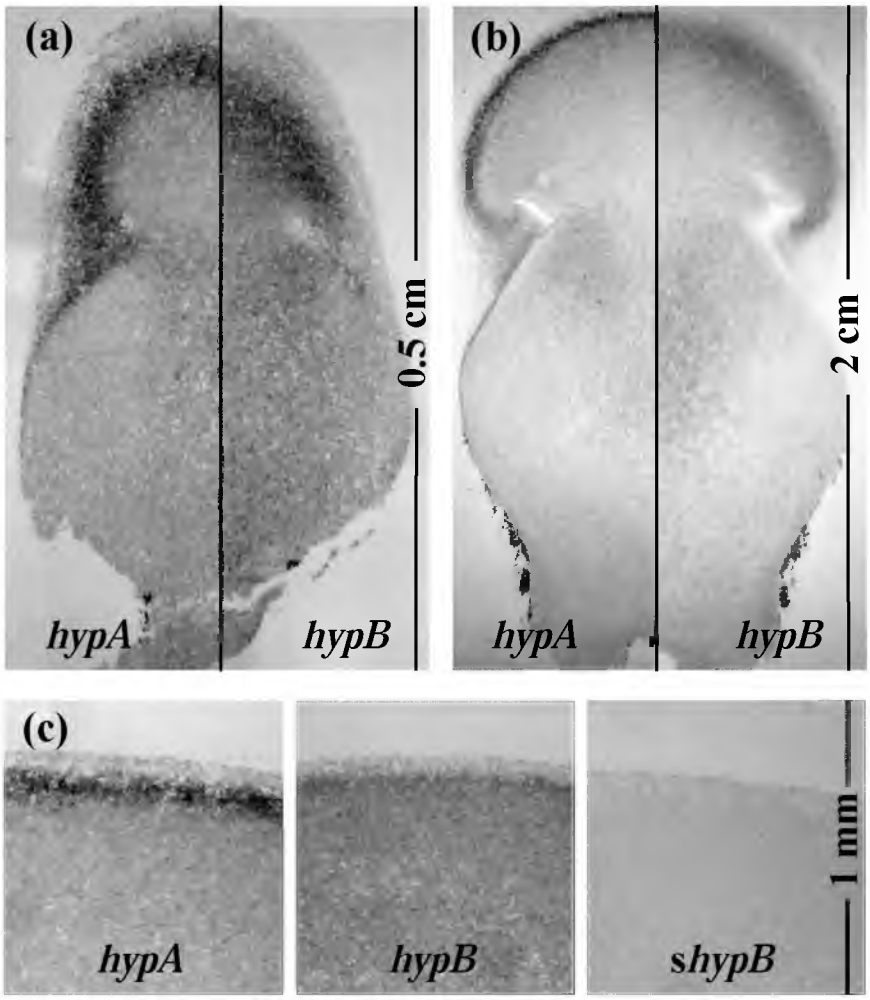


Figure 4.5. *In situ* Northern analyses showing the spatial expression of *hypA* and *hypB* during fruit body development. (a) Longitudinal sections of whole fruit body at stage 1 and (b) at stage 2 of development. (c) Longitudinal sections of the upper part of the cap a mature mushroom. Paraffin embedded fruit body tissues were hybridised with the non-coding strand of *hypA* and *hypB* and, as a negative control, with the coding (sense) strand of *hypB* (s*hypB*).

layers. In the future stipe *hypB* messengers are also clearly present in the inner regions but at a lower level whereas *hypA* mRNA in the stipe is hardly detectable. As negative controls, primordia slides were probed with the sense strand of *hypA* and *hypB* and no hybridisation

signals were obtained (results not shown). At stage 2, *hypA* messengers clearly accumulate in the pileipellis and veil hyphae that cover the future cap and highest *hypB* mRNA levels are again found in cells located more inward in the fruit body cap. At stage 4, the mushrooms are too large to be analysed as a whole, and we therefore analysed the outer (top) region of a mushroom cap (Figure 4.5(c)). In this region, a thin layer of cells show high expression of *hypA* whereas *hypB* mRNA levels in the outer region of the mushroom cap are low. Again, no signals were found after probing with the sense strands of *hypA* and *hypB* (Figure 4.5(c)). These results are consistent with the results obtained by the conventional Northern analysis presented in Figure 4.4. However, the *in situ* analysis also demonstrated that the high levels of *hypA* mRNA in the pileipellis are not produced by the outermost cell layers of the mushroom caps but by cells that are located more inward as can be seen clearly in Figures 4.5(a) and 4.5(c).

Discussion

The results presented here show that the gene corresponding to cDNA clone pDG47 belongs to the fungal family of hydrophobin genes. This *hypB* gene is, after the tandemly linked *hypA* and *hypC* genes (De Groot *et al.*, 1996), the third fruit body specific hydrophobin encoding gene that has been found in *A. bisporus*. In contrast to the expression levels of *hypA* and *hypB*, those of *hypC* are low, suggesting that the HYPC protein is probably not indispensable for normal fruit body development. The duplicated region comprising *hypC* is located downstream of *hypA* and starts 310 bp upstream of the putative translation start of *hypC*. This indicates that fruit body specificity of transcription is determined by the duplicated promoter region present in both *hypC* and *hypA*, whereas an element responsible for the high expression levels of *hypA* in certain tissues of the fruit body might be located further upstream in the *hypA* promoter. Comparison of the *hypA* and *hypC* promoter regions with the *hypB* promoter did not reveal similar sequences which could be indicative of regulating elements in these fruit body specific promoters.

The identification of multiple hydrophobins of *A. bisporus* of which two are expressed at high levels in fruit bodies led us to investigate whether these proteins have dissimilar functions. The presence of multiple hydrophobin genes is not unique to *A. bisporus* but has also been shown for three other basidiomycetes, *S. commune* (Schuren & Wessels, 1990; Wessels *et al.*, 1995), *C. cinereus* (Wessels, 1997) and *P. tinctorius* (Tagu *et al.*, 1996) and the ascomycetes *Aspergillus nidulans* (Stringer *et al.*, 1991; Stringer & Timberlake, 1995)

and *Trichoderma reesei* (Nakari-Setälä *et al.*, 1996, 1997). Differences in expression of the hydrophobins of most of these fungi indicate that the different hydrophobins contribute to surface hydrophobicity of morphologically different structures. Although this may be true for all filamentous fungi, especially in the case of the Basidiomycetes which often produce large and complex developmental structures, the presence of multiple surface-active hydrophobins seems necessary. For example, of the four hydrophobin encoding genes discovered in *S. commune*, *Sc3* was only expressed in vegetatively growing mycelium of both mono- and dikaryons, whereas *Sc1*, *Sc4* and *Sc6* were dikaryon specific and associated with fruit body formation. Expression patterns obtained with *in situ* hybridisations of the dikaryon specific genes indicate that regulation of *Sc4* differs from *Sc1* and *Sc6* (Ruiters & Wessels, 1989). Separate functions were also suggested by the low level of sequence identity between the *S. commune* hydrophobins and from the fact that the secondary mycelium of *Sc3*⁻ deletion strains produced aerial hyphae that were hydrophilic (Van Wetter *et al.*, 1996). The expression patterns observed with Northern analyses of *hypA* and *hypB* show that the two genes are differently regulated in time and space and indicates that each of these *A. bisporus* hydrophobins has its specific role in development.

The highest transcript levels of *hypB* are found during the early stages of fruit body development. During these stages the primordium differentiates into a distinctive cap and stipe. Using *in situ* Northern hybridisation techniques it was shown that in primordia highest levels of *hypB* messengers are found in the inner regions of the future cap structure. In mature mushrooms that show a threefold overall decrease of *hypB* mRNA levels, a strong accumulation of *hypB* mRNA was found only in the transitional zone using conventional Northern analysis. These results suggest that the HYPB hydrophobin is instrumental in the differentiation process and that HYPB could be part of a mono- or bilayer that is formed between fruit body caps and stipes. This might relate to the fact that bacterial infections, starting in peel tissue, often are unable to invade the mushroom caps (Umar & Van Griensven, 1995). Another possibility is that, analogous to the proposed function of the Sc4p hydrophobin of *S. commune* (Wessels *et al.*, 1995), HYPB is functional in coating air channels in the (stipe of the) fruit body.

Messenger levels of the *hypA* gene during development are more or less constant. At the primordial stage of development, high expression of *hypA* is observed in the universal veil covering the whole "undifferentiated" primordium. In mature fruit bodies, *hypA* mRNA is mainly found in the pileipellis, the partial veil and the annulus. These fruit body parts consist of so-called white hyphae, which are suggested to represent a lower grade of differentiation than the hyphae of the cap, stipe and gills (Umar & Van Griensven, 1997).

In a previous paper (De Groot *et al.*, 1996) in which the cloning and regulation of the *hypA* gene was described, elevated expression of this gene was also observed in gill tissue. However, the gill fraction used in that paper was probably contaminated with partial veil tissue in which *hypA* is highly expressed. After improving our method of isolating spore-bearing gills, we now have obtained a more pure gill fraction that showed low expression of *hypA* and no expression of *hypB*, whereas *tefA* mRNA levels in gills were equal to the mRNA levels in other parts of mature fruit bodies. Whether this is a general phenomenon in Basidiomycetes is unknown. The hydrophobins discovered in the ascomycetes *A. nidulans* and *Neurospora crassa* are part of the rodlet layer surrounding conidiospores and contribute to the hydrophobicity of the spore surface. Using a different approach of *in situ* hybridisation, hybridisation on fixed tissue prior to embedding in the glycol methacrylate resin Technovit 7100, we could analyse expression of the *hypA* gene in gill tissue. In preliminary experiments, we detected *hypA* mRNA only in subhymenial cells (results not shown), which could indicate that these cells produce HYPA monomers that may be part of a hydrophobic layer surrounding the basidiospores.

The *in situ* Northern analyses also showed that the *hypA* mRNA levels in the outermost layer of hyphae are low compared to cell layers located more inward (Figures 4.5(a) and 4.5(c)). This implies that HYPA protein, which was previously shown to surround the mushroom caps (De Groot *et al.*, 1996; Lugones *et al.*, 1996) is mainly produced by these inner layers of hyphae and that self-assembly of HYPA monomers does not take place before these molecules have reached the interface between the mushroom surface and the air. This supports the idea that *in vivo* HYPA specifically self-assembles at hydrophobic-hydrophilic interfaces. In agreement with these results, hot-SDS resistant HYPA layers are formed when aqueous solutions of purified HYPA monomers are brought in contact with hydrophobic surfaces (Lugones *et al.*, 1996; Gunning *et al.*, 1998) and this behaviour is similar to the interfacial self-assembly observed for Sc3p (Wösten *et al.*, 1994a, 1994b). The reason for the relatively low HYPA production in the outermost layer of hyphae of the pileipellis compared to the connecting zone is not clear. Perhaps the HYPA layer surrounding these cells hampers secretion of HYPA monomers.

Acknowledgements

The authors thank Drs J. Baars and A. Sonnenberg of the Mushroom Experimental Station for their expertise in culturing the *A. bisporus* strains and for fruitful discussions.

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Atomic Force Microscopy of a hydrophobin protein from the edible mushroom *Agaricus bisporus*

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Journal of Colloid and Interface Science **201,** 118-126 (1998)

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Atomic Force Microscopy (AFM) has been used to study the fungal hydrophobin protein HYPA produced by the mushroom *Agaricus bisporus*. The protein was adsorbed from dilute solution onto highly oriented pyrolytic graphite (HOPG), a hydrophobic and atomically flat substrate, and the resulting self-assembled layers imaged under n-butanol. The nature of the self-assembled layer was found to be influenced by the protein concentration in the solution. At relatively high protein concentrations (20 μg ml^{-1}) the monolayer formed contained randomly-oriented protein molecules. However, at lower protein concentrations (2 μg ml^{-1}) a highly ordered monolayer was formed with a higher level of surface coverage. The protein molecules appear to assemble end-to-end to form short rods with an average length of 80 nm. The thickness of the ordered monolayer is uniform and around 3.6 nm. Finally, the effect of washing the adsorbed protein layers with solutions of sodium dodecyl sulphate, leading to partial removal of the HYPA monolayer and bilayer formation, and of washing with trifluoroacetic acid which strongly disrupts the adsorbed monolayer, were also examined.

Introduction

Hydrophobins are small proteins which have been found to be secreted from various filamentous fungi (De Groot *et al.*, 1996; Lugones *et al.*, 1996; Wessels, 1997). The characteristic features of these proteins are the presence of eight cysteine residues, whose spacing is conserved, and a typical hydrophobicity pattern. Hydrophobins seem to contribute to surface hydrophobicity, which is important for processes such as adhesion of hyphae in reproductive structures, dispersal of aerial spores and adhesion of pathogenic fungi to host structures (Wessels, 1994; Wösten *et al.*, 1994, 1995). In the edible mushroom *Agaricus bisporus* the HYPA hydrophobin was proposed to be instrumental in the formation of a protective coat around the cap of its fruit bodies which might protect them from bacterial infection and other environmental effects (De Groot *et al.*, 1996; Lugones *et al.*, 1996). Transmission electron microscopy (TEM) studies of the well studied Sc3p hydrophobin, secreted by *Schizophyllum commune*, have illustrated its ability to self-assemble at water-solid and water-gas interfaces (Wösten *et al.*, 1993). It was proposed that the monomeric Sc3p units form insoluble rodlike particles at such interfaces, although individual proteins were not resolved within the rods (Wösten *et al.*, 1993). The layer so formed was typically either highly hydrophobic, in the case of adsorption to a hydrophilic surface such as the aerial hyphae of a fungus (Wösten *et al.*, 1993), or moderately hydrophilic when the protein was adsorbed to a hydrophobic surface such as

teflon (Wösten *et al.*, 1994). Detailed studies of the adsorption of Sc3p to hydrophobic surfaces have been carried out demonstrating a strong correlation between amount of adsorbed protein and surface hydrophobicity (Wösten *et al.*, 1995). HYPA is a similar hydrophobin protein and has a molecular mass of 8 - 9 kDa calculated from its amino acid sequence and estimated on SDS-PAGE (De Groot *et al.*, 1996). Comparison of the HYPA sequence with protein bank databases showed the highest level of homology for HYPA with the hydrophobin family of the basidiomycete *S. commune* (Schuren & Wessels, 1990; De Groot *et al.*, 1996) and like the hydrophobins Sc3p and Sc4p, it forms a hot SDS resistant aggregate which can be dissolved by trifluoroacetic acid (Wösten *et al.*, 1993; De Groot *et al.*, 1996). In a more recent study the HYPA hydrophobin secreted by *Agaricus bisporus* (named ABH1 by these authors) has been shown to self-assemble at hydrophobic-hydrophilic interfaces, producing a typical rodlet layer pattern (Lugones *et al.*, 1996). The present study reports the results of atomic force microscopic examination of the adsorption of HYPA to a hydrophobic graphite surface.

Atomic force microscopy (AFM) has been used to visualise many proteins (Droz *et al.*, 1994; Kouyama *et al.*, 1994; Mastrangelo *et al.*, 1994; Morris, 1994; Fritz *et al.*, 1995; Han *et al.*, 1995; Mazzola *et al.*, 1995) including some in the form of interfacial films (Fare *et al.*, 1992; Birdi *et al.*, 1994; Yamada *et al.*, 1994; Boury *et al.*, 1995; Dubreuil *et al.*, 1995; Gunning *et al.*, 1996; Von Nahmen *et al.*, 1997). AFM is most powerful when scanning relatively flat samples. A protein monolayer is just such an ideal sample meaning that molecular resolution is relatively straightforward to obtain (Gunning *et al.*, 1996). AFM can provide images which are at least comparable in resolution to TEM, but has the great advantage of being able to image the sample directly without the need for staining/fixation, coating or freeze-fracture replication normally employed in conventional electron microscopy. Because of this AFM can sometimes provide higher resolution images than TEM since the sample surface is not obscured by metal grains. In the present study, a significant advantage of AFM over electron microscopy is that the samples are not subjected to any derivatisation steps which may alter structure and introduce artefacts, since the AFM images were obtained under liquid at ambient temperature and pressure. The importance of minimising sample preparation for weak networks such as self-assembled protein films or protein films formed using Langmuir-Blodgett techniques cannot be overstated since their detailed structure is highly susceptible to adverse environmental effects such as complete dehydration in vacuo or freezing (Brooker, 1993). Great care must therefore be used in the interpretation of electron microscopic images of such samples. Thus, AFM has the ability to image interfacial proteins at extremely high resolution under

far more natural conditions than electron microscopy and so represents a complementary and powerful tool.

Materials and Methods

Atomic force microscopy

An East Coast Scientific (ECS Ltd. Cambridge, UK) AFM was used in this study, operated in the constant force mode. The sample was contained in a liquid cell and imaged under redistilled butanol. Working under butanol allowed very low imaging forces to be attained, and these were typically in the range 0.3-0.5 nN. We found it necessary to use the minimum imaging force possible with a set-point for the cantilever very close to the point at which the tip will jump off the surface. This meant that the tip would often jump off the surface during a scan but with patience good images could be obtained. If the force was increased to aid stability during imaging then this always resulted in a loss of image quality, to the extent that the individual protein molecules were not clearly resolved. Tips used were Nanoprobe™ Si_3N_4 sharpened levers with a quoted force constant of 0.38 $N.m^{-1}$ (Digital Instruments, Santa Barbara, USA). The AFM was calibrated in the Z piezo direction using Ultrasharp™ calibration gratings TGZ series (NT-MDT Co., Moscow, Russia). These provided a three point height calibration (25.5 nm, 106 nm and 512 nm).

Image analysis

Several areas of the sample were examined to ensure that the coverage was the same everywhere prior to washing, since it was not possible to scan exactly the same location on the substrate after the washing step. Furthermore, the same AFM tip was used to ensure consistency in the images after successive washes. Protein area determination was carried out using Visilog v.4.1.3 rev 6 (Noesis, Velizy France). The images were thresholded to produce a binary image (i.e. two grey levels, black for the substrate and white for the protein layer) and then the white (protein areas) quantified by counting the pixels. The threshold parameters were constant for all images compared to ensure consistency.

Values calculated by such measurements are only comparable as an estimate of protein coverage level when a monolayer is present, since area determination uses only the x and y dimensions of the image, and therefore images of areas containing bilayers (present after the hot SDS wash) were not used and no estimate of coverage after TFA washing was carried out.

Protein purification

Mature *A. bisporus* Horst®U1 fruit bodies were obtained using commercial cultivation conditions (Van Gils, 1988). Peel tissue from fruit body caps was collected, frozen into liquid nitrogen and fragmented using a Waring blender. After addition of ten volumes of a 1% SDS solution, the suspension was kept at 100°C for 10 minutes and subsequently centrifuged. The SDS washing step was repeated twice with the pellet which was then washed five times with 20 volumes of water and freeze-dried. The pellet was resuspended in trifluoroacetic acid (TFA) and after overnight incubation at 4°C, insoluble material was removed by centrifugation. Under the conditions used the TFA would not hydrolyse or extract cell wall polysaccharides and any such contaminants would be removed by the centrifugation step. TFA was removed from the supernatant by evaporation under a stream of air. The TFA extracted proteins were dissolved in distilled water and solids were removed by centrifugation. The protein samples were freeze dried and stored at 4°C until use. Protein solutions

were prepared by gently dissolving the freeze-dried sample in distilled water in slowly rotating tubes filled completely with degassed distilled water to avoid assembly at an air/water interface. Protein samples were analysed on SDS-PAGE to confirm that only 9 kDa monomers were present and concentrations of 20 μg ml^{-1} and 2 μg ml^{-1} were used for deposition experiments, the second more dilute solution being prepared by dilution of the first.

Deposition of HYPA solutions

In order to study the adsorption of HYPA to hydrophobic surfaces the substrate highly-oriented pyrolytic graphite (HOPG) was chosen because it is extremely hydrophobic, crystalline and easily cleavable. When freshly-cleaved it produces atomically flat, clean surfaces which provide an excellent substrate for imaging small molecules by AFM provided that they are hydrophobic. Protein adsorption was achieved by incubating pieces of freshly-cleaved graphite in the HYPA solutions in a glass cuvette. The cuvette was completely filled with solution and then sealed with a glass coverslip (placed over the top of the liquid to exclude any air). Previous studies carried out by Wösten and co-workers (Wösten *et al.*, 1994) demonstrated a concentration-dependent adsorption of the Sc3p protein of *S. commune* to teflon surfaces. The similarity in function and behaviour of HYPA with the *S. commune* family of hydrophobins, led us to assume that HYPA would also exhibit concentration-dependent adsorption and therefore different time courses were used for the adsorption experiments with HYPA solutions of different concentrations. At the higher concentration of 20 μg ml^{-1}, the graphite was incubated for 15 minutes and at the lower concentration of 2 μg ml^{-1} incubation was for 16 hours. After incubation, the substrates were removed and washed in distilled water for about 5 minutes and any excess water was removed from the graphite using the edge of a filter paper. The samples were air-dried for 10 minutes prior to imaging under butanol.

SDS and TFA washes

Sodium dodecyl sulphate (SDS) and TFA were purchased from Sigma Chemicals Ltd. (Dorset, UK). Washing was carried out after an initial water rinse by incubating a 20 μg ml^{-1} sample in cold (20°C) and subsequently hot (98-100°C) 2% SDS solution for 10 minutes. After both SDS washes the sample was rinsed thoroughly with distilled water, air-dried and imaged by AFM. The TFA wash was performed by incubation of a fresh 20 μg ml^{-1} sample in undiluted TFA for 15 minutes and subsequently rinsing and air-drying the sample.

Results and Discussion

Typical images obtained for fast deposition of HYPA onto graphite from the higher concentration 20 μg ml^{-1} solution are shown in Figure 5.1. The images obtained confirm the affinity of HYPA for hydrophobic surfaces, since it is highly unusual for biopolymers (e.g. proteins or polysaccharides) to stick to HOPG well enough to allow imaging by AFM, especially if rinsing is carried out (Droz *et al.*, 1994). Indeed HOPG is normally considered

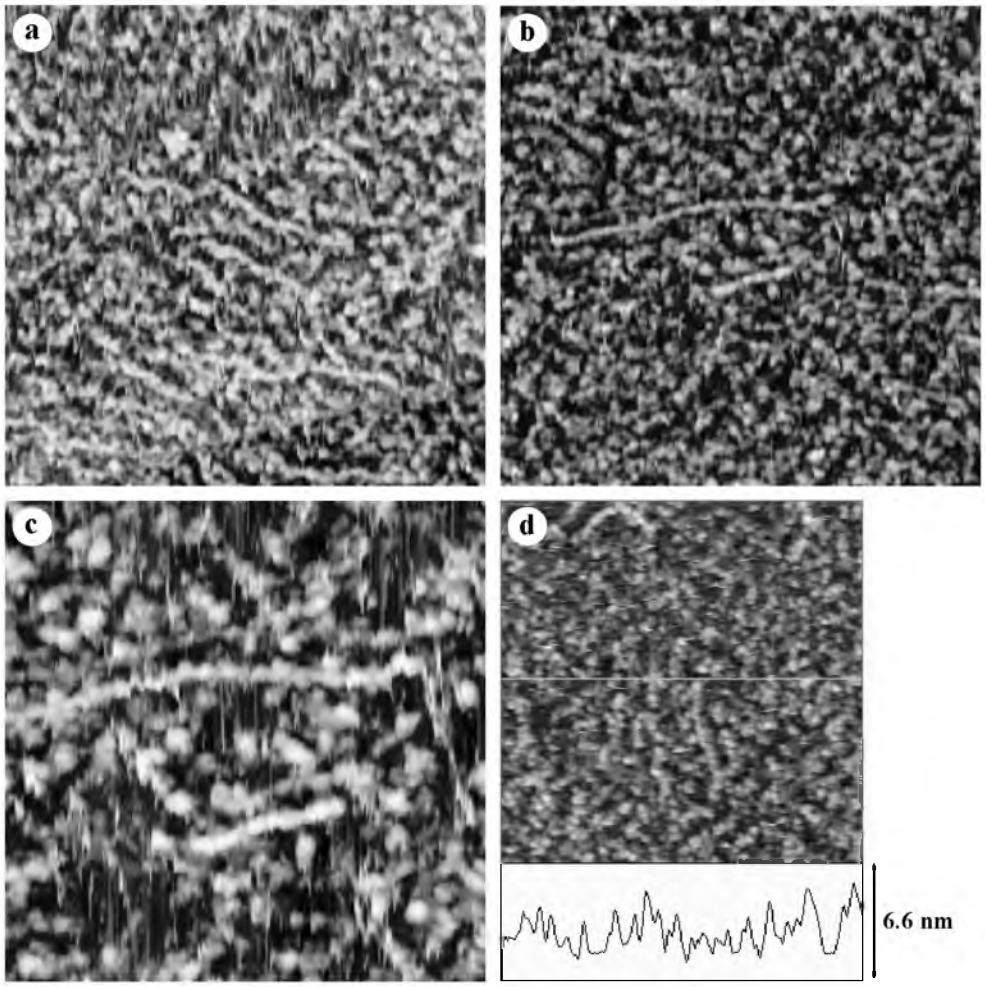


Figure 5.1. HYPA adsorbed onto graphite from a 20 μg ml^{-1} solution. (a) Typical area. Scan size: 800 x 800 nm, height: 7.4 nm (black to white). (b) String-like assemblies. Scan size: 800 x 800 nm, height: 7.5 nm (black to white). (c) String-like assemblies. Scan size: 400 x 400 nm, height: 6.2 nm (black to white). (d) Profile across molecular layer (peak height 3.9 nm). Scan size 600 x 600 nm.

a poor substrate for imaging proteins, most of which are hydrophilic (Droz *et al.*, 1994). Figure 5.1(a) shows a fairly uniform coverage of globular features over large areas, and height measurements taken across the images (Figure 5.1(d)) indicate that the features are approximately 1.7-3.9 nm high and the widths range from 21-28 nm. Widths measured by AFM are often larger than the actual width of the object being measured due to an effect known as probe-broadening (Kirby *et al.*, 1996). This arises because the AFM tip is large (typically 10-40 nm radius) compared to the object being imaged and so different regions of the tip interact with the sample as it is scanned under the tip. The result is to 'smear out' or broaden the profile of the object. Measured heights are unaffected by this process and give a more reliable indication of particle size. AFM has a distinct advantage over electron microscopy in that it can measure sample heights directly and with great accuracy. Theoretical dimensions calculated for HYPA, based on a molecular mass of 8 - 9 kDa and assuming it to be spherical with a partial specific volume of 0.74 x 10^{-6} $m^3.g^{-1}$, suggest a diameter of 2.6-2.8 nm. Measured heights of the globular features seen in Figure 5.1(d) range from 1.7-3.9 nm, suggesting that they are single HYPA molecules but are probably non-spherical in shape and randomly oriented on the graphite surface. Thus the AFM has provided molecular resolution of the HYPA proteins. This observation is consistent with the work of Wösten *et al.* (1993) who found that a 10 nm thick monolayer of Sc3p, a 14.5 kDa protein, was formed on hydrophobic surfaces. The HYPA molecules appear to be randomly orientated on the substrate which may account for the variation in their heights, particularly if they are not perfect spheres.

When the deposit is examined in more detail (Figures 5.1(b, c)) long string-like structures are often visible, suggesting that the molecules have some capacity for self-association. This is consistent with the proposed self-assembly of hydrophobins at interfaces suggested by Wösten *et al.* (1993), although from these images it is not possible to be sure whether this happens only at the liquid/solid interface upon adsorption or whether the string-like objects were present in the HYPA solution prior to their adsorption. However, the images which were obtained for the more dilute HYPA solution provide experimental evidence supporting the former explanation.

Figures 5.2(a - g) show typical examples of the images seen after the graphite substrate was incubated in the more dilute (2 $\mu g\ ml^{-1}$) HYPA solution. The deposit is strikingly different to that obtained for the high concentration/fast deposition experiment shown in Figures 5.1(a - d). The HYPA molecules appear to have adsorbed uniformly over the substrate (Figures 5.2(a, b)) and the features are rod-like. Figures 5.2(a - g) are typical of every area examined. The adsorption is much more ordered as can be seen in Figures

5.2(c, d). Image analysis indicates a high efficiency of HYPA coverage of the graphite surface of the order of 89%. Furthermore, the HYPA layer exhibits a very uniform height or thickness of 3.5 - 3.8 nm (Figure 5.2(e)), which is close to the highest value obtained from the fast deposition onto graphite (Figure 5.1(d)). The much smaller variation in the heights of the features, as compared to the fast deposition with the high concentration HYPA solution, might be expected for a more ordered layer (Kirby *et al.*, 1996). The measured heights of the molecules are larger than the calculated theoretical height for spherical HYPA molecules (2.6-2.8 nm) which suggests that bound HYPA molecules are not spherical. Furthermore, the uniformity of height over large areas suggests that the molecules are bound in a specific orientation in which protein orientation with the major dimension perpendicular to the surface would optimise packing, presumably with the apolar side to the hydrophobic substrate surface. These results are consistent with the Sc3p adsorption experiments of Wösten *et al.* (1993), and indicate that the images represent monolayer coverage of the HOPG surface.

The widths of the rod-like features measured in Figure 5.2(e) are 16-23 nm which is smaller than those measured in Figure 5.1(d). This is to be expected due to the reduction in probe-broadening when scanning close-packed particles and probably represents a more realistic estimate of molecular width. The lengths of the rods are fairly uniform; a typical value being 80 nm. There are no long 'string-like' rods similar to those seen in the higher concentration sample (Figures 5.1(a - d)) and the observed uniformity suggests that rod-length is governed by the space available on the substrate. This in turn suggests that molecular association does not happen in solution prior to deposition, and that self-assembly of the HYPA molecules occurs at the liquid-solid interface as suggested for Sc3p by Wösten *et al.* (1993). Further evidence for this conclusion comes from the fact that, once associated, the HYPA molecules become insoluble and are only broken up by treatment with TFA (De Groot *et al.*, 1996; Lugones *et al.*, 1996), and since the low concentration solution was diluted from the higher concentration the absence of long string-like rods in the images provides strong evidence that assembly takes place at the interface and not in solution. Careful examination of Figures 5.2(c, d) reveals that each rod appears to be composed of several subunits, presumably individual HYPA molecules, linked side by side. Figure 5.2(g) is a high magnification image of the monolayer showing the orientational order of the HYPA molecules. It is apparent in Figures 5.2(a, d) that there are a few molecules on top of the layer. This is illustrated in Figure 5.2(f) where the line profile indicates an approximate doubling of height (3.4 nm to 6.0 nm for the peak shown). This doubling of height is always observed when line profiles are run across these brighter

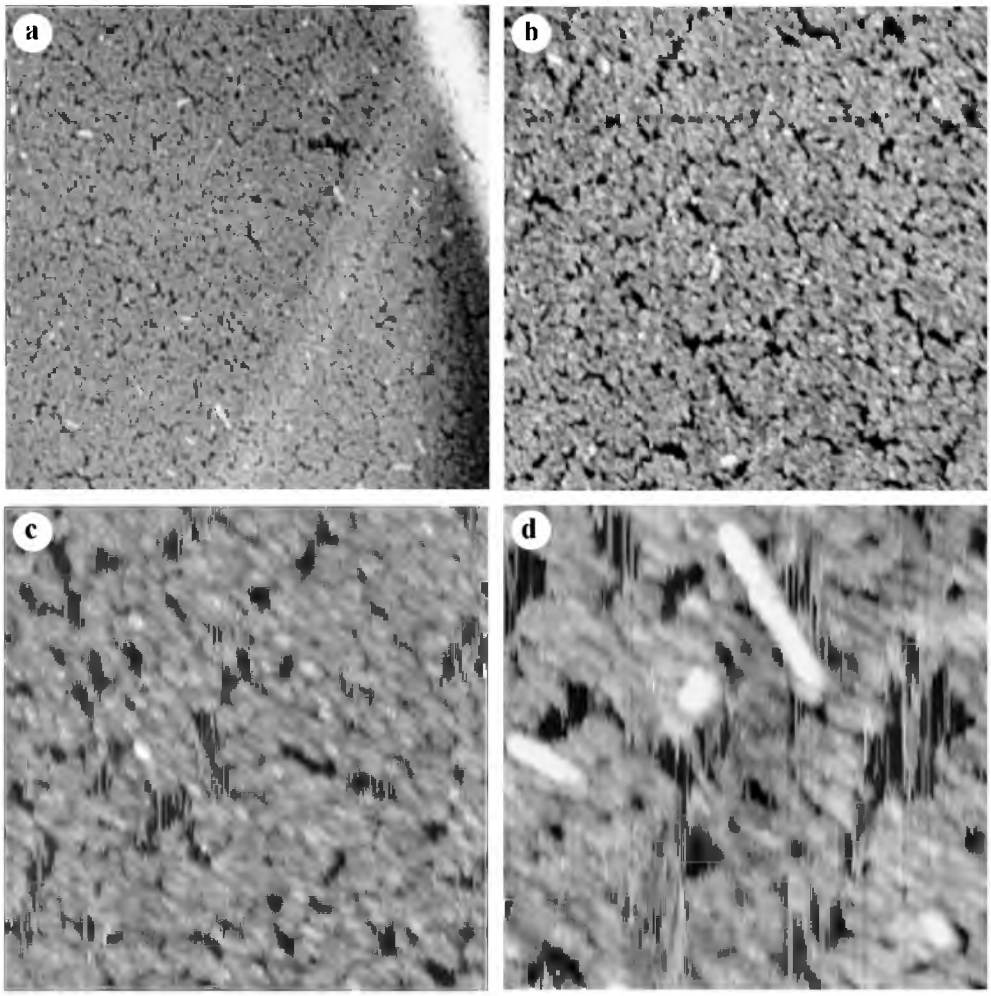
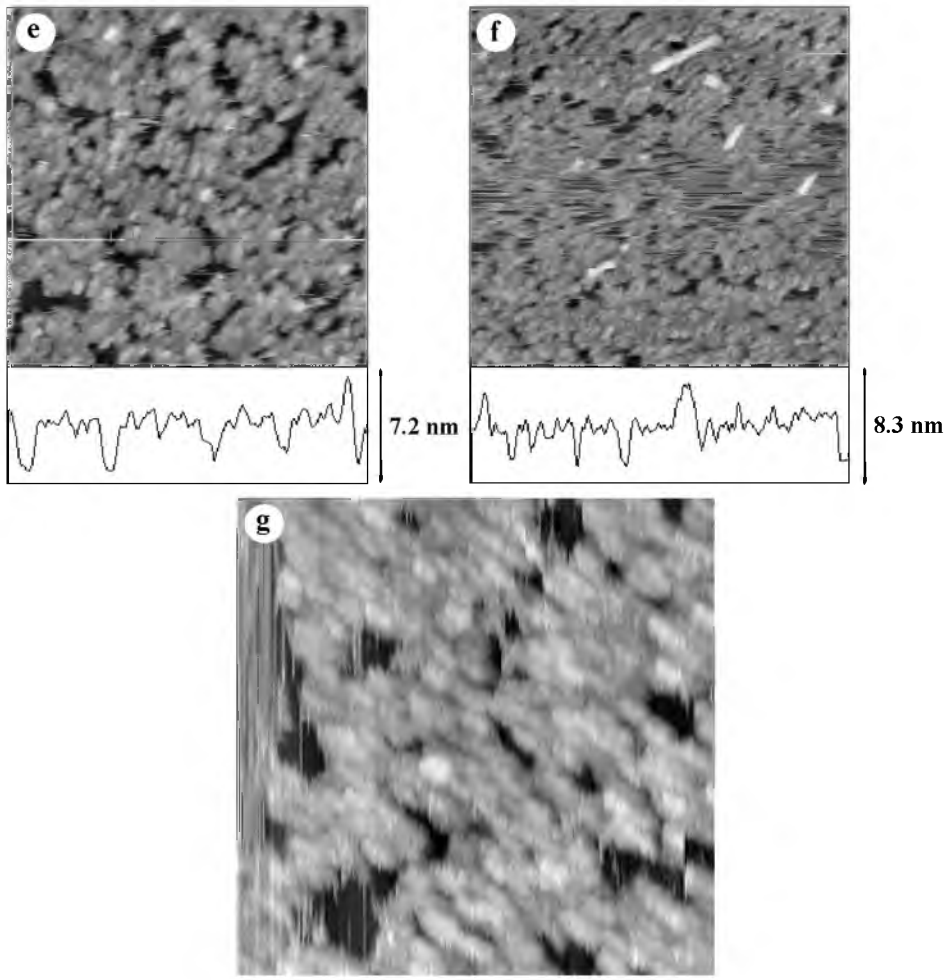


Figure 5.2. HYPA adsorbed onto graphite from a 2 μg ml^{-1} solution. (a) Low magnification image of an ordered layer. Scan size: 2.4 x 2.4 μm, height: 13.3 nm (black to white). (b) Intermediate magnification image. Scan size: 1.4 x 1.4 μm, height: 7.5 nm (black to white). (c) Higher magnification image revealing molecular order. Scan size: 600 x 600 nm, height: 7.4 nm (black to white). (d) Image illustrating bilayer formation. Scan size: 400 x 400 nm, height: 8.3 nm (black to white). (e) Profile over molecular layer (peak height 3.8 nm). Scan size: 600 x 600 nm. (f) Profile across molecular bilayer (peak height 6.0 nm). Scan size: 600 x 600 nm. (g) High magnification image of molecular packing. Scan size: 300 x 300 nm, height: 7.3 nm (black to white).

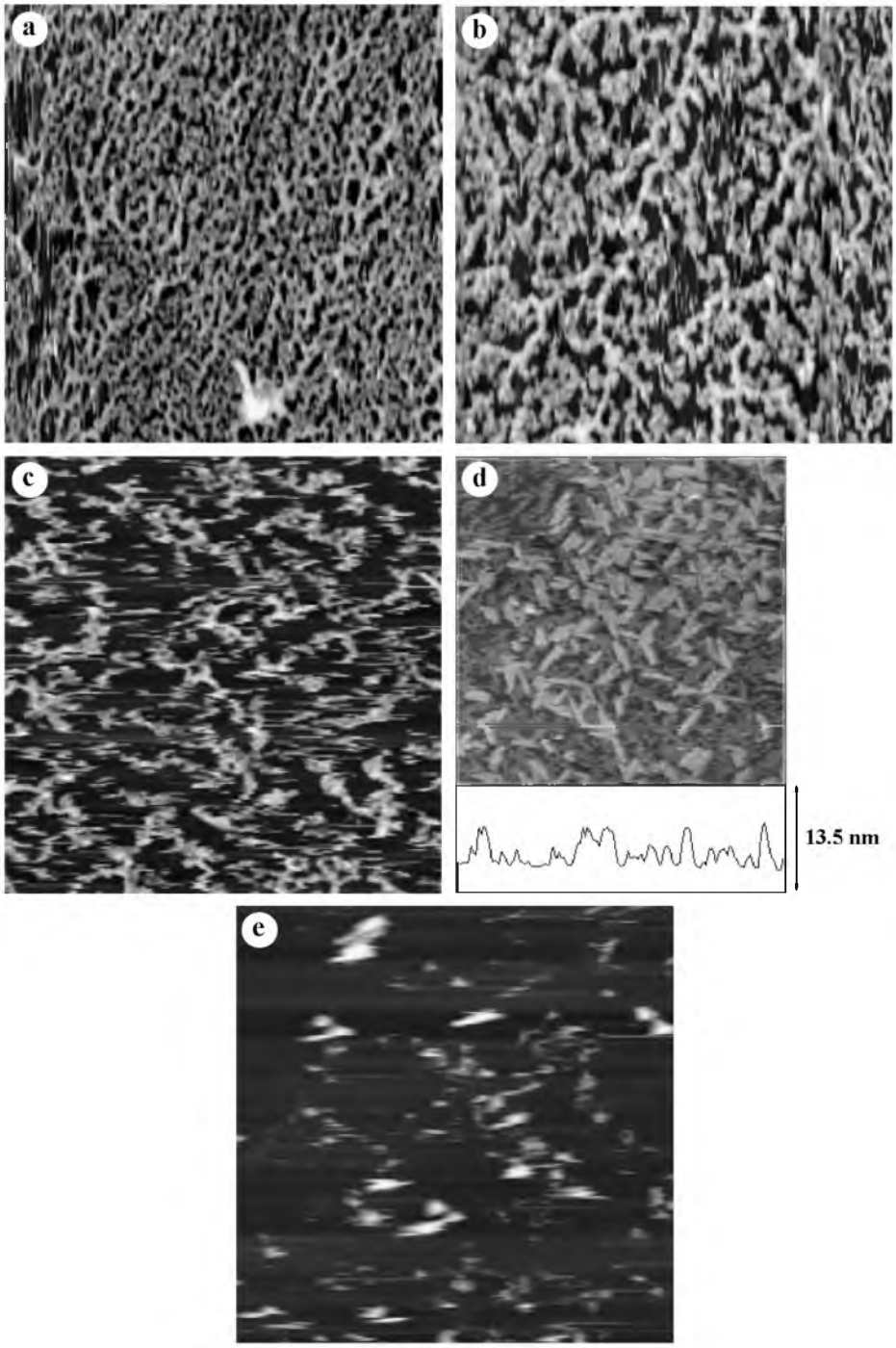


rods suggesting that they represent areas of bilayer coverage. This may well be due to insufficient washing after incubation and agrees with recent work by Van der Vegt *et al.* (1996), who predict bilayer formation of Sc3p if washing of the incubated substrate is not carried out. It is noteworthy that where a bilayer is present the rods of the second layer are generally longer, possibly because their growth is not restricted as would be the case in the initial monolayer. The greater detail seen in the present high resolution AFM images of the HYPA layers presented here has revealed that there is an interesting and previously unreported difference in the nature of the self-assembled layer dependent on the

concentration of HYPA in the deposition solution. At lower resolution and with less image contrast the long string-like features seen in the higher concentration deposited samples will dominate image contrast and render differentiation between the two cases impossible which may account for the failure of previous electron microscopy studies to report the difference presented here (Lugones *et al.*, 1996). Thus the use of AFM is clearly of great importance in characterising more fully such self-assembled protein films.

The results of washing experiments are shown in Figures 5.3(a - e). These were done on a freshly prepared sample adsorbed at the higher concentration of 20 μg ml^{-1}. Figure 5.3(a) is an image of the sample prior to SDS washing. There is fairly dense and random coverage as was seen previously (Figure 5.1). The stringlike features seen in Figure 5.1 appear to dominate Figure 5.3(a). Image analysis indicated a level of 74% protein coverage of the graphite surface before washing. Figure 5.3(b) shows a typical image, at the same magnification as Figure 5.3(a), obtained after the first washing step with cold 2% SDS solution. The coverage of HYPA appears to be quite similar to the unwashed sample (Figure 5.3(a)) and image analysis gives a value of 58% protein coverage, i.e. a loss of 16% compared to the unwashed sample. After washing the sample with hot 2% SDS solution there is a marked decrease in the density of HYPA coverage (Figure 5.3(c)), indicating significant removal of the protein from the graphite surface, and the coverage level drops to 38% of the scan area, representing a loss of 36% compared to the unwashed sample. Wösten and co-workers have carried out similar SDS washing experiments for radio-labelled Sc3p adsorbed onto teflon surfaces and monitored its removal by counting the intensity of the radiation. Loss of Sc3p from the teflon after hot SDS washing was measured to be only 13% (Wösten *et al.*, 1994). Comparison of these values indicates that HYPA may not be as resistant to SDS washing as Sc3p, although the substrates are not identical in the two experiments and only areas of HYPA monolayer could be used to estimate coverage levels. Furthermore the present values represent the result of a two step sequential wash with cold and hot SDS solutions.

Figure 5.3. Effects of washing the HYPA layer. HYPA adsorbed onto graphite from a 20 μg ml^{-1} solution. (a) Prior to washing. Scan size: 800 x 800 nm, height: 11.3 nm (black to white). (b) After cold (20°C) SDS wash. Scan size: 800 x 800 nm, height: 9.5 nm (black to white). (c) After hot (98-100°C) SDS wash. Scan size: 800 x 800 nm, height: 8.0 nm (black to white). (d) Bilayer formation after hot SDS wash. Profile across molecular bilayer (peak height 6.0 nm) Scan size: 1.2 x 1.2 μm, height: 13.7 nm (black to white). (e) Effect of TFA washing of HYPA layer. Scan size: 1.6 x 1.6 μm, height: 20.8 nm (black to white).


a
b
c
d
13.5 nm
e

Wösten *et al.* (1994) also related loss of Sc3p from teflon to decreased wettability using contact angle measurements and similar experiments were performed with ABH1 (or HYPA) (Lugones *et al.*, 1996). When Sc3p or ABH1 is adsorbed onto a hydrophobic surface such as teflon, only the hydrophilic part of the molecule is left exposed and therefore the treated surface becomes hydrophilic. This is opposite to the situation found in nature, where hydrophobins are attached with their hydrophilic side to hydrophilic hyphae/fruit body caps, and their hydrophobic side is exposed to the air. Water contact angles were noted to decrease after the teflon was coated with Sc3p or ABH1, but after removal of Sc3p or ABH1 by washing with hot SDS the water contact angles increased again, indicating a partial restoration of original hydrophobicity of the teflon (Wösten *et al.*, 1994; Lugones *et al.*, 1996). For HYPA Figure 5.3(d) illustrates that removal of protein might not be the only mechanism which causes increased hydrophobicity, since after hot SDS washing several areas of bilayer coverage appeared.

The features seen in Figure 5.3(d) are unlikely to be unremoved SDS since this effect was not seen after cold SDS washing and the heights of the bright features (6 nm) are double those of the HYPA molecules in the first layer and are the same as the bilayers observed in the low concentration sample (Figure 5.2(f)). Furthermore, subsequent AFM measurements carried out on pure SDS monolayer gave thickness values of approximately 0.6-0.75 nm.

Since the interaction between the first and second layer of HYPA molecules is hydrophilic, the uppermost surface is again hydrophobic when a bilayer forms. Thus the hydrophobicity of a HYPA treated surface after washing with hot SDS solution may well increase by a larger amount than would be predicted simply by quantifying the area of exposed substrate. It seems that during the washing step HYPA removed from the substrate surface re-adsorbs by hydrophilic interaction onto the protein layer still present. It seems likely that the higher concentration HYPA solution gives rise to a more randomly bound monolayer and the initial random orientation of the molecules prevents bilayer formation. Thus, when improperly bound HYPA molecules are washed they may re-adsorb to form a bilayer. The bilayer seen in Figure 5.3(d) has a degree of orientational order similar to that seen for the lower concentration ordered deposition which is consistent with specifically bound HYPA molecules in the first layer determining the positional orientation of the bilayer. This suggests that the bilayer seen in Figure 5.2(d) is formed by hydrophilic interaction between the layers and is not simply due to random stacking of the HYPA molecules. It is possible that the initial monolayer of HYPA molecules is influenced by the crystal structure of the graphite surface and that the deposition is epitaxial since the relative orientation of the features is approximately 60 degrees. This is consistent with the work of

Van der Vegt *et al.* (1996) who report an increase in hydrophobicity after adsorption of Sc3p to fluoroethylene-propylene-teflon if a washing step to remove unbound protein molecules was omitted, which they attribute to formation of bilayers. However, it is not clear why the patches of HYPA bilayer in the present study can resist the subsequent water rinses. Finally, the effect of washing the HYPA layer with TFA is shown in Figure 5.3(e). This produces severe disruption of the HYPA layer, although even after 15 minutes incubation in pure TFA there is still some protein left on the surface. It seems that once adsorbed it is very difficult to remove all of the HYPA from the graphite. This is in contrast to the findings for Sc3p, however, removal of Sc3p in that study was monitored by restoration of hydrophobicity of the teflon (Wösten *et al.*, 1994) which is clearly not such a sensitive measure as AFM imaging.

Conclusions

The hydrophobin protein HYPA produced by *Agaricus bisporus* has been shown to adsorb from aqueous solutions to HOPG surfaces to form a self-assembled monolayer. The use of AFM has allowed the monolayer to be imaged at molecular resolution for the first time. Such high resolution imaging has revealed the previously unreported result that the nature of the adsorbed protein film depends upon the protein concentration in the solution. The difference in the protein films obtained has been demonstrated qualitatively with high-resolution AFM images and analysed quantitatively by area measurement. At relatively high protein concentration (20 $\mu g\ ml^{-1}$) a monolayer is formed on the graphite surface which is typified by randomly oriented molecules, with some string-like assemblies and a total surface coverage of ≈74%. When the protein is adsorbed from a lower concentration solution (2 $\mu g\ ml^{-1}$) highly ordered monolayers are seen and the surface coverage increases to ≈89%. Protein removal from the monolayer by washing with cold and subsequently hot solutions of SDS has been quantified at 16% and 36% respectively, by analysing images obtained before and after each washing step. After washing with hot SDS solution, an unexpected formation of protein bilayers was observed. Finally, TFA washing failed to remove completely the adsorbed protein layer.

Acknowledgements

The authors thank Gerard F.H. Kramer for setting up the link which led to this research being carried out. The research at IFR was supported by the BBSRC through a CSG grant.

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6

Analysis of the structure of housekeeping genes of *Agaricus bisporus* and their spatial expression in fruit bodies

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Manuscript in preparation

Analysis of the structure of housekeeping genes of *Agaricus bisporus* and their spatial expression in fruit bodies

The *A. bisporus* housekeeping genes coding for 3-phosphoglycerate kinase (*pgkA*) and pyruvate kinase (*pkiA*) were cloned using heterologous hybridisation techniques. Identification of the *aldA* gene coding for aldehyde dehydrogenase resulted from random sequencing of a number of cDNA clones. The three genes are spiked with numerous small intervening sequences and the positions of these introns do not correlate with positions which among ascomycetes are conserved. Phylogenetic analysis based on amino acid sequence comparisons also show that *A. bisporus* has diverged early from the group of ascomycete and deuteromycete fungi. Analysis of the codon usage of 17 *A. bisporus* genes encoding 5135 amino acids in total shows a preference for C or T in the third base position of the codons. The three newly cloned housekeeping genes and the *A. bisporus* genes encoding translation elongation factor 1α and ribosomal protein S15a were then used to monitor different metabolic routes in fruit bodies by studying their expression levels in various parts of mature mushrooms. The results indicate the existence of defined regions of different metabolic activity in the mushroom and provide basic molecular knowledge essential for understanding mushroom development.

Introduction

During commercial cultivation, mushrooms appear synchronously in three to five successive flushes. Fruit body development and inflation poses a logistic problem. It requires massive production, transport and use of metabolites and implies that the mycelium and the fruit bodies have a different metabolism. A number of enzymes which are associated with carbon metabolism appear to oscillate co-ordinated with the crop flushing pattern. For the enzymes glucose-6-phosphate dehydrogenase, glucose phosphate isomerase and mannitol dehydrogenase highest activities were found at the onset of fruiting (Hammond, 1981; Hammond & Wood, 1985). The mannitol concentration in fruit body initials is higher than in the surrounding mycelium and continues to increase in fruit bodies until maturation (Hammond & Nichols, 1976). Trehalase and glycogen phosphorylase on the other hand clearly exhibited maximum activities coincident with the peak of the flush (Wells *et al.*, 1987), indicating an increased use of the corresponding storage carbohydrates during sporophore inflation.

Genes coding for essential enzymes of the carbon metabolism are highly conserved between species and can be used as well-characterised markers for the construction of a genomic map of *A. bisporus* (Schaap *et al.*, 1995b; Sonnenberg *et al.*, 1996; De Groot *et al.*, 1998b). Analysis of the structures of such housekeeping genes will provide basic knowledge of the structures of *A. bisporus* genes and their amino acid sequences are also often used to

study evolutionary relationships between species. Harmsen *et al.* (1992) reported cloning of two *gpd* genes encoding glyceraldehyde-3-phosphate dehydrogenase and used the active copy of the gene for phylogenetic contemplations. In this paper, we describe the cloning and characterisation of the genes coding for 3-phosphoglycerate kinase (PGK: EC 2.7.2.3), pyruvate kinase (PKI: EC 2.7.1.40) and aldehyde dehydrogenase (ALDH: EC 1.2.1.3) and use their amino acid sequences for phylogenetic analyses.

Mature mushrooms are the result of a differentiation process and consist of morphologically different cell types and tissues. On the genetic level, aspects of mushroom development have only been studied by isolating and characterising genes that are specifically expressed in developing fruit bodies (De Groot *et al.*, 1997). The expression levels of these genes in the morphologically different parts of the fruit body can differ considerably. The *hypA* gene, which is one of the three hydrophobin encoding genes that were cloned, is expressed at very high levels in the pileipellis (peel) of the mushroom caps (De Groot *et al.*, 1996). The second hydrophobin gene (*hypB*) and the *sepA* gene encoding a septin on the other hand showed a strong accumulation of their messengers in the transitional zone between cap and stipe tissue (De Groot *et al.*, 1998a, 1999).

Therefore, we wanted to investigate whether the differentiated hyphal cells of mushrooms also differ in their metabolism. The housekeeping genes were therefore also used in Northern analyses to screen for metabolic differences between various parts of the fruit body. Included in this analysis was the *s15A* gene which encodes a ribosomal protein (Schaap *et al.*, 1995a).

Materials and Methods

Agaricus strains, bacterial strains and DNA techniques

Agaricus bisporus Horst®U1 and its homokaryotic constituents, strains H39 and H97, were used from the collection of the Mushroom Experimental Station (Horst, The Netherlands). For DNA isolation these strains were cultured on DT80 glucose medium according to Sonnenberg *et al.* (1988). Genomic DNA was isolated from those cultures according to De Graaff *et al.* (1988). Vegetative mycelium and different parts of mature mushrooms of strain Horst®U1 used for Northern analysis were obtained as described by De Groot *et al.* (1999).

Escherichia coli LE392 (Promega, Madison, USA) was used for phage amplification and λ DNA isolation. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, MD, USA) was used for plasmid transformation and propagation. Standard DNA manipulations were carried out essentially as described in Sambrook *et al.* (1989). DNA sequences were determined from both strands using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) and an ALF automated sequencer (Pharmacia Biotech, Uppsala, Sweden).

Cloning and identification of the genes encoding phosphoglycerate kinase, pyruvate kinase and an aldehyde dehydrogenase

The *A. bisporus* genes were isolated from a genomic library of *A. bisporus* strain H39 by screening of about 5 genome equivalents of strain H39 with various probes. For isolation of the gene encoding PGK, a 1.4 kb *Eco*RI-*Hin*dIII fragment comprising the complete *pgkA* gene from *Saccharomyces cerevisiae* (Hitzeman *et al.*, 1982) was used as a probe. For isolation of the gene encoding PKI, a 1.9 kb *Sma*I fragment encoding a major part of the *pkiA* gene from *Aspergillus niger* (De Graaff *et al.*, 1988) was used as a probe. DNA probes were labelled with [α-^{32}P] dATP using the method of Feinberg & Vogelstein (1983). Hybridisation was performed in standard hybridisation buffer (SHB; 6 x SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0), 5 x Denhardt's solution, 0.5% SDS and 100 µg ml^{-1} denatured herring sperm DNA) using heterologous hybridisation conditions (the filters were washed twice in 4 x SSC, 0.1% SDS at 56°C). Hybridising plaques were purified by standard techniques and analysed by Southern analysis using the same conditions. Hybridising fragments were cloned in the vector pUC19 (Yanisch-Perron *et al.*, 1985), sequenced and analysed by database searches using the GCG programmes BLAST (Gish & States, 1993) and BESTFIT (Devereux *et al.*, 1984).

The corresponding cDNA clones of *pgkA* and *pkiA* were isolated from a cDNA expression library constructed from pin stage Horst®U1 mushrooms (De Groot *et al.*, 1996), using the corresponding genomic *A. bisporus* fragments as probes and stringent hybridisation conditions (65°C and final washing in 0.1 x SSC, 0.1% SDS) according to Sambrook *et al.* (1989).

A cDNA clone specifying an aldehyde dehydrogenase was identified while constructing the cDNA expression library. This clone was used as a probe for the isolation of *aldA* genomic sequences from the H39 genomic library using stringent hybridisation conditions.

A cDNA fragment of the *echA* gene was generated by RT-PCR. First strand cDNA was synthesised by standard primer extension methods as described by Sambrook *et al.* (1989) using RNA, treated with DNase (Promega), from mature fruit bodies as template. The first strand cDNA was subsequently used as template in standard PCR reactions to generate double stranded cDNA fragments that were cloned in pGEMT and sequenced. The oligonucleotides 5'-GGCAAACTCGGGTGTCC-3' (in antisense direction) and 5'-GGTTCTCGCCTCAAGCC-3' (sense direction) were used for RT-PCR and were designed against regions that showed high levels of homology with ECHA proteins from human and rat.

CHEF gel analysis and assignment of genes to chromosomes

Isolation of intact chromosomal DNA and separation by clamped homogeneous electric field (CHEF) electrophoresis were performed as described by Sonnenberg *et al.* (1996). Separated chromosomes were blotted onto Hybond-N membranes (Amersham International plc., Amersham, UK) and hybridised with genomic fragments of *pgkA* and *pkiA* and the cDNA of *aldA* in SHB using stringent hybridisation conditions. For further localisation of *pgkA* linkage analysis was performed as described by Sonnenberg *et al.* (1996) using a set of 86 homokaryotic offspring of Horst®U1 and chromosome III and chromosome IV specific markers.

Northern analyses

Total RNA was isolated from fruit body and mycelium samples using TRIzol™ Reagent (BRL). The concentration of the isolated RNA was determined spectrophotometrically. Total RNA samples were denatured in 10 x SSC and 6.15 M formaldehyde and spotted on Hybond-N membrane or denatured by standard techniques using glyoxal and DMSO, separated on a 1.6% (w/v) agarose gel

and transferred to a Hybond-N membrane. Transcript sizes were determined by co-electrophoresis of RNA molecular weight markers (BRL) which were stained after transfer with methylene blue (Sambrook *et al.*, 1989). The membranes were hybridised with various cDNA probes at 42°C in SHB to which 50% (v/v) formamide and 10% (w/v) dextran sulphate was added. Washing was performed at 65°C using stringent conditions. All membranes were stripped and rehybridised with a 900 bp *Eco*RI fragment comprising part of the 3' end of the 28S ribosomal DNA (Schaap *et al.,* 1996) to provide a loading control. Signal intensities were quantified by scanning the autoradiograms with an Ultroscan XL laser densitometer (LKB).

Phylogenetic analyses

Protein sequences were extracted from the OWL non-redundant protein database (Bleasby & Wootton, 1990). For optimal alignment and tree building the Blosum series of matrices and the program Clustal W were used (Thompson *et al.*, 1994). Trees were drawn using the Drawtree program from the PHYLIP package (Felsenstein, 1988).

Results and Discussion

Cloning and genomic organisation of the genes

Southern analysis of *Agaricus bisporus* genomic DNA, using the entire phosphoglycerate kinase encoding gene of *Saccharomyces cerevisiae* as a probe, indicated that a putative *A. bisporus* homologue hybridised if conditions of low stringency were used (result not shown). The *A. bisporus* homologue, designated *pgkA,* was isolated by screening of a λEMBL4 genomic library of the homokaryotic strain H39 using the same low stringency hybridisation conditions. Sequence analysis of a hybridising 1.0 kb *Sal*I λ fragment strongly suggested that the *A. bisporus* homologue was isolated and that the fragment comprised a large portion of the coding sequence of *pgkA.* Southern analysis with total DNA of the homokaryotic strains H39 and H97 using this 1.0 kb *Sal*I λ fragment as a probe revealed that in strain H39 the entire *pgk*A gene is located on two adjacent *Hin*dIII fragments of 6.5 kb and 2.5 kb (not shown). No additional copies of *pgk* were found in the *A. bisporus* genome. The entire sequence of the *pgkA* ORF and 713 bp upstream and 317 bp downstream non-translated sequences were determined from both strands. A genomic map of the gene is depicted in Figure 6.1(a).

A similar strategy was used for cloning of the PKI encoding gene of *A. bisporus* using the *Aspergillus niger pkiA* gene as a probe. The *A. bisporus pkiA* gene also appears to be present as a single copy gene and the entire gene is located on a 3.3 kb *Eco*RI fragment. A partially overlapping *Pst*I fragment of 2.9 kb was used to sequence upstream non-translated

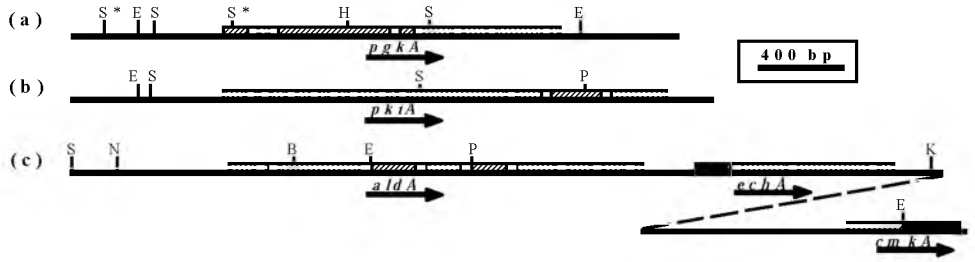

Figure 6.1. Molecular maps of (a) the *pgkA* gene, (b) the *pkiA* gene and (c) the *aldA* gene locus. The coding regions of the genes are represented by hatched boxes. Introns in the coding region of the genes are shown by open boxes and not determined extensions of *echA* and *cmkA* are indicated by black boxes. Arrows indicate direction of transcription. The following restriction sites are indicated: (B) *Bam*HI; (E) *Eco*RI; (H) *Hin*dIII; (K) *Kpn*I; (N) *Nco*I; (P) *Pst*I; (S) *Sal*I; (S*) *Sal*I not found in H97. If the 1 kb *Sal*I genomic fragment of the *pgkA* gene locus of H39 is used as a probe, a 5 kb *Sal*I fragment is detected in H97. This RFLP was used for linkage analysis.

sequences. The sequence of the *pkiA* ORF and 764 bp upstream and 174 bp downstream non-translated sequences were determined and a genomic map of *pkiA* is depicted in Figure 6.1(b).

A cDNA clone encoding an aldehyde dehydrogenase was fortuitously cloned while constructing a directional cDNA expression library of primordia. In order to verify the directional orientation of cDNA inserts from this library we previously sequenced a few randomly selected cDNA clones at their presumed 5' and 3' end (Schaap *et al.*, 1995a). The deduced amino acid sequence of one of these cDNAs, designated *aldA*, showed high homology with aldehyde dehydrogenases which are primarily involved in the conversion of ethanol to acetate. Preliminary Northern analysis suggested that compared to compost grown mycelium, expression of *aldA* is strongly enhanced in fruit bodies and therefore this gene was analysed further. Southern analysis of genomic DNA of strain H39 using the *aldA* cDNA as a probe revealed that the entire gene is located on two adjacent *Eco*RI fragments

of 3.5 kb and 5.5 kb. These two adjacent *Eco*RI fragments were isolated from the strain H39 genomic library, cloned and subjected to sequence analysis.

A database search using the BLAST algorithm (Gish & States, 1993) and the 5' and 3' non-translated regions of *aldA* as query sequences revealed that the 5.5 kb *Eco*RI fragment harboured two additional ORFs (Figure 6.1(c)). One was located immediately downstream of *aldA* on the same DNA strand and showed significant similarity with peroxisomal enoyl-CoA hydratases (ECH) of human and rat (Fitzpatrick *et al.*, 1995). The conceptual translation of the *A. bisporus echA* ORF reads the sequence serine-lysine-leucine, at its extreme C-terminal end (Figure 6.2) which in many organisms is a C-terminal peroxisomal import signal (Borst, 1989). The homologous hydratases in human and rat are directly involved in fatty acid metabolism in glycosomes and appear to be specific for unsaturated fatty acids.

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Human MAAGIVASRR LRDLLTRRLT GSNYPGLSIS LRLTGSSAQE EASGVALGEA PDHSYESLRV TSAQKHVLHV QLNRPNKRNA MNKVFWREMV  90
Rat   MATAMTVSSK LLGLLMQQLR GTRQLYPNVS LRSLSSSAQE -ASKRIPEEV SDHNYESIQV TSAQKHVLHV QLNRPEKRNA MNRAFWRELV  89

Human ECFNKISRDA DCRAVVISGA GKMFTAGIDL MDMASDILQP KGD---DVAR ISWYLRDIIT RYQETFNVIE RCPKPVIAAV HGGCIGGGVD 177
A.bis               VLASSL SKMFTAGLDL NE-ASNLVEE TADMKRDGAR TSLSMHKLIR RFQEAITKPD QAPFPVIAAV HGPVPGLGVD
Rat   ECFQKISKDS DCRAVVVSGA GKMFTSGIDL MDMASDILQP PGD---DVAR IAWYLRDLIS RYQKTFTVIE KCPKPVITAI HGGCIGGGVD 176

Human LVTACDIRYC AQDAFFQVKE VDVGLAADVG TLERLPKVIG NQSLVNELAF TAHKMMADEA LDSGLVSRVF PDKEVMLDAA LPLAPEISSK 267
A.bis IISSCDIRYA AENSIFAIKE VDVGLAADIG SLAYLPKITG NQSLIRELAY TGRAFSANEA EKLGLVSRII PGSREEVVKA ALELAKTIAG
Rat   LISACDIRYC TQDAFFQVKE VDVGLAADVG TLQRLPKVIG NRSLVNELTF TARKMMADEA LDSGLVSRVF P-DKDVMLNA AFALAADISS 265

Human TTVLVQSTKV NLLYSRDHSV AESLNYVAS- WNMSMLQTQD LVKSVQPTTE NKELKTVTF- ---SKL 327
A.bis KSPIAVSGSK RLISHARDHS VSENLVYTQT WNAHAIVTKD MAECATS-MK EKRTPEFAPL KVPSKL
Rat   KSPVAVQGSK INLIYSRDHS VDESLDYMAT WNMSMLQTQD IIKSVQAAME KKDSKSITF- ---SKL 327
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Figure 6.2. Alignment of the partial amino acid sequence of the inferred *A. bisporus* enoyl-CoA hydratase with those of human and rat. Amino acids identical to the *A. bisporus* sequence are indicated by asterisks. The C-terminal peroxisomal import signal and the active site residues (D^{176} and E^{196}) are indicated by underlining the rat sequence.

The second ORF was localised around the 3' distant *Eco*RI site and the sequenced part of this ORF showed significant similarity with serine/threonine protein kinases of many organisms. Highest homology was found with calmodulin dependent protein kinases (CMK) of *Saccharomyces. cerevisiae* (Pausch *et al.*, 1991) and *Emericella nidulans* (Kornstein *et al.*, 1992). This ORF was therefore provisionally designated *cmkA*. The sequences of the *pgkA* gene, the *pkiA* gene and of the *aldA* gene locus are available in the EMBL/GenBank databases under the accession numbers X97580 (*pgkA*), X97579 (*pkiA*), and Y17825 (*aldA* and *echA*).

The chromosomal location of *pgkA*, *pkiA* and *aldA* was determined by Southern analysis of Clamped Homogeneous Electric Field (CHEF) resolved chromosomes of the homokaryotic strains H39 and H97. The *pkiA* gene is located on the largest chromosome (I)

and the *aldA* gene is located on chromosome XII of both strains. The *pgkA* gene was assigned to either chromosome III or IV of both strains. Since these two chromosomes were not separable by CHEF-analysis, definite assignment of *pgkA* was achieved by linkage analysis. Using Southern analysis, we detected a polymorphism between *Sal*I digested DNA of H39 and H97 (Figure 6.1(a)). Linkage analysis using this polymorphism and a set of 86 homokaryotic offspring of Horst®U1 indicated that *pgkA* was completely linked to markers that were previously assigned to chromosome III, whereas no linkage to chromosome IV specific markers was found. This unambiguously proved that the *pgkA* gene is located on chromosome III.

Characterisation of the *A. bisporus* genes

Analysis of the *pgkA* nucleotide sequence indicated that the (ORF) is interrupted by seven putative introns. These introns were confirmed by comparison of the genomic sequence with the sequence of *pgkA* specifying clones isolated from a cDNA library. The cDNAs end with a poly(A) tail 60 nt downstream of the TGA stop codon. Similarly, analysis of the *pkiA* genomic sequence indicated that the open reading frame of *pkiA* is interrupted by eleven small introns which were confirmed by sequencing *pkiA* specifying cDNA clones. A poly(A) tail is located 69 nt downstream of the TAA stop codon of *pkiA*. Comparison of *aldA* cDNA and genomic sequences revealed that the open reading frame is interrupted by nine introns. The poly(A) tail of the *aldA* cDNA is located 86 nt downstream of the TAA stop codon. For the putative *echA* gene screening of the cDNA library did not result in the isolation of cDNA clones. However, with RT-PCR techniques using RNA isolated from fruit bodies as template a cDNA fragment was amplified which confirmed the three most 3' introns and also showed that the gene is expressed in fruit bodies albeit at a low level.

Thus, as was found for all other *A. bisporus* genes for which the genomic organisation was resolved, the housekeeping genes are heavily spiked with intervening sequences. The sequences around the exon-intron and intron-exon splice boundaries of all introns detected in *pgkA*, *pkiA*, *aldA* and *echA* are conform the consensus sequences for spliceosomal introns. The 30 newly found introns are all between 48-60 bp in length and so far larger introns have only been reported for *cel1* (Raguz *et al.*, 1992), the *gpd* genes (Harmsen *et al.*, 1992), *gdhA* (Schaap *et al.*, 1996) and *hypB* (De Groot *et al.*, 1999).

The 5' non-translated regions of *pgkA*, *pkiA* and *aldA* were searched for the commonly found CCAAT and TATA boxes, representing core promoter elements (reviewed by Nussinov, 1990). All three 5' non-translated regions contain such boxes (Table 6.1).

Table 6.1. Commonly found promoter elements

Gene	TATAAA-element	CCAAT element	Distance to ATG
aldA	TATATAAA		39
pgkA	TATATATA		29
pkiA	TATATAAG		48
pkiA		ATTGG	448

For analysis of the codon usage of *A. bisporus* genes we analysed the genes that are currently available in the databases and classified them into three groups: housekeeping genes, genes involved in utilisation of compost and fruit body specific genes (Table 6.2). The codon usage of the third base positions of the listed amino acids is: G 13.1%, A 12.6%, T 37.9%, C 36.4% (housekeeping genes); G 19.8%, A 20.0%, T 29.8%, C 30.5% (compost utilisation genes); G 7.5%, A 13.5%, T 45.1%, C 33.8% (fruit body specific hydrophobins). Thus, for all three groups of genes a preference was found for C or T in the third base position. Arginine codons are six fold degenerate, and 73% of its codons use a C at the first base position, similarly 79% of the leucine codons also use a C at the first base position. For serine, 76% of the codons start with TC. For future research this information may be particularly helpful when designing degenerative oligonucleotides.

Phylogenetic analysis

Gene structures of ascomycete and deuteromycete fungi often show conservation with respect to the positions of their introns. For the *A. bisporus pgkA* gene, the positions of the seven introns do not coincide with the positions of the two introns shared by the ascomycete and deuteromycete PGK encoding genes. The intron positions of the *pgk1* and *pgk2* genes of the zygomycete *Rhizopus niveus* also deviate from ascomycete and deuteromycete fungi and, interestingly, the position of the second intron in *R. niveus* coincides with the position of the fourth intron in the *A. bisporus pgkA* gene (Figure 6.3(a)). Similarly, the positions of the nine introns of the *A. bisporus aldA* gene do not coincide with the positions of the introns of the ALDH encoding genes of *A. niger* and *E. nidulans*. For *pkiA*, only two of the eleven introns are found at the same position as introns of the PKI encoding genes of *A. niger* and other ascomycetes. Of these, the position of the second intron in *pkiA* coincides with the first intron in PKI encoding genes of other fungi as well as organisms belonging to other phylogenetic kingdoms (Figure 6.3(b)).

Table 6.2. Codon usage of *Agaricus bisporus* for amino acids that are encoded by at least four codons

Amino acid	Codon	Codon usage (numbers)		
		Housekeeping genes (2340)	Compost utilisation genes (2564)	Fruit body specific genes (231)
Alanine	GCG	24	27	3
	GCA	32	35	5
	GCT	76	57	12
	GCC	65	42	4
Arginine	CGG	6	18	0
	CGA	6	21	0
	CGT	50	22	0
	CGC	39	23	1
	AGG*	14	30	0
	AGA*	5	21	0
Glycine	GGG	21	25	0
	GGA	38	41	9
	GGT	84	79	13
	GGC	57	52	3
Leucine	CTG	24	49	1
	CTA	9	33	0
	CTT	50	45	9
	CTC	69	70	7
	TTG*	22	51	5
	TTA*	3	18	0
Proline	CCG	10	47	0
	CCA	13	53	0
	CCT	42	49	7
	CCC	37	51	6
Serine	TCG	25	39	0
	TCA	12	34	1
	TCT	41	43	5
	TCC	38	51	2
	AGT*	14	27	3
	AGC*	18	24	4
Threonine	ACG	21	35	4
	ACA	22	32	1
	ACT	50	76	8
	ACC	47	62	7
Valine	GTG	27	31	2
	GTA	19	24	2
	GTT	63	36	6
	GTC	86	66	15

Analysed housekeeping genes are *pkiA*, *pgkA*, *aldA*, *gpdII* (M81728), *L41A* (X94189), *rs13A* (X94704), *s15A* (X97405), *htbA* (X94188) and *hhfA* (X94189). Analysed genes encoding compost degrading enzymes are *cel1* (M86356), *cel2* (Z50094), *cel3* (L24519), *cel4* (Z50095), *xlnA* (Z83199) and *lcc1* (L10664). Analysed fruit body specific genes are *hypA* (X89242) and *hypB* (Y15941). The total number of amino acids that are searched in each group are indicated between brackets. * These codons were not included for calculation of relative third base usage.

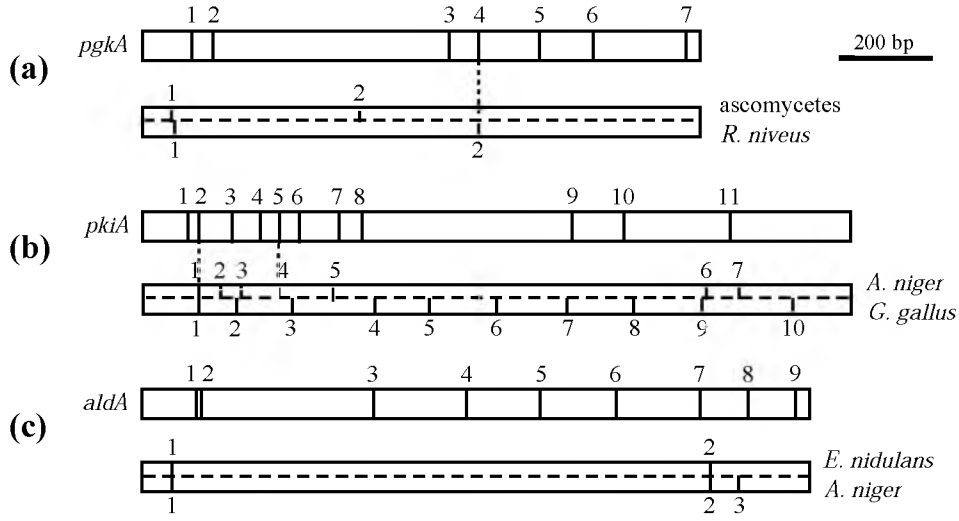

Figure 6.3. Comparison of the intron positions in the PGK, PKI and ALDH encoding genes of *A. bisporus* and related fungi. (a) The positions of the seven introns in the *A. bisporus pgkA* gene are compared with the positions of the two introns in PGK encoding genes of ascomycetes and the zygomycete *R. niveus*. (b) Positions of the eleven introns in the *A. bisporus pkiA* gene are compared with the positions of the seven introns and ten introns in the PKI encoding genes of *A. niger* and *G. gallus* (chicken), respectively. (c) Positions of the nine introns in the *A. bisporus aldA* gene are compared with the positions of the two and three introns in the ALDH encoding genes of *E. nidulans* and *A. niger*, respectively. Open boxes indicate coding regions and vertical bars indicate intervening sequences.

The deduced amino acid sequences of *A. bisporus* PGK, PKI, and ALDH were aligned with the amino acid sequences of homologous proteins of other fungi (Table 6.3). The levels of sequence identity between the *A. bisporus* proteins and their functional homologues from other fungi are consistently lower than among these other fungi. Consequently, and in agreement with the lack of conservation in intron positions, phylogenetic analyses using these amino acid sequence alignment data places the basidiomycete *A. bisporus* outside the group of ascomycete fungi and their likes (Figure 6.4). For CLUSTAL analysis of PGK proteins, the *Bacillus stearothermophilus*, the chicken and the mouse testis specific phosphoglycerate kinase were used as outgroups. Routed from the outgroups, the first evolutionary split in the fungal species analysed was apparently into yeasts and filamentous fungi. The second split separated ascomycete and deuteromycete fungi from the basidiomycete *A. bisporus* and the zygomycete *Rhizopus niveus*.

The ascomycete and deuteromycete branch is further divided into plectomycetes (*E. nidulans, Aspergillus oryzae* and the *Penicillium* species) and pyrenomycete-like imperfect fungi (*Trichoderma reesei* and. *Trichoderma viride*). The true pyrenomycete *Neurospora crassa* branches off separately. The finding that *A. bisporus* and the zygomycete *R. niveus* have a common origin is in agreement with a phylogenetic analysis based upon the structure of the orotidine 5'-monophosphate decarboxylase gene (Radford, 1993). In their analysis, the analysed fungi are classified into three major groups. One group consisted of ascomycete fungi and ascomycete-like imperfect fungi, the second group of ascomycete yeasts and the third group consisted of the zygomycetes *Phycomyces blakesleeanus* and *Mucor circinelloides,* the basidiomycete yeast *Ustilago maydis* and the basidiomycete fungus *Schizophyllum commune*. Only at a later stage the two basidiomycetes branch off

Table 6.3. Phosphoglycerate kinase, pyruvate kinase and aldehyde dehydrogenase sequences used for phylogenetic analysis

Species	Abbreviation	Accession number		
		Phosphoglycerate kinase	Pyruvate kinase	Aldehyde dehydrogenase
Agaricus bisporus	agabis	X97580	X97579	Y17825
Alternaria alternata	altal			P42041
Aspergillus niger	aspng		Q12669	P41751
Aspergillus oryzae	aspor	P41756		
Bacillus stearothermophilus	bacst	P18912	Q02499	P42329
Candida albicans	canal	P46273		
Candida maltosa	canma	P41757		
Cladosporium herberum	clahe			P40108
Emericella nidulans	emeni	P11977	P22360	P08157
Gallus gallus	chick	P51903	P00548	P27463
Kluyveromyces lactis	klula	P14828		
Homo sapiens	human		P30613	P00352
Mus musculus	mouse	P09401		
Neurospora crassa	neucr	P38667		
Penicillium citrinum	penci	P33161		
Penicillium chrysogenum	pench	P09188		
Saccharomyces cerevisiae	yeast	P00560	P00549	P54115[3]
Schizosaccharomyces pombe	schpo		Q10208	Z99262
Ustilago maydis	ustma			U74468
Trichoderma reesei	trire	P14228	P31865	
Trichoderma viride	trivi	P24590		
Rhizopus niveus	rhini	P29405[1]		
		P29406[2]		
Yarrowia lipolytica	yarli		P30614	
Xenopus laevis	xenla		Q92122	

[1] PGK1 and [2] PGK2 proteins of *R. niveus*; [3] cytosolic DHA6 protein of *S. cerevisiae*.

from the zygomycetes. Also in this case there is a conservation of intron position between a basidiomycete (*S. commune*) and zygomycetes whereas the analysed filamentous ascomycetes share a common intron at a different position.

The CLUSTAL analysis of pyruvate kinase is limited by the number of sequences available in the databases. Routed from the two outgroups *B. stearothermophilus* and the metazoa three main groups are formed: *A. bisporus* which branches off as a single species, the filamentous fungi and the yeasts. Again, the pyrenomycete-like imperfect fungus *T. reesei* branches off early from the group of filamentous fungi. The tree is thus showing a topology similar to the one for phosphoglycerate kinase.

CLUSTAL analysis of aldehyde dehydrogenase is complicated by the large number of homologous aldehyde dehydrogenase genes from *S. cerevisiae*. If the cytosolic *S. cerevisiae* aldehyde dehydrogenase DHA6 which shows highest sequence identity with the *A. bisporus* aldehyde dehydrogenase is used in the analysis, the tree topology is similar to the ones obtained with PGK and PKI. The filamentous ascomycetes split off in plectomycetes and loculoascomycetes, and *A. bisporus* and the basidiomycete yeast *U. maydis* branch off together.

Spatial expression of *pgkA*, *pkiA*, *aldA* and *s15A* in mature fruit bodies

With the newly cloned genes *pgkA*, *pkiA* and *aldA*, and *s15A* encoding a ribosomal protein (Schaap *et al.*, 1995a), we monitored spatial expression patterns in mature mushrooms with spore-bearing, reproductive organs. This was done by dissecting mature mushrooms into morphologically different parts prior to RNA isolations. The following parts were sampled: the pileipellis (outer peel), a connecting zone (tissue breaching the outer peel), veil + peel, plectenchyma (inner cap tissue), gills, the annulus, the transitional zone (upper stipe) and mid-stipe. The two stipe fractions are 3 mm horizontal sections after removal of the annulus. The veil fraction contains both veil and peel tissue since it is difficult to isolate those separately. Gill tissue was used without separating the gills into tramal, subhymenial and meiotic hymenial cells.

Northern analysis showed that each of the *pgkA*, *pkiA*, and *aldA* probes specifically hybridise with a transcript of about 1600 nt and that the *s15A* gene hybridises with a transcript of about 550 nt. Previously, we analysed the *tefA* mRNA levels in the various parts of the fruit bodies, showing that the expression level in the differentiated fruit body cells is comparable to the expression level observed in vegetatively growing cells (see Chapter 4). Since *tefA* expression was found to correlate with cell growth and proliferation of a variety of systems (Krieg *et al.*, 1989; Sanders *et al.*, 1992; Liu *et al.*, 1996) this

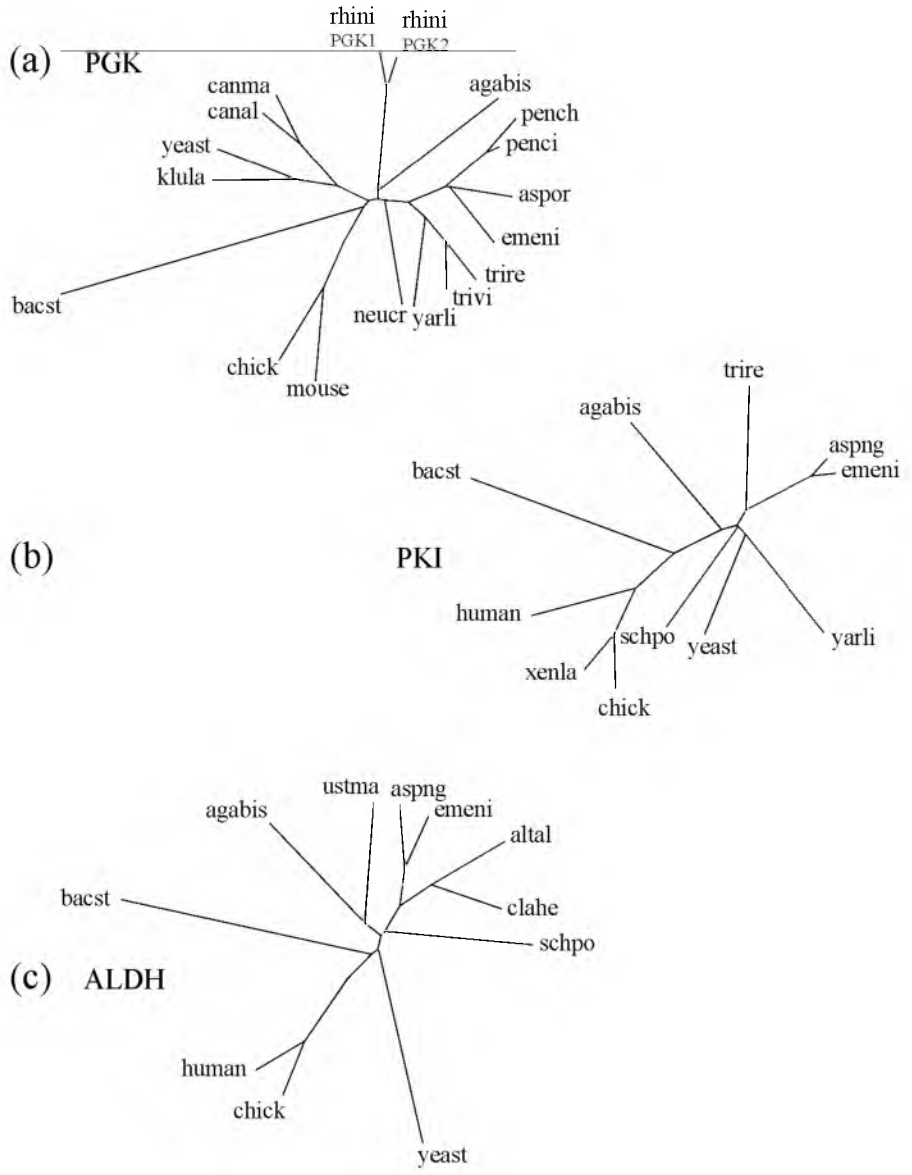

Figure 6.4. Phylogenetic analysis using (a) PGK, (b) PKI and (c) ALDH amino acid sequence comparisons. Phylogenetic analyses are based upon multiple sequence alignments generated with the program CLUSTAL W using the proteins that are listed in Table 6.3. The length of the branches between two ends is a measure of the evolutionary distance between two proteins.

suggests that all assayed parts of the mushroom are metabolically active. In all fruit body samples detectable mRNA levels were also found using *pgkA* and *pkiA* as probes, although the *pgkA* mRNA levels in stipe tissue fractions was threefold lower than in other parts of the mushroom suggesting a lower flux through glycolysis in these cells. The limited number of probes, however, does not allow firm conclusions about that.

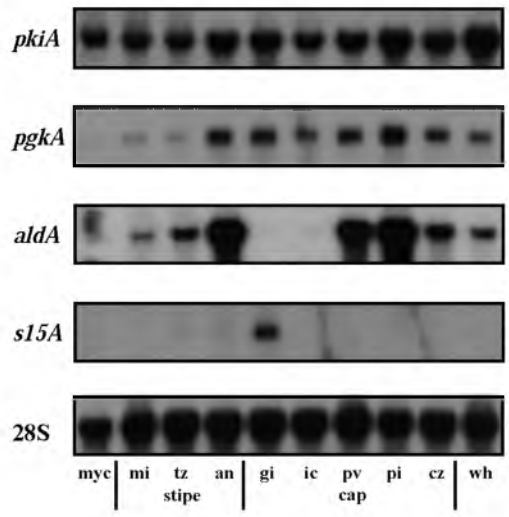

Figure 6.5. Spatial expression of housekeeping genes in mature fruit bodies. Northern analyses showing the relative mRNA levels of the studied genes in different parts of mature mushrooms and in mycelium grown on sterilised compost. The different tissues that are analysed are: myc, compost grown mycelium; mi, mid-stipe; tz, transitional zone; an, annulus; gi, gills; ic, inner cap; pv, pileipellis + partial veil; pi, pileipellis; cz, connecting zone; wh, whole mushroom.

Comparison of their messenger levels in compost grown mycelium and whole mature fruit bodies showed that expression of *pgkA* and *aldA* is strongly elevated in fruit bodies. For *aldA*, within the fruit bodies high expression levels were found in peel, veil + peel and annulus tissue fractions (Figure 6.5). These tissues all consist of white hyphae which are cells of a lower grade of differentiation (Umar & Van Griensven, 1997). In gills and inner cap tissue, *aldA* expression was hardly detectable. The pattern of expression is very similar to that of the *hypA* gene encoding a fruit body specific hydrophobin (Chapter 4). This gene is also predominantly expressed in the peel layer of mushroom caps and shows a low level

of expression in the gill fraction. A clear difference between the spatial expression patterns of *aldA* and *hypA* is found in the zone connecting the pileipellis and the cap (plectenchyma) where expression of *hypA* is at a very high level and the relative expression level of *aldA* is much lower.

Aldehyde dehydrogenase encoding genes are often induced by ethanol and/or osmotic stress (Miralles & Serrano, 1995; Basse *et al.*, 1996) which might both also provide an explanation for the high expression level of *aldA* in fruit bodies. Interestingly, Adams & Timberlake (1990) showed that artificially induced asexual development of *E. nidulans* repressed the coordinately controlled expression of alcohol and aldehyde dehydrogenase encoding genes which is in agreement with our observation that high levels of *aldA* expression are restricted to the less differentiated white hyphae surrounding the mushroom caps.

The *s15A* gene was incorporated in the Northern analysis to investigate whether cap expansion and stipe elongation, processes which involve cell division as well as cell elongation (Craig *et al.*, 1979) also require massive ribosome biogenesis. While cap, peel, veil + peel and stipe cells displayed a low expression level of *s15A*, much higher expression levels were found in the gill which may reflect a high incidence of cell division in that tissue. Alternatively, the high expression level could be a reflection of an unique feature of the subhymenial cells of the gills, which have an additional outer cap around the dolipore, densely packed with ribosomes (Craig *et al.*, 1977).

Comparison of the mRNA levels in the two stipe fractions (mid-stipe and the transitional zone), for the genes analysed in this Chapter, did not reveal marked differences between these fractions. This is in contrast to the expression patterns obtained with the *hypB* gene encoding a fruit body specific hydrophobin (De Groot *et al.*, 1999) and the *sepA* gene encoding a septin (De Groot *et al.*, 1998a; Chapter 3). The latter genes specifically accumulate in the transitional zone, which is the part of the stipe that forms the connection with the plectenchyma (inner cap tissue). For the housekeeping genes, four regions of different metabolic activity were found. These are the gills, the plectenchyma, the stipe, and the white hyphae of the peel, veil and annulus.

We also followed the temporal expression of *pgkA*, *pkiA*, *aldA* and *s15A* in whole fruit body tissue during fruit body development (results not shown). For this, fruit bodies were picked daily from the first visual appearance of primordia until they expose their spore-bearing gills and are considered mature. In a previous paper, we found that fluctuations of the expression levels of fruit body specific genes correlated with morphological changes of the fruit bodies during fruit body development (De Groot *et al.*, 1997). For the

housekeeping genes no significant changes in temporal expression were detected, indicating that in this case analysis of whole fruit body tissue is not sensitive enough to detect metabolic changes that take place during development of the primordium into a fully differentiated mushroom.

Conclusions

The *A. bisporus* genes coding for 3-phosphoglycerate kinase, pyruvate kinase and aldehyde dehydrogenase were cloned and characterised. The three genes are spiked with numerous small intervening sequences, whose positions as a rule do not correlate with intron positions that show conservation among ascomycetes. Phylogenetic trees based on amino acid sequence comparisons of PGK, PKI and ALDH proteins were very comparable and they all showed that *A. bisporus* has diverged early from the better studied group of ascomycete and deuteromycete fungi. Analysis of the codon usage of available *A. bisporus* genes indicates that *A. bisporus* has a small preference for C or T in the third base position.

Northern analysis of the spatial expression patterns of the cloned housekeeping genes in mature fruit bodies showed that all assayed parts of the mushrooms are metabolically active and with these genes four metabolically different regions of mature mushrooms could be distinguished. For *pgkA* and *aldA*, the mRNA levels in (specific parts of the) mushrooms are clearly elevated compared to compost grown mycelium, and the spatial expression pattern of *aldA* in mature mushrooms is very similar to that of the fruit body specific hydrophobin encoding *hypA* gene. The *aldA* gene might therefore provide a valuable tool to study the transcriptional regulation of fruit body development.

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7

An endo-1,4-ß-xylanase encoding gene from *Agaricus bisporus* is regulated by compost specific factors

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Journal of Molecular Biology **277,** 273-284 (1998)

An endo-1,4-ß-xylanase encoding gene from *Agaricus bisporus* is regulated by compost specific factors

Compost is the preferred substrate for growth of the edible fungus *Agaricus bisporus*. Utilisation of compost requires the production of enzymes involved in degradation of lignocellulolytic components. For molecular characterisation of these processes we are isolating the encoding genes. By applying heterologous screening techniques, we have cloned such a gene which is specifically induced on compost encoding an endo-1,4-ß-xylanase (*xlnA*) belonging to glycosyl hydrolase family 10. The gene encodes a pre-protein of 333 amino acid residues with a predicted molecular mass of 34,946 for the mature protein. The open reading frame is interrupted by ten introns of which introns 5 and 6 are separated by an exon of only two basepairs. High expression of the *xlnA* gene was observed in vegetative mycelium grown on sterilised compost while *xlnA* messengers were not detected in fruit bodies. Addition of glucose or xylose to compost repressed *xlnA* expression. When glucose-grown colonies were transferred to a medium containing cellulose, xylan or xylose as sole carbon source, the organism responded by expressing *xlnA* at a high level for a short period. Transfer from glucose to compost yielded a much stronger and constant *xlnA* induction. A similar pattern of expression was found for the *cel3* gene encoding a cellulase, suggesting that these genes are induced by compost specific factors rather than by the substrates they act upon. Antiserum raised against XLNA protein, which was heterologously expressed in *Escherichia coli*, detected when the fungus was grown on compost, an extracellular protein of 33 kDa with endoxylanase activity.

Introduction

Fruit bodies of the basidiomycetous fungus *Agaricus bisporus* are produced in large quantities for human consumption on a specific compost covered with a casing layer. Commercially available compost, which is selective for growth of *A. bisporus* mycelium (Gerrits, 1988), is usually prepared from wheat straw, straw-bedded horse manure, chicken manure and gypsum in a two phase humification process (Van Gils, 1988).

After composting, the substrate consists of two major components, a lignocellulose fraction and a microbial biomass. A broad range of activities involved in the degradation of the lignocellulose fraction including cellulose-, hemicellulose-, and lignin-degrading activities (Durrant *et al.*, 1991; Bonnen *et al.*, 1994) was found to be associated with *A. bisporus*. Using differential screening, a number of cellulose-induced genes were identified. Of these cellulose induced genes three were directly involved in the breakdown of cellulose and these encoded a cellulase (Chow *et al.*, 1994), a cellobiohydrolase and a mannanase (Yagüe *et al.*, 1997). Regulation studies of these genes showed that they were repressed by

the easily metabolised carbon sources glucose, fructose and lactose (Chow *et al.*, 1994; Yagüe *et al.*, 1997).

The major constituent of hemicellulose, the second most abundant polysaccharide in plant cell walls, is xylan. Xylan is a complex polymer consisting of ß-1,4-linked xylosyl residues, which can be acetylated or can have covalently linked arabinosyl and glucuronic acid side-groups. Complete enzymatic conversion of xylan into monomeric sugars requires the concerted action of several enzymes including endo-1,4-ß-xylanase, ß-xylosidase, α-L-arabinofuranosidase, α-glucuronidase and a number of esterases. Sequences of xylan-degrading enzymes, classified as members of different glycosyl hydrolase families, have been reported for a large number of fungi and prokaryotes (Henrissat & Bairoch, 1993). For a number of fungi such as *Aspergillus* species different endo-xylanase activities were found and the corresponding genes were cloned (Ito *et al.*, 1992a, 1992b; De Graaff *et al.*, 1994; MacCabe *et al.*, 1996). However, in the field of basidiomycetous fungi knowledge of the primary structures of these enzymes is very poor and only the protein sequence of an endo-1,4-ß-xylanase from *Schizophyllum commune*, belonging to glycosyl hydrolase family 11, has been reported (Oku *et al.*, 1993).

In order to gain a better understanding of the role of the hemicellulolytic activities associated with *A. bisporus* in the utilisation of compost, we have cloned a xylanase encoding gene. The regulation of the gene was studied by growth of *A. bisporus* colonies on milled compost and/or defined carbon sources and was compared with *cel3* expression. Furthermore, the substrate specificity of the *xlnA* gene product was analysed by activity measurements and Western analysis showing that the XLNA protein has endo-1,4-ß-xylanase activity.

Materials and Methods

Strains and culture conditions

Aspergillus kawachii (strain CBS 120.80) was used as source for generation of the *xynA* probe by polymerase chain reaction (PCR).

For the isolation of total DNA of homokaryotic *Agaricus bisporus* strains H39 and H97, these strains were grown as described by De Groot *et al.* (1996). The commercially widely used strain Horst®U1 was used for expression studies and protein analyses. Fruit body primordia and mature mushrooms were obtained during a ten day flushing cycle (Van Gils, 1988). Mycelium, pre-grown for 14 days as colonies on solidified DT80 medium (Sonnenberg *et al.*, 1988) supplemented with 3% (w/v) glucose, was used as inoculum for cultures in which the mycelium was grown for seven days on different media on top of cellophane sheets in Petri dishes. All mycelium samples consisted of 25 inoculates grown in five Petri dishes. The media contained either DT80 or 7.5% (w/v) sterilised Phase II compost (Van Gils, 1988) powder, which was prepared by sieving (mesh size 1 mm) dried and

milled compost, and were solidified with 1.5% (w/v) agar. These media were supplemented with 3% (w/v) glucose, 0.1% (w/v) carboxymethylcellulose (CMC, Sigma), 0.1% (w/v) oat spelts xylan (Sigma) or 0.1% (w/v) birchwood xylan (Sigma). Shift experiments were done as follows. Mycelium was grown for seven days on compost agar supplemented with 3% (w/v) glucose after which the mycelium, using the cellophane sheet as carrier, was transferred to Petri dishes containing DT80 medium or DT80 medium supplemented with 7.5% (w/v) compost, 1% (w/v) CMC, 1% (w/v) oat spelts xylan, 1% (w/v) xylose or 1% (w/v) glycerol. Mycelium was harvested 0, 1, 2, 4, 8, 16 and 24 hours after transfer. Similarly, mycelium grown on compost agar medium was transferred to compost agar or compost agar supplemented with 1% (w/v) glucose or 1% (w/v) xylose and harvested after 24 hours. All samples were immediately frozen into liquid nitrogen and stored at -70°C.

Escherichia coli SolR cells (Stratagene) were used for *in vivo* excision of the cloned cDNA insert. *E. coli* LE392 (Promega) was used for phage amplification and λ DNA isolation. *E. coli* DH5α (Bethesda Research Laboratories) was used for plasmid transformation and propagation. Strain SG13009 (Qiagen) was used for heterologous expression of XLNA in *E. coli*.

Recombinant DNA techniques and enzymes

Amplification of PCR products was achieved using 30 cycles of one minute of denaturation at 94°C, one minute of annealing at 60°C and one minute of extension at 72°C, followed by a final cycle of five minutes at 72°C. For the cloning of PCR products the pGEM-T vector system of Promega was used. Plasmid pUC19 (Yanisch-Perron *et al.*, 1985) was used as cloning vector for genomic DNA fragments. Plasmid pQE60 (Qiagen) was used as *E. coli* expression vector. Standard DNA manipulations were carried out essentially as described by Sambrook *et al.* (1989). Restriction enzymes and other enzymes used for DNA manipulations were purchased from BRL and used according to the supplier's instructions.

Cloning and sequencing of *xlnA*

An *A. kawachii* probe, comprising 741 bp of the *xynA* gene and starting 89 bp downstream of the translation start, was generated by standard PCR techniques using total DNA, followed by labelling with [α-^{32}P]dATP according to the method of Feinberg & Vogelstein (1983). The *A. bisporus xlnA* gene was isolated from a λEMBL4 genomic library of *A. bisporus* strain H39 using standard methods. Hybridisations were performed at 56°C in standard hybridisation buffer (SHB: 6 x SSC, 5 x Denhardt's solution, 0.5% (w/v) sodium dodecyl sulphate (SDS) and 100 μg ml^{-1} denatured herring sperm DNA) with final washing in 2 x SSC (SSC; 0.15 M NaCl, 0.015 M trisodium citrate, pH 7), 0.1% (w/v) SDS. Cloned hybridising fragments, cloned PCR products and cDNA clones were sequenced by the method of Sanger *et al.* (1977) using a phage T7 sequencing Kit (Pharmacia) and standard and sequence specific oligonucleotides.

Isolation of *xlnA* cDNA and primer extension

A cDNA expression library obtained from Horst®U1 primordia was screened using a genomic *A. bisporus* 790 bp *Bam*HI-*Hin*dIII *xlnA* fragment as a probe. The membranes were hybridised overnight at 65°C and washed stringently (65°C, 0.1 x SSC, 0.1% (w/v) SDS). The cloned insert from the only hybridising phage was rescued as a pBluescript plasmid and sequenced. Additional cDNA fragments were generated by RT-PCR. First-strand cDNA was synthesised by standard primer extension methods as described by Sambrook *et al.* (1989) using RNA, treated with DNase (Promega), from mycelium grown on compost as template. Three different reactions were performed using 24-mer primers in antisense direction, two of which were positioned over previously deduced

introns (Figure 7.2). The first strand cDNA was subsequently used as template in standard PCR reactions to generate double strand cDNA fragments that were cloned in pGEM-T and sequenced. Similar primer extension techniques were applied to determine the transcription starting point(s) of *xlnA* using a primer positioned 56 bp downstream of the putative translation start-codon (Calzone *et al.*, 1987).

Total RNA isolation and Northern analysis

Total RNA was isolated from mycelium samples using TRIzol™ Reagent (BRL). The concentration of the RNA samples was determined spectrophotometrically and 10 µg RNA was denatured using glyoxal and dimethyl sulphoxide by standard techniques, separated on a 1.6% (w/v) agarose gel and transferred to Hybond-N membrane (Amersham). The size of *xlnA* messenger was determined by co-electrophoresis of RNA molecular mass markers (BRL), which were stained with methylene blue after transfer to Hybond-N (Sambrook *et al.*, 1989). Hybridisation was executed at 42°C in SHB to which 10% (w/v) dextran sulphate and 50% (v/v) formamide was added and the membranes were stringently washed (65°C, 0.1 x SSC, 0.1% (w/v) SDS). For Northern analysis of *xlnA*, the 524 bp partial *xlnA* cDNA clone was used as probe (unless stated otherwise). For Northern analysis of *cel3* (Chow *et al.*, 1994) and *s15A* (Schaap *et al.*, 1995) full-length cDNAs were used as probes, and to provide a loading control all blots were rehybridised with part of the 28S rDNA repeat (Schaap *et al.*, 1996). When appropriate signal intensities were compared by scanning the autoradiograms with an Ultroscan XL laser densitometer (LKB).

Heterologous expression in *E. coli*

A cDNA fragment specifying the entire predicted mature protein starting at glutamine residue 18 was synthesised by RT-PCR, cloned in pGEM-T vector and sequenced. The two primers used for amplification contained a *Bgl*II site at their 5' terminus which enabled cloning of the cDNA insert into expression vector pQE60 (Qiagen). The recombinant protein contained six histidine residues at its C terminus, allowing a one-step purification using Ni^{2+}-NTA resin (Qiagen) and denaturing conditions. Antibodies against XLNA were raised in Balb/c mice following the procedure described by Van der Veen *et al.* (1991).

Western analysis, protein purification and activity measurements

Protein samples were analysed by SDS-PAGE using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS according to the method of Laemmli (1970). The gels were either stained directly using Coomassie Brilliant Blue R250 or separated proteins were electroblotted onto nitrocellulose. Standard protein molecular mass markers were obtained from Serva. For Western analysis of complete colonies, mycelium was blotted directly onto nitrocellulose. Western blots were probed with anti-XLNA antiserum and XLNA was visualised with alkaline phosphatase-labelled goat anti-mouse IgG as described by the supplier (Bio-Rad).

Protein extracts from mycelium grown on compost and compost/glucose agar plates were obtained by grinding and extracting the mycelium in 10 mM bisTris (pH 7.0). A protein extract from full-grown compost was obtained by washing the compost with water, precipitation with 80% (w/v) ammonium sulphate of the soluble fraction with a molecular mass exceeding 10 kDa and resuspension in 10 mM bisTris, pH 7.0. Proteins from compost agar plates were purified by loading the extract on a Resource Q column (Pharmacia) and eluting with a 0 to 1.0 M NaCl gradient. Xylanase and cellulase activity measurements were done as described by Bailey *et al.* (1992). Incubations were performed at 37°C with 1% (w/v) birchwood xylan or 0.5% (w/v) CMC in 10 mM

potassium phosphate at pH 6.8. Xylan breakdown products were analysed by high performance anion exchange chromatography. Samples (10 µl; 0.2 to 2 mU) were incubated for 60 minutes at 37°C with 0.5 ml 1% (w/v) birchwood xylan. A fivefold diluted heat-inactivated culture filtrate was loaded on a CarboPac PA-100 column (Dionex). Internal standards used were D-xylose to xylopentaose (Megazyme) oligomers. Elution was performed with a gradient of 0.05 M to 0.90 M NaOH and monitored by amperometrical detection. Protein concentrations were determined using the bicinchoninic acid method (Sigma).

Isolation of total genomic DNA and Southern blot analysis

Total DNA from strains H39 and H97 was isolated according to the method of De Graaff *et al.* (1988). CHEF electrophoresis of intact chromosomal DNA of H39 and H97 was performed as described by Sonnenberg *et al.* (1996) using a Biorad CHEF DrII system followed by capillary blotting to Hybond-N membrane. The membranes were hybridised overnight at 65°C in SHB using the 524 bp partial *xlnA* cDNA clone as probe. The membranes were washed stringently at 65°C and exposed.

Nucleotide and protein sequence comparisons

The nucleotide sequences reported in this paper have been deposited with the Genbank/EMBL Databases under the accession numbers Z83199 for *xlnA* cDNA and Z83310 for *xlnA* genomic sequences.

The predicted XLNA amino acid sequences were compared with translated sequences from GenBank and EMBL using the University of Wisconsin Genetics Computer Group (GCG) program BLASTX (Gish & States, 1993). Homology calculations and amino acid alignments were made using the GCG programs BESTFIT, PRETTY and PILEUP.

Results

Identification of the *xlnA* gene

For the cloning of *A. bisporus* genes that are involved in the breakdown of hemicellulose components present in compost we screened a genomic library of the homokaryotic strain Horst H39 using non-stringent hybridisation conditions with various *Aspergillus* genes encoding hemicellulolytic enzymes as probes (Ito *et al.*, 1992a, 1992b: *xynA*, *xynC*; De Graaff *et al.*, 1994: *xlnA*; MacCabe *et al.*, 1996: *xlnC*). Only hybridisation with an 841 bp PCR fragment specifying a structural part of the *A. kawachii xynA* gene encoding an endo-1,4-ß-xylanase (Ito *et al.*, 1992a) resulted in the isolation of a hybridising lambda phage.

To investigate whether the smallest hybridising fragment from this phage, a 0.8 kb *Bam*HI-*Hin*dIII fragment, specified a gene or gene fragment that is expressed on compost, we used this as a probe in a Northern analysis. We compared RNA isolated from mycelium of the commercial strain Horst®U1 grown on milled compost with RNA isolated from fruit

body primordia and mature mushrooms from the same strain (Figure 7.1(a)). In compost, the fragment hybridised with a messenger of 1300 nt, a size that could specify a glycosyl hydrolase of family 10, the protein family to which the *A. kawachii* xylanase was assigned (Henrissat & Bairoch, 1993). No expression was detectable in the two fruit body samples. Together with hybridisation of the probe with the *A. kawachii xynA* gene, these results strongly suggested that in this way a putative xylanase gene was cloned, hereafter designated *xlnA*, the product of which is involved in compost utilisation.

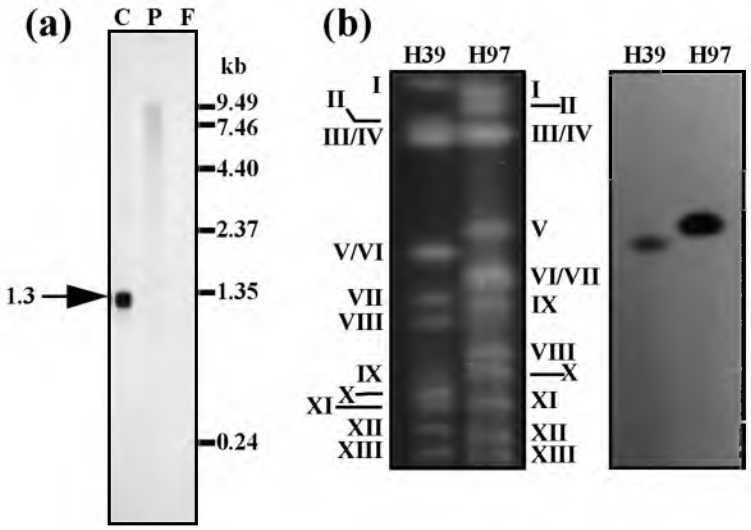

Figure 7.1. Analysis of the genomic *A. bisporus* 0.8 kb *Bam*HI-*Hin*dIII fragment that hybridised with the *A. kawachii xynA* gene. (a) Northern analysis. Lanes were loaded with 10 µg total RNA isolated from mycelium grown on sterilised compost (C), fruit body primordia (P) and mature fruit bodies (F). The sizes of the RNA molecular mass markers are indicated (in kb). The 28S rRNA loading control verified equal loading (not shown). (b) CHEF analysis of *A. bisporus* strains H39 and H97. Chromosomes of the parental lines of Horst®U1 separated by CHEF gel electrophoresis after staining with ethidium bromide (left panel) and after hybridisation with the 0.8 kb *Bam*HI-*Hin*dIII fragment (right panel). Chromosomes are indicated by Roman numerals according to Sonnenberg *et al.* (1996).

Southern analysis of total DNA from the homokaryotic strains H39 and H97, both constituents of the cultivar Horst®U1, probed with the 0.8 kb *Bam*HI-*Hin*dIII *xlnA* fragment, indicated that a single copy of this gene is present in the *A. bisporus* genome (not

shown). This was confirmed by Southern analysis of intact chromosomes of the same homokaryotic strains separated by contour-clamped homogeneous field electrophoresis (CHEF) (Figure 7.1(b)). CHEF analysis and linkage analysis showed that the putative *xlnA* gene is located on chromosome V of both strains (Sonnenberg *et al.*, 1996).

Subsequently, a 6 kb *Eco*RI fragment from the hybridising phage was cloned and the gene and its surroundings were sequenced from both strands (Figure 7.2). Alignment of the conceptual translation of *xlnA* with *A. kawachii* XYNA suggested the presence of numerous short intervening sequences in the *A. bisporus xlnA* gene. We therefore screened a directional Horst®U1 cDNA library (De Groot *et al.*, 1996) with the 0.8 kb *Bam*HI-*Hin*dIII fragment. This resulted in a partial cDNA of 524 bp, with a poly(A) track at its 3' end. By sequencing this cDNA we could confirm the positions of two intervening regions in the 3' terminal region of the *xlnA* gene. The remaining intervening sequences were confirmed by reverse transcription/polymerase chain reaction (RT-PCR) techniques using total RNA from compost-grown mycelium as template. Sequencing of overlapping cDNA fragments revealed ten introns in total, varying in length from 47 to 56 bp. All introns appear to have normal fungal splice sites (Unkles, 1992). Interestingly, between introns 5 and 6 an exon of only two basepairs was found. Using the same primer extension techniques and an oligonucleotide that was positioned over the presumed translation start codon, two transcription initiation sites were found, 217 and 68 bp upstream of the translation start. Further upstream, at position -238 relative to the start codon, a CCAAT box characteristic of fungal promoter sequences was found, but a TATA box, which is often absent in fungal promoters, was not detected in the promoter region (Unkles, 1992). No known consensus sequence for eukaryotic polyadenylation sites was found in the region surrounding the polyadenylation site (Humphrey & Proudfoot, 1988). Taken together the analysis confirmed that the transcribed region is about 1300 bp in size and that the *xlnA* gene encodes a peptide of 333 amino acid residues.

Amino acid sequence analysis

A highly hydrophobic amino terminal sequence with a predicted α-helix in the translated amino acid sequence of XLNA, suggested the existence of a 17 amino acid residues containing signal sequence for secretion (Von Heijne, 1986). The mature protein would then encode a protein of 316 amino acid residues, with a calculated molecular mass of 34,946, similar in size to that of other fungal family 10 glycosyl hydrolases (Figure 7.3). The pre-protein is 43% and 46% identical to XYNA of *A. kawachii* and the catalytic

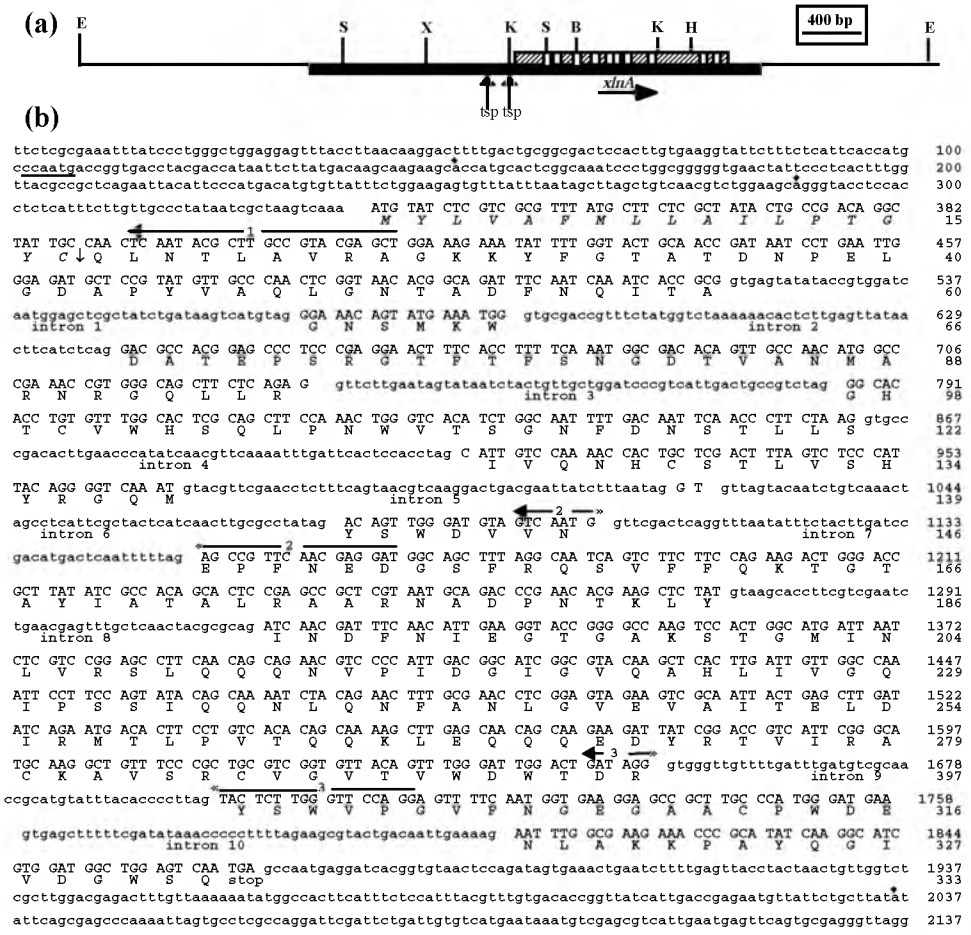

Figure 7.2. Genomic organisation of *xlnA*. (a) Molecular map of the *xlnA* gene. The sequenced region is marked by a filled box, the coding regions are represented by hatched boxes and introns in the coding region of *xlnA* are represented by open boxes. The horizontal arrow indicates orientation of transcription. The vertical arrows mark the transcription starting points (tsp). The following restriction sites are indicated: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; S, *Sst*I: X, *Xho*I. (b) Nucleotide sequence of the *xlnA* gene and its flanking regions and the deduced amino acid sequence of the XLNA protein. Coding regions of *xlnA* are shown in upper case letters, flanking regions and intervening sequences in lower case letters. The two transcription starting points and the polyadenylation site are indicated by diamonds. A putative CCAAT box in the *xlnA* promoter region is underlined. Primers used for transcription starting point determination (primer 1) and RT-PCR experiments (primers 1, 2 and 3) are indicated by arrows above the sequence. The XLNA amino acid sequence is given underneath the coding regions in standard one-letter code. The putative signal sequence is shown in italics and a potential signal peptidase cleavage site is indicated by a vertical arrow.

Figure 7.3. Comparison of the deduced XLNA amino acid sequence with other family 10 glycosyl hydrolases. The deduced *xlnA* amino acid sequence was aligned with those of *C. fimi cex* (O'Neill *et al.*, 1986), *T. alba xylA* (EMBL accession number Z81013), *F. oxysporum Ffam1* (Sheppard *et al.*, 1994) and *A. kawachii xynA* (Ito *et al.*, 1992a). Gaps were introduced for optimal alignment and are indicated by dots. The proposed N terminal residue of the mature *A. bisporus* protein is marked by an asterisk. The consensus sequence is composed of residues shared by at least four of the (predicted) mature proteins.

domain of *Fusarium oxysporum* Ffam1, respectively, both of which are fungal xylanases classified as family 10 glycosyl hydrolases. Unexpectedly, the protein was more homologous to the catalytic domains of two bacterial cellulases from this family; it showed 49% identity with CEX from *Cellulomonas fimi* and 47% identity with XYLA from *Thermomonospora alba*. A cellulose binding domain, often present in glycosyl hydrolases as an extension in either the C or N terminus was not found in the *A. bisporus* protein.

The *xlnA* gene is induced on compost and repressed on glucose

To study the relation between the carbon source used for growth of the fungus and the expression of the *xlnA* gene, *A. bisporus* strain Horst®U1 was grown for seven days on various carbon sources. Highly induced mRNA levels of *xlnA* were observed in mycelium grown on milled compost agar and the gene was also abundantly expressed on compost agar to which 0.1% (w/v) CMC was added (Figure 7.4). In contrast, *xlnA* expression was repressed on compost agar to which 3% (w/v) glucose was added. In cultures that were grown in solidified DT80 medium supplemented with 3% (w/v) glucose, 0.1% (w/v) CMC, 0.1% (w/v) oat spelts xylan or 0.1% (w/v) birchwood xylan no *xlnA* expression was observed, suggesting an inducing effect on the *xlnA* gene which is specific for compost and which cannot be mimicked by any of these carbon sources.

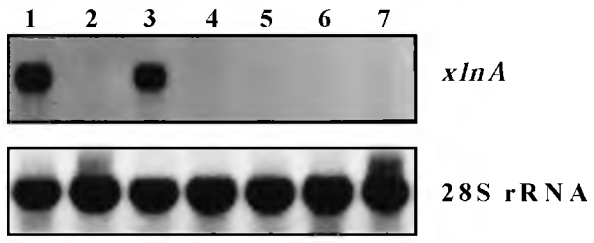

Figure 7.4. Transcript levels of *xlnA* in mycelium grown on solidified media. Northern analysis of *xlnA* mRNA levels in Horst®U1 mycelium that was grown for seven days on cellophane sheets on solidified media supplemented with different carbon sources. Lane 1, 7.5% (w/v) compost; lane 2, 7.5% (w/v) compost + 3% (w/v) glucose; lane 3, 7.5% (w/v) compost + 0.1% (w/v) carboxymethylcellulose (CMC); lane 4, 3% (w/v) glucose; lane 5, 0.1% (w/v) CMC; lane 6, 0.1% (w/v) oat spelts xylan; lane 7, 0.1% (w/v) birchwood xylan. The blot was rehybridised with the 28S rDNA repeat to provide for a loading control.

In a shift experiment *xlnA* expression was induced from a repressed state by exchanging the repressing carbon source glucose by possible inducing carbon sources (Figure 7.5(a)). Horst®U1 mycelium grown for seven days on compost agar supplemented with glucose, showing almost no *xlnA* expression, was transferred to DT80 medium supplemented with 7.5% (w/v) compost or 1% (w/v) CMC, oat spelts xylan or xylose. Colonies that were transferred from compost/glucose to compost showed a clear induction of the *xlnA* gene and mRNA levels increased constantly during the time course of the experiment. For mycelium transferred to CMC, a transient increase in *xlnA* mRNA with highest levels 16 hours after transfer was observed, but its peak mRNA levels are a twofold lower than the level reached on compost 24 hours after transfer. On xylan and xylose also a transient increase of *xlnA* mRNA was observed, reaching highest levels eight hours after transfer of the mycelium. The same blots were hybridised with the *A. bisporus s15A* gene which encodes a small subunit ribosomal protein (Schaap *et al.*, 1995) and with the 28S rDNA repeat to provide for a loading control. For none of the tested carbon sources: compost, CMC, xylan and xylose, were differences in *s15A* mRNA levels more than twofold detected (not shown), confirming the observed induction of *xlnA* on these carbon sources. To test whether the transient character of the expression could be due to derepression as a result of glucose depletion in these samples, mycelium was also transferred to solidified DT80 medium supplemented with 1% (w/v) of the neutral carbon source glycerol (Figure 7.5(a)) and DT80 without any carbon source (not shown). In both cases, the low *xlnA* expression levels observed prior to shifting disappeared after transfer.

***XlnA* and *cel3* are co-expressed**

Since glycosyl hydrolase family 10 harbours both xylanases and cellulases and compost-grown mycelium shows both xylanase and cellulase activity, we compared the regulation of *xlnA* with that of the *cel3* gene encoding a cellulase (Chow *et al.*, 1994) by Northern analysis of the same shift experiment. On all carbon sources the *cel3* expression patterns were very similar to that of *xlnA* (Figure 7.5(a)).

Repressing effects on *xlnA* and *cel3* expression were studied by transferring mycelium, in which *xlnA* and *cel3* were induced by growth on compost agar, to compost agar supplemented with 1% (w/v) glucose or xylose (Figure 7.5(b)). In both cases, *xlnA* and *cel3* showed a decrease in their mRNA levels 24 hours after transfer. The strongest repression was observed on compost/xylose, showing mRNA levels that were as low as levels in mycelium grown for seven days on compost/glucose. The comparable results obtained with *xlnA* and *cel3* gene again indicate that the two genes are co-regulated.

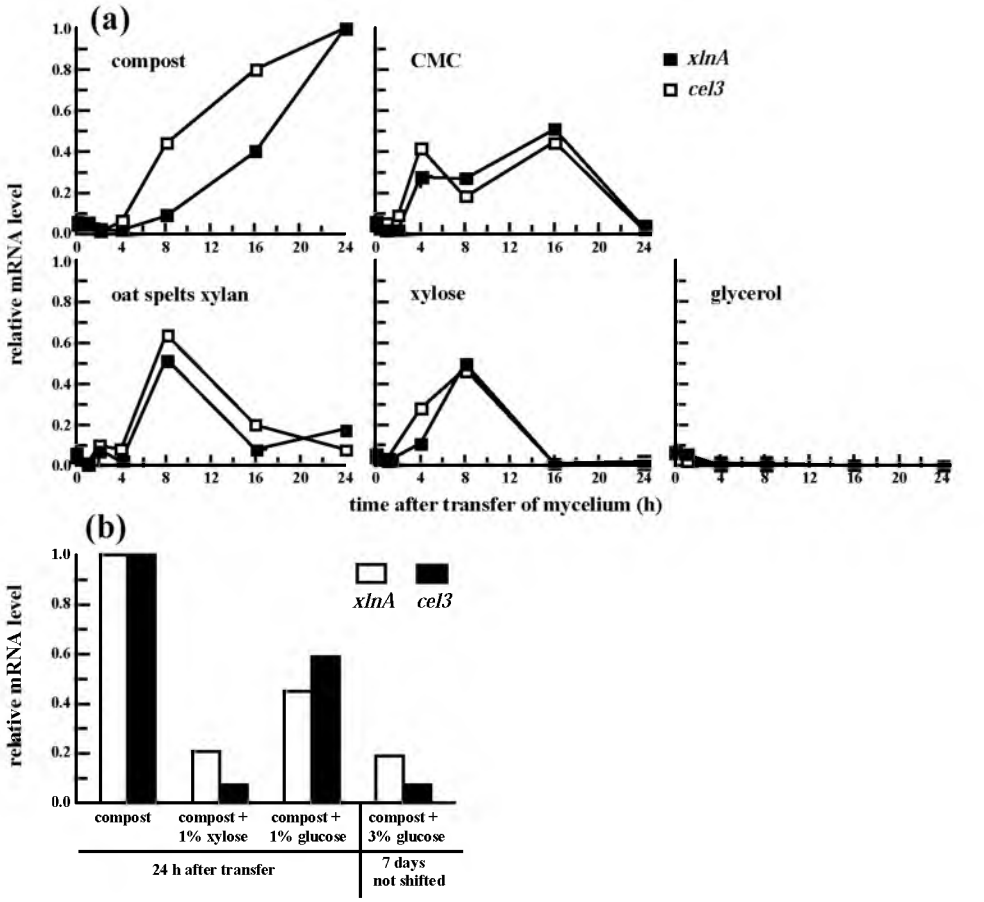

Figure 7.5. Northern analyses of *xlnA* and *cel3* using shift experiments. (a) Graphs of relative mRNA levels of *xlnA* and *cel3* after transfer from compost/glucose to various carbon sources. Horst®U1 colonies were pre-grown for seven days on 7.5% (w/v) compost agar + 3% (w/v) glucose (t = 0) and transferred to solidified DT80 medium supplemented with 7.5% (w/v) compost, 1% (w/v) CMC, 1% (w/v) oat spelts xylan, 1% (w/v) xylose or 1% (w/v) glycerol. Transcript levels were normalised against 28S ribosomal RNA and the mRNA levels obtained for mycelium 24 hours after transfer to compost were set to 1.0. (b) Repression of *xlnA* and *cel3* after transfer from compost to compost supplemented with xylose or glucose. Horst®U1 colonies were pre-grown for seven days on 7.5% (w/v) compost and transferred to solidified medium containing compost, compost + 1% (w/v) xylose or compost + 1% (w/v) glucose. The *xlnA* and *cel3* mRNA levels in mycelium grown for seven days on compost + 3% (w/v) glucose is shown on the right for comparison. Transcript levels were normalised against 28S ribosomal RNA and the mRNA levels obtained for mycelium 24 hours after transfer to compost were set to 1.0.

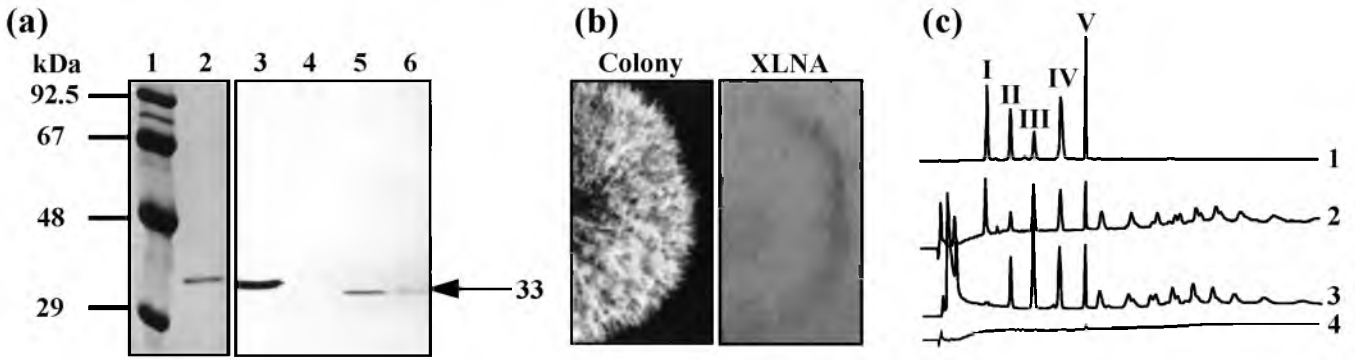

Figure 7.6. Characterisation of XLNA. (a) SDS-PAGE and Western analysis of the XLNA protein. Left panel, SDS-PAGE. Lane 1, molecular mass marker proteins; lane 2, purified recombinant XLNA. Right panel, Western analysis probed with antiserum raised against the recombinant XLNA; lane 3, Purified recombinant XLNA; lane 4, cell-free extract from compost/glucose-grown mycelium; lane 5, cell free extract from compost-grown mycelium; lane 6, protein extract from full-grown compost. (b) Western analysis of whole colonies grown on compost agar. Left panel, visualisation of a part of a colony. Right panel, Western analysis of the same colony probed with anti-XLNA antiserum. (c) High performance anion exchange chromatography elution profiles. 1, xylo-oligomer standards. Peak I to V: D-xylose to D-xylopentaose; 2, birchwood xylan after incubation for 60 minutes with cell-free extract from compost-grown mycelium; 3, birchwood xylan after incubation for 60 minutes with purified XLNA; 4, birchwood xylan (control).

The *xlnA* gene encodes an endo-1,4-ß-xylanase

For analysis of the characteristics of the gene product, *xlnA* was heterologously expressed in *Escherichia coli*. The *xlnA* coding region was amplified using RT-PCR starting with the first nucleotide of the glutamine residue at position 18, and cloned into the *E. coli* expression vector pQE60. This resulted in the production of an inactive XLNA protein having six histidine residues at its extreme C terminal end and a molecular mass, estimated from SDS-PAGE, of about 34 kDa (Figure 7.6(a)). Anti-XLNA antiserum raised against the purified recombinant protein detected a slightly smaller protein band of about 33 kDa in compost-grown mycelium and in a protein extract of full-grown compost, compost that is fully colonised with vegetative *A. bisporus* mycelium and is used for mushroom production. Consistent with the results of the Northern analyses, anti-XLNA antiserum did not react with mycelium grown on compost/glucose. Western analysis of intact colonies showed the presence of extracellular XLNA protein at the outer edge of colonies growing on compost agar (Figure 7.6(b)).

In mycelium grown on compost agar high levels of xylanase activity were measured compared to mycelium grown on compost/glucose agar (Table 7.1). Xylanase activity was also detectable in full-grown compost. In order to obtain evidence that at least part of the xylanase activity in protein extracts from mycelium grown on compost agar results from *xlnA* expression, we purified xylanase activity using a Resource Q anion column. Two fractions were collected in which xylanase activity was detectable. Ninety per cent of the xylanase activity was associated with the non-bound protein fraction and this activity was 11-fold purified. The remaining 10% of the activity was collected after salt elution and eluted with 0.3 M NaCl. Western analysis of the two fractions showed that XLNA was only present in the non-bound protein fraction (not shown). Cellulase activity, low levels of which were found in compost-grown mycelium and full-grown compost, was not detected in the same non-bound protein fraction. To establish the mode of action of the *xlnA* gene product, we incubated birchwood xylan with a protein extract from mycelium grown on compost agar and with the partially purified xylanase and analysed the reaction products by HPLC analysis. The total protein extract is able to degrade xylan into oligomeric and monomeric sugars. The xylanase-containing protein fraction on the other hand only produced oligomeric sugars (Figure 7.6(c)). Taken together the results confirm that the *xlnA* gene encodes an endo-1,4-ß-xylanase that is specifically expressed in compost.

Table 7.1. Partial purification of XLNA

Source	Total xylanase act. (units)[a]	Specific xylanase act. (units/mg protein)	Purification (-fold)
compost agar	41.8	1.1	1
non-bound protein	37.6	12.3	11
elution with 0.3 M NaCl	4.1	2.6	
compost + glucose		0.05	
full-grown compost		0.1	

[a] One unit xylanase activity is defined as the amount of enzyme that produces one µmol reducing sugars min^{-1}.

Discussion

By heterologous screening a xylanase encoding gene was cloned that was found to have homology with members of glycosyl hydrolase family 10. Family 10 comprises both bacterial and fungal enzymes that can hydrolyse cellulose, xylan or both (Gilkes *et al.*, 1991). Sequence analysis of the *xlnA* gene revealed that the gene was spiked with numerous introns as has been found for almost all other *A. bisporus* genes reported to date. For introns 1 to 4 and 7 to 10 normal intron boundaries, GT...AG (Unkles, 1992), were found. If we also assume normal intron-exon boundaries for the sequence comprising introns 5 and 6, these introns are separated by an exon of only two basepairs and the GT at the indicated position in Figure 7.2(b) is the only possible candidate exon. A similar arrangement has also been reported for the *tef1* gene of *Trichoderma reesei* (Nakari *et al.*, 1993).

The size of the deduced, mature *A. bisporus* XLNA protein is comparable to the size of *A. kawachii* xylanase A. Other glycosyl hydrolases belonging to family 10 have N or C-terminal extensions comprising a cellulose-binding domain (CBD) linked by a hinge region. CBDs facilitate binding of the cellulose substrate by the hydrolysing enzyme but are not indispensable for catalytic activity (Gilkes *et al.*, 1991). However, CBDs are also present in several xylanases and the absence of such a sequence in XLNA does not therefore give an indication of the substrate preference of this enzyme.

All *A. bisporus* mycelium that was cultured for *xlnA* regulation studies was grown on solidified medium covered with a cellophane sheet. This enabled a quick and selective transfer of mycelium (plus sheets) to fresh medium. To study actual *xlnA* expression in compost during cultivation of the mushroom crop, sampling of compost at different times is required. However, isolation of RNA from compost is technically difficult whereas shift experiments are impossible. Therefore we used *A. bisporus* colonies grown on a sterilised milled compost solidified with agar.

Mycelium grown for seven days on compost agar plates showed high *xlnA* mRNA levels. Addition of glucose to the compost agar had a strong, negative effect on *xlnA* expression but repression was not observed when CMC was added. In mycelium grown on glucose, CMC, birchwood xylan or oat spelts xylan as sole carbon sources no *xlnA* expression was observed, which clearly demonstrates the strong inducing effect of the compost on *xlnA* expression. Maximal colony size and mycelial density were observed on media containing compost, which reflects the preference of the fungus for using this fermented substrate as carbon source for growth.

Releasing glucose repression by transferring the mycelium from compost/glucose agar to compost agar showed a strong and constant induction of *xlnA*. Again, this illustrates that compost contains compounds that strongly induce expression of the *xlnA* gene. A transient and much lower induction of *xlnA* was observed in mycelium that was transferred to CMC, oat spelts xylan or xylose. A transient increase in *xlnA* mRNA levels, however, was not found after transfer to glycerol or to a medium without a carbon source and this suggests that *xlnA* expression is also induced by xylose, oat spelts xylan and CMC. The long-term low levels of *xlnA* mRNA observed on those carbon sources might be caused by an increased intracellular concentration of glucose and xylose. Thus, low concentrations of xylose and glucose would induce *xlnA* expression but when a certain intracellular threshold concentration is reached glucose and xylose repress *xlnA* expression. Recently, a similar mechanism of regulation, induction by low and repression by high levels of glucose, was found for the *SUC2* gene encoding invertase of *Saccharomyces cerevisiae* (Özcan *et al.*, 1997). Comparison of *cel3*, encoding a cellulase, and *xlnA* expression patterns showed that, although these genes and their promoters do not have significant sequence similarities, their expression patterns are very similar. Moreover, in agreement with the observed *xlnA* and *cel3* expression patterns, Manning & Wood (1983) reported glucose repression of endocellulase production and optimal activity at low cellulose concentrations. The intracellular glucose generated by addition of 0.1% (w/v) CMC to compost (Figure 7.4) might not have reached repressing concentrations. Alternatively, the growth stimulatory effect of compost might outcompete the repressing effect of cellulose as carbon source.

The *cel2* and *cel4* genes, encoding a cellobiohydrolase and a mannanase respectively, are co-expressed with *cel3* (Yagüe *et al.*, 1997) and this suggests that at least a number of the genes encoding enzymes involved in utilisation of the lignocellulolytic complex present in compost are co-regulated with the *xlnA* gene. The long term low expression level of *cel3* on CMC in this study supports recent findings by Yagüe *et al.* (1997) who found no induction of *cel2*, *cel3* and *cel4* by this carbon source. In their report, it was concluded that crystalline cellulose is necessary for induction of the *cel* genes. However, the observed co-regulation between *cel3* and the *xlnA* gene together with the strong induction of both genes on compost, considering the complex structure of this substrate, do not support this conclusion.

Effects of growing the *A. bisporus* colonies on top of cellophane sheets which can be used as a carbon source by this fungus, on *xlnA* and *cel3* expression could not be excluded. However, the observed high mRNA levels found on compost together with undetectable messenger levels on glycerol suggest that the cellulolytic components of the sheets used seem neither to repress nor to induce the expression of both genes.

Antiserum raised against the XLNA protein produced in *E. coli* detected an extracellular protein of 33 kDa in mycelium grown on compost. The size of this protein is slightly smaller than that of the recombinant XLNA protein, which is extended with six histidine residues and agrees well with the size of the predicted mature XLNA protein. This also suggests that the *A. bisporus* protein is not glycosylated. Partial purification together with Western analysis confirmed that XLNA has xylanase rather than cellulase activity and therefore seems to be involved in the release of sugars from the hemicellulolytic fraction in the compost.

The studies on the regulation of the *A. bisporus xlnA* and *cel3* genes presented in this paper demonstrate that their expression is regulated by components of the natural fermented substrate for growth rather than by the substrates they act upon and suggest an adaptation of the fungus towards the requirements of its ecological niche. Characterisation of compost fractions and their effects on *xlnA* expression will, hopefully, enable us to identify agent(s) involved in the regulation of lignolytic enzymes of *A. bisporus* and related basidiomycetous fungi.

Acknowledgements

The authors thank Dr. J.J.P. Baars of the Mushroom Experimental Station for culturing *A. bisporus* and H. Kester of MGIM for his assistance with the protein analysis. The *cel3* probe was kindly provided by Dr C.F. Thurston (King's College, London, UK).

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8

Isolation of expressed sequence tags of *Agaricus bisporus* and their assignment to chromosomes

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Applied and Environmental Microbiology **62,** 4542-4547 (1996)

Isolation of expressed sequence tags of *Agaricus bisporus* and their assignment to chromosomes

The genome of the cultivated basidiomycete *Agaricus bisporus* Horst®U1 and of its homokaryotic parents has been characterised by using an optimised method of pulsed field gel electrophoresis. Expressed sequence tags obtained as expressed cDNAs from a primordial tissue derived cDNA library and a number of previously isolated genes were used to identify the individual chromosomes of the parental lines of Horst®U1. The genome consists of 13 chromosomes, and its total size is 31 Mb. For those chromosomes that could not be resolved by contour-clamped homogeneous electric field electrophoresis, the segregation of marker genes was studied in a set of 86 homokaryotic offspring of Horst®U1. At least two markers were assigned to each individual chromosome. In this way, all individual chromosomes were unequivocally identified. The large size difference observed between the homologous chromosomes IX, harbouring the rDNA repeat, was shown to be largely due to a higher copy number of rDNA in parental strain H97 than in parental strain H39.

Introduction

The annual world production of the edible mushroom *Agaricus bisporus* (Lange) Imbach amounted to 1,950,000 tonnes in 1995 (Boon, 1995). Although it is an economically important crop, the breeding activity of *A. bisporus* was based only on chance selection until the late 1970s. This was mostly caused by the secondarily homothallic life cycle of *A. bisporus* (Raper *et al.*, 1972), which renders breeding difficult and rather unprofitable. Most of the basidia produce two spores which preferentially inherit two non-sister nuclei and retain heterozygosity for most of the parental markers (Summerbell *et al.*, 1989). Only approximately 2% of the basidia produce three or four spores, which form predominantly homokaryotic mycelia upon germination (Kerrigan & Ross, 1987).

The first systematic breeding approach was taken by Fritsche in The Netherlands, and this led to the introduction of the "hybrid" strains Horst®U1 and Horst®U3 (Fritsche, 1983). By the late 1980s most of the cultivated strains in the world were identical to or derived from those strains (Loftus *et al.*, 1988), leaving the industry with a crop that was genetically very limited and had a large risk of sensitivity to disease. Recently, a large collection of *A. bisporus* strains collected from the wild, designated the *Agaricus* Resource Program collection, has been established by Kerrigan (1991), forming a sound basis for further breeding activity. Also, some studies were carried out on the meiotic behaviour of *A. bisporus* (Summerbell *et al.*, 1989; Kerrigan *et al.*, 1993; Xu *et al.*, 1993) and on chromosome characterisation (Sonnenberg *et al.*, 1991; Royer *et al.*, 1992). A few genes of

A. bisporus have been isolated and were characterised, i.e., the glyceraldehyde-3-phosphate dehydrogenase I and II (Harmsen *et al.*, 1992), cellulase 1 (Raguz *et al.*, 1992) and cellulase 3 (Chow *et al.*, 1994), and laccase I and II (Perry *et al.*, 1993) genes.

Although the use of restriction fragment length polymorphism (RFLP) markers for the identification of homokaryotic offspring and for the confirmation of crosses between compatible homokaryons (Castle *et al.*, 1987) has already accelerated breeding, a detailed genetic linkage map in which important commercial traits are linked to well-defined genetic markers is needed for a more efficient process.

The first genetic map for *A. bisporus* was produced by Kerrigan and co-workers (Kerrigan *et al.*, 1993) and showed that homologous chromosomes segregate conventionally but that crossing over is infrequent. Although a step forward, this first map was incomplete and thus of limited practical use. A complete correspondence of linkage and genetic structure was not achieved. Also, for a number of loci, randomly amplified polymorphic DNA markers, which do not always give consistent results (Ellsworth *et al.*, 1993; Quiros *et al.*, 1995) were used. We therefore started a genome mapping programme in which well-defined genetic markers, preferentially genes, were to be used (Schaap *et al.*, 1995b). An additional advantage of this approach was that isolation of genes would supply tools for further study of the molecular biology of *A. bisporus*.

We identified a number of high copy cDNAs from a primordial cDNA library and used them as expressed sequence tags (ESTs) in mapping. Besides these newly isolated *A. bisporus* genes, previously identified genes and RFLP markers were used to identify homokaryotic offspring and homologous chromosomes of the parental lines. In addition, 86 homokaryotic offspring were obtained so that the segregation of markers on those chromosomes that could not be resolved by pulsed field electrophoresis could be studied. The same homokaryons were paired to the parental lines to determine the location of the mating type gene.

The present study forms a solid basis for the further development of a gene-based linkage map.

Materials and Methods

Strains and DNA manipulation

The commercial strain *A. bisporus* Horst®U1 and its parental strains, H39 and H97, were obtained from the culture collection of the Mushroom Experimental Station, Horst, The Netherlands, and maintained at 4°C on slant tubes of wheat extract agar (64 g of wheat boiled for 2 h in tap water, filtered to remove the grains, and sterilised together with 40 g of agar). Basidiospores of a fruit body

of strain Horst®U1 were germinated on MMP medium {1% malt extract, 0.5% mycological peptone, 10 mM MOPS [3-(*N*-morpholino)propanesulphonic acid], pH 7.0}. Single-spore cultures were isolated as described by Fritsche (1978), and their homokaryotic nature was identified by the loss of heteroallelism of two markers (*gdhA* and *cel1*), both located on the same chromosome.

Escherichia coli LE392 (Promega, Madison, Wis.) was used for phage amplification and λ DNA isolation. *E. coli* DH5α (GIBCO BRL Life Technology, Gaithersburg, MD.) was used for plasmid transformation and propagation. *E. coli* XL1-blue cells (Stratagene, La Jolla, Calif.) were used for *in vivo* excision of cloned cDNA inserts.

Standard DNA manipulations were carried out essentially as described by Sambrook *et al.* (1989). Restriction enzymes and other enzymes used for DNA manipulations were purchased from GIBCO BRL Life Technology and used according to the supplier's instructions. Probes were labelled with [α-^{32}P]dATP by the method of Feinberg & Vogelstein (1983) or with digoxigenin by using the Dig DNA labelling kit (Boehringer, Mannheim, Germany). Hybridisation was carried out overnight at 65°C in standard hybridisation buffer (6 x SSC [1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5 x Denhardt's solution, 0.5% sodium dodecyl sulphate, and 100 μg ml^{-1} denatured herring sperm DNA). The filters were washed at 65°C under stringent conditions, dried, and exposed on Kodak X-Omat AR films (Eastman Kodak Co., Rochester, NY) at -70°C with intensifying screens. When digoxigenin labelled probes were used, hybridisation and detection of hybrids were carried out according to the conditions recommended in the Dig Chemiluminescent Detection Kit (Boehringer). DNA sequences were determined using a Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Buckinghamshire, United Kingdom) and an ALF automated sequencer (Pharmacia Biotech, Uppsala, Sweden). Genomic clones of the *htbA* and *hhfA* genes were obtained by screening a λEMBL4 genomic library of *A. bisporus* strain H39 by standard methods with cDNA probes.

Generation and identification of ESTs

ESTs were generated by random excision of a cDNA library made from primordia and small fruit bodies (De Groot *et al.*, 1996) and were analysed by sequence analysis combined with a database search using the program Blast (Gish & States, 1993). ESTs that could be identified were preferentially used for mapping.

Chromosome-sized DNA preparations

For the isolation and separation of intact chromosomes, protoplasts were isolated from strains H39 and H97 (parental strains of Horst®U1) according to the system of Sonnenberg *et al.* (1988). Protoplasts were diluted with 2% InCert agarose (FMC Corporation, Rockland, Maine) at 42°C to a final concentration of 0.7% agarose and (1 - 1.5) x 10^9 protoplasts per ml. After transfer to a pre-warmed mold (40°C), agarose was allowed to solidify on ice for 30 min. The resulting plugs were incubated in NDS buffer (0.5 M EDTA, 0.01 M Tris-HCl [pH 9.5], 1% *N*-lauroylsarcosine) containing 1 mg ml^{-1} of proteinase K for 25 h at 50°C. Plugs were washed three times in 50 mM EDTA (pH 8.0) at room temperature and stored in 50 mM EDTA - 0.2% NaN_3 at 4°C until used. Before electrophoresis, plugs were incubated in electrophoresis buffer for 0.5 to 1 h.

Pulsed field electrophoretic separation of chromosomes

Pulsed field electrophoresis was optimised for the highest resolution of chromosomal bands. The modifications were based on the method previously described (Sonnenberg *et al.*, 1991). Gels were run at 14°C in 0.8% agarose (SeaKem; FMC) in 0.5 x TBE (1 x TBE is 0.089 M Tris-borate and

0.0025 M EDTA, pH 8.3) with a CHEF-DR II apparatus (BioRad, Hercules, Calif.). The electrophoretic parameters used were a ramped switching interval of 400 to 800 s at 100 V during 96 h followed by a ramped switching interval of 1,800 to 3,300 s at 50 V during 115 h. The electrophoresis buffer was replaced once after 96 h. *Sal*I fragments of chromosomes were separated by contour-clamped homogeneous electric field (CHEF) electrophoresis on a 1% agarose gel. The electrophoretic parameters used were a ramped switching interval of 150 to 240 s at 100 V for 24 h followed by a ramped switching interval of 50 to 103 s at 100 V for 96 h.

Ethidium bromide stained gels were photographed and capillary transferred to Hybond-N membranes (Amersham).

Mating tests

The collection of spores and the isolation of single-spore cultures were done as previously described (Fritsche, 1978). Pairings of the single-spore isolates with the parental homokaryons were made on compost agar (7.5% dried and powdered peak-heated compost in tap water, 1.5% agar). A positive mating, usually seen as fluffy mycelial growth along the junction zone, was transferred twice to new MMP medium before analysis.

Linkage analysis

The chi-square analysis was used to test for the hypothesis of no linkage between four markers assigned to chromosomes that could not be resolved by pulsed field electrophoresis (chromosomes III and IV). Because the alleles of the markers showed a statistically significant deviation from the expected 1:1 segregation, the expected ratios of the four progeny genotypes were corrected for the observed segregation distortion.

Nucleotide sequence accession numbers

The sequence data from this article have been deposited with the Genbank and EMBL data libraries under the following accession numbers: *htbA*, X94188; *hhfA*, X94189; *rs13*, X94704; *l41*, X94764; *rpaB*, X94765; *tefA*, X97204.

Results

Electrophoretic separation of chromosomes

Chromosome-sized DNA of strains H39 and H97 was separated by CHEF electrophoresis. Chromosomes of both lines were separated into 11 distinct bands (Figure 8.1(a)). Ethidium bromide staining revealed that in each karyotype two bands were stained more intensely than the others, indicating that these bands represented two chromosomes similar in length, i.e., chromosomes III and IV and chromosomes V and VI in strain H39 and chromosomes III and IV and chromosomes VI and VII in strain H97. This is supported by the assignment of markers and linkage analysis described below. Thus both strains

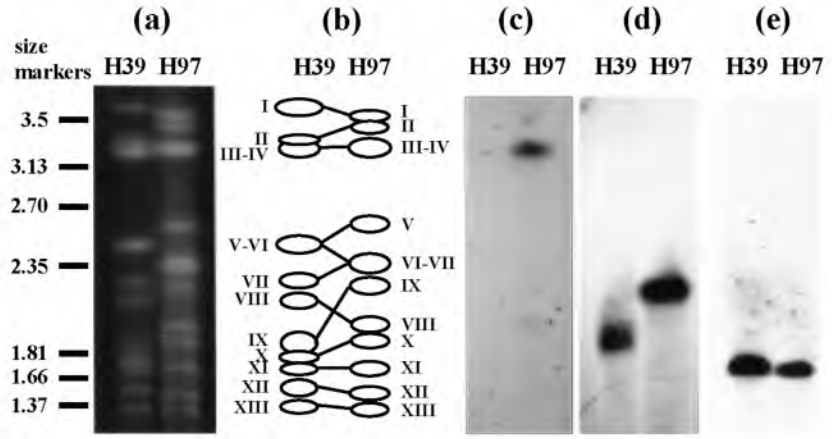

Figure 8.1. CHEF analysis of *A. bisporus* H39 and H97. (a) Chromosomes of the parental lines of strain Horst®U1 separated by CHEF gel electrophoresis. Strains are indicated above the panels. The positions of chromosomal size markers (chromosomes of *S. pombe* and *H. wingei*, in megabases) are indicated on the left. (b) Diagrammatic interpretation of the karyotypes. Chromosomes are indicated by Roman numerals. Homologous chromosomes are connected with lines. (c-e) Southern analysis of chromosomes separated by CHEF. CHEF blots were hybridised with the *htbA* gene (c), with the rDNA probe (d), or with the *Icc1* gene (e). Different CHEF gels were used in various experiments, accounting for the difference in signal strength observed.

probably have 13 chromosomes. The estimated sizes of chromosomal DNAs, calculated by using chromosome size standards from *Hansenula wingei*, ranged from 1.40 to 3.65 Mb (Table 8.1).

Cloning and identification of ESTs

For rapid isolation of *A. bisporus* ESTs, a cDNA library constructed from poly(A)-enriched RNA isolated from primordia of the strain Horst®U1 was used. From this library a number of randomly excised clones were analysed by sequence analysis. A database search using the programme Blast (Gish & States, 1993) identified a number of the ESTs, and these tags were preferentially used as probes.

Two ESTs within this collection were identified as apparently full length cDNAs of genes encoding the core histones H2B and H4 and were named *htbA* and *hhfA*, respectively. Both open reading frames have an A at the third position preceding the ATG start codon, which conforms to the optimal consensus sequence proposed by Kozak (1989).

Table 8.1. Estimated sizes of chromosomes of the parental lines of Horst®U1[a]

H39		H97	
Chromosome no.	Size (Mb)	Chromosome no.	Size (Mb)
I	3.65	I	3.60
II	3.14	II	3.44
III/IV	3.06	III/IV	3.06
V/VI	2.49	V	2.57
VII	2.23	VI/VII	2.32
VIII	2.11	VIII	1.90
IX	1.86	IX	2.13
X	1.75	X	1.81
XI	1.68	XI	1.66
XII	1.56	XII	1.51
XIII	1.45	XIII	1.40
Total	31	Total	31

[a] Sizes of chromosomes were estimated by using the chromosomes of *S. pombe* and *H. wingei*, run on the same gel, as size markers. The electrophoretic mobility's of the smallest chromosome of *S. pombe* (3.5 Mb) and of chromosomes of *H. wingei* (ranging from 3.13 to 1.37 Mb) were plotted against the chromosome sizes, and the resulting curve was used to estimate the sizes of the chromosomes of H39 and H97.

Also, as was found for histone genes from other fungi (Osley, 1991), both cDNAs end with a poly(A) tail. The *A. bisporus* H4 protein is identical to H4 of the basidiomycete *Phanerochaete chrysosporium* and has 90 to 93% identity with H4 proteins from a number of ascomycetous fungi. The homology between *A. bisporus* H2B and other fungal H2B proteins is lower: 78% identity with *Aspergillus nidulans*, 77% identity with *Saccharomyces cerevisiae*, and 74% identity with *Schizosaccharomyces pombe*, respectively. This is partly caused by insertions of 10 and 13 amino acids in the N-terminal part of the *A. nidulans* and *A. bisporus* H2B proteins relative to the *S. cerevisiae* H2B proteins. Both histone genes appear to be duplicated in the genome. For *htbA*, this is shown by CHEF analysis (Figure 8.1(c)). For *hhfA*, the existence of a second gene on chromosome VI (see below) was observed when the complicated restriction patterns obtained by Southern analysis of genomic DNA of strains H39 and H97 (Figure 8.2) were compared with the simple restriction patterns obtained by Southern analysis of an individual lambda clone harbouring a single copy of the *hhfA* gene (not shown).

Two ESTs were identified as apparent full length cDNA clones encoding ribosomal proteins. This group of about 80 different proteins is highly conserved between species, and in eukaryotes their genes are scattered throughout the genome. One cDNA clone

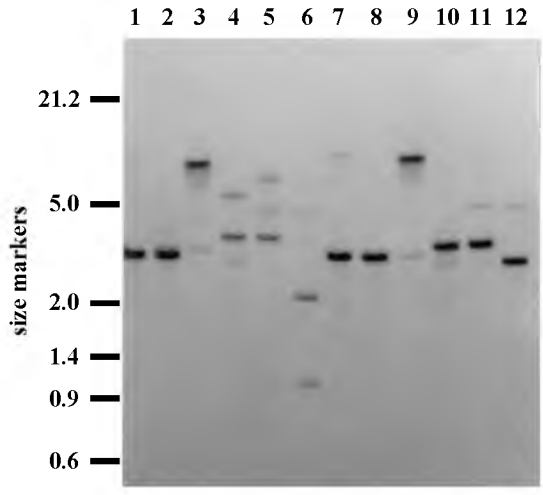

Figure 8.2. Southern analysis of genomic DNA of the parental lines of Horst®U1 with the histone H4 gene (*hhfA*). Digested DNA fragments of strains H39 (lanes 1 through 6) and H97 (lanes 7 through 12) were separated on a 0.8% agarose gel. The following digestions were performed: *Bam*HI (lanes 1 and 7), *Eco*RI and *Bam*HI (lanes 2 and 8), *Eco*RI (lanes 3 and 9), *Eco*RI and *Hin*dIII (lanes 4 and 10), *Hin*dIII (lanes 5 and 11), and *Bam*HI and *Hin*dIII (lanes 6 and 12). Molecular size markers (in kilobases) are indicated on the left.

encoded RS13, a small subunit ribosomal protein of the S15 protein family; this protein was found to have 80% identity with rat and human ribosomal protein RS13, while the identity with the yeast members of the S15 family was 73%. Another apparent full length clone encoded L41, one of the smaller proteins of the large subunit ribosomal protein family L41E. This protein was found to be 84% identical to *Gossypium hirsutum* (upland cotton) L41 and 80% identical to *S. cerevisiae* and human L41. A third ribosomal protein encoding cDNA that we used as a tag encodes S15a (gene designation, *s15A*) and is described elsewhere (Schaap *et al.*, 1995a).

Two other ESTs that we could identify were apparent partial cDNAs of about 800 bp. One of these cDNAs showed high homology with subunit B of the DNA-directed RNA polymerase and was named *rpaB*. The other, *tefA*, probably encodes a translation elongation factor similar to the Eflα of various origins.

Another cDNA clone was identified as a nearly full length cDNA of the gene encoding the δ-subunit of the mitochondrial ATP synthase (gene designation, *atpD*). This protein is

similar to isofunctional proteins of the yeast *S. cerevisiae* and the fungus *Neurospora crassa* (De Groot *et al.*, 1995).

The last EST was identified as encoding a pyrroline-5-carboxylate dehydrogenase and was named *pruA* (for proline utilisation) (Schaap *et al.*, 1997). A phosphoglycerate kinase-encoding gene, named *pgkA*, was previously isolated by heterologous hybridisation (Schaap *et al.*, 1995b).

Unidentified cDNA clones that were used to complete the identification of homologous chromosomes were named EST1 to EST6.

Chromosome assignment of genetic markers and identification of homologous chromosomes

The newly isolated ESTs and some previously isolated genes were used to identify homologous chromosomes of strain Horst®U1. Chromosomes were numbered according to the sizes of the chromosomes of strain H39.

With *htbA*, a strong signal was obtained for the unresolved chromosomes III and IV, whereas a weaker hybridisation signal was visible for chromosome VIII, indicating that another copy of *htbA*, named *htbB*, is located on the latter chromosome (Figure 8.1(c)). Every other EST probe on a gene gave a clear hybridisation signal with only 1 of the 11 chromosome bands for strains H39 and H97 (Table 8.2).

Chromosomes V and VI of strain H39 and chromosomes VI and VII of strain H97 were not separable by CHEF analysis. However, when the distinct hybridisation patterns obtained for both strains were combined, it appeared that the two homokaryons have different-sized copies of chromosomes V, VI and VII, as indicated in Figure 8.1(b). We could, therefore, unambiguously assign EST2 to chromosome V, both copies of the histone H4 encoding genes to chromosome VI, and EST3 to chromosome VII.

A 900-bp probe encoding part of the 28S rDNA (Schaap *et al.*, 1996) hybridised strongly with chromosome IX of strain H39, indicating that the rDNA repeat is located on that chromosome (Figure 8.1(b)). This chromosome also hybridised with two other ESTs encoding ribosomal proteins, *l41* and *s15A*, and with the unidentified marker EST1. All four probes hybridised also to a single chromosome in strain H97, and although this chromosome (2.13 Mb) differs considerably in size from its homologue in strain H39 (1.86 Mb), it was also labelled IX. In a number of fungi it has been shown that size differences between homologous chromosomes harbouring the rDNA cluster are due to differences in copy numbers of the rDNA unit (McCluskey & Mills, 1990; Talbot *et al.*, 1991; Iwaguchi *et al.*, 1992; Morales *et al.*, 1993; Rustchenko *et al.*, 1993). A difference between the

Table 8.2. Chromosome assignment of *A. bisporus* genes

Gene	Gene product or function	Chromosome	Reference
tefA	Translation elongation factor	I	This article
MAT	Mating type	I	This article, Xu *et al.*, 1993
atpD	ATP synthase d-subunit (mitochondrial)	II	De Groot *et al.*, 1995
pgkA	3-Phosphoglycerate kinase	III	Schaap *et al.*, 1995b
rpaB	DNA-directed RNA polymerase I subunit B	IV	This article
pruA	Pyrroline-5-carboxylate dehydrogenase	IV	Schaap *et al.*, 1997
hypA	Hydrophobin A	III/IV	De Groot *et al.*, 1996
htbA	Histone H2B-1	III/IV	This article
EST2	Not identified	V	This article
hhfA	Histone H4-1	VI	This article
hhfB	Histone H4-2	VI	This article
gdhB	NAD^+-glutamate dehydrogenase	VII	Schaap, unpublished data
EST3	Not identified	VII	De Groot *et al.*, 1996
htbB	Histone H2B-2	VIII	This article
rs13	Ribosomal protein RS13	VIII	This article
s15A	Ribosomal protein S15a	IX	Schaap *et al.*, 1995a
l41	Ribosomal protein L41	IX	This article
EST1	Not identified	IX	This article
28S rDNA	Large-subunit rRNA	IX	Schaap *et al.*, 1996
gdhA	$NADP^+$-glutamate dehydrogenase	X	Schaap *et al.*, 1996
cel1	Cellulase	X	Raguz *et al.*, 1992
lcc1	Laccase	XI	Perry *et al.*, 1993
EST5	Not identified	XI	This article
EST4	Not identified	XII	De Groot *et al.*, 1996
gpd	Glyceraldehyde-3-phosphate dehydrogenase	XIII	Harmsen *et al.*, 1992
EST6	Not identified	XIII	This article

hybridisation signal strength of the rDNA probe on chromosomes IX of strains H39 and H97 and to the signal strength of a single copy gene supported the idea of a difference in rDNA copy number. The rDNA probe hybridised to high molecular weight DNA on Southern blots of genomic DNA digested with *Sal*I, indicating that this restriction enzyme does not cut within the rDNA unit. Total chromosome size DNA of both strains was therefore digested with *Sal*I and separated by CHEF electrophoresis, and this revealed a large fragment of approximately 625 kb in strain H97 (Figure 8.3(a)). In this region no band was observed for strain H39. Hybridisation of the gel with the partial 28S rDNA probe showed that the large band observed for strain H97 hybridised strongly (Figure 8.3(b)). In addition, a number of weakly hybridising signals were seen in the lower molecular weight region. For strain H39 multiple hybridising bands varying in length between 185 and 240 kb were obtained. Assuming that the rDNA units reside on chromosome IX in a tandem array, most of the rDNA on chromosome IX of strain H97 is located in one large array. The

hybridisation signals in the lower molecular weight region may indicate the presence of smaller rDNA clusters with a lower copy number. The hybridisation signals in strain H39 also suggest the presence of rDNA clusters of different lengths. Since the size difference between the intact homologous chromosomes IX of the two strains is large compared with those of other homologous pairs, it seems likely that it is largely due to the difference in length between the rDNA clusters in the two strains. Assuming that the length of the *A. bisporus* rDNA unit is 9.2 kb (Hintz *et al.*, 1989), the number of rDNA repeats on chromosome IX of strain H97 is approximately 68. The number of rDNA units on chromosome IX of strain H39 varies between 20 and 26.

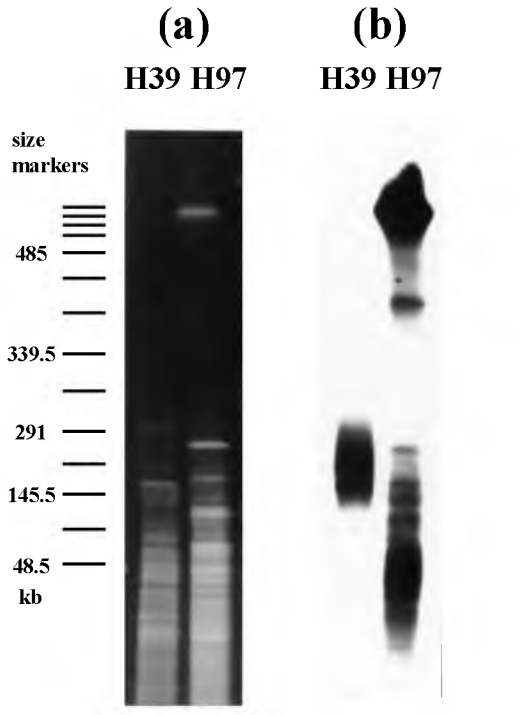

Figure 8.3. Analysis of the rDNA repeat on chromosome IX. (a) Chromosomes of strains H39 and H97 digested with *Sal*I and separated CHEF by electrophoresis. (b) Hybridisation of the gel with the rDNA probe. Molecular size markers consist of concatemers of λ DNA of 48.5 kb (BioRad).

Other currently available *A. bisporus* genes, not previously assigned to a chromosome, were located by CHEF analysis. The NAD^+-glutamate dehydrogenase encoding gene (Kersten *et al.*, 1997) was located on chromosome VII. The *cel1* gene, encoding an *A. bisporus* cellulase (Raguz *et al.*, 1992) and the *lcc1* gene, encoding an *A. bisporus* laccase (Perry *et al.*, 1993), were located on chromosomes X and XI, respectively (Figure 8.1(e) and Table 8.2).

On the basis of the intensity of staining with ethidium bromide, the third largest band in CHEF gels of both strains was suspected to represent two chromosomes, i.e., chromosomes III and IV. To confirm the presence of two chromosomes, the segregation of four markers, which were previously assigned to these chromosomes and for which polymorphism was found between strains H39 and H97, was studied. Two markers (*pruA* and *rpaB*) showed no recombination and thus are located on the same chromosome. The recombination fraction (recombinant genotypes/parental genotypes) between the third marker p33n5 and these two markers was the same, i.e., 0.41. After correction to account for the observed segregation distortion, a chi-square value of 5.04 indicated no significant deviation from the expected 1:1:1:1 ratio of genotypes. This indicates that p33n5 is located on a different chromosome.

Additionally, we have paired the same set of homokaryotic offspring with each parental strain in order to determine the mating type of each progeny. All matings gave a positive interaction on compost agar with only one of the parental strains, i.e., fluffy growth was observed along the junction zone. The presence of both parental alleles of EST *tefA* confirmed the heterokaryotic nature of the mycelia obtained after each pairing of two homokaryons. Since Xu *et al.* (1993) had reported that the mating type gene was located on chromosome I of strain AG93b, we used EST *tefA*, which was assigned to chromosome I (Table 8.2), to see if this marker and the mating type were linked. The mating type allele segregated from the parental allele of *tefA* in only 5 of the 86 homokaryotic offspring, indicating that in H39 and H97 the mating type is indeed located on chromosome I.

To confirm the presented identification of homologous chromosomes of strains H39 and H97, and to enable comparison between our data and the previously published map (Kerrigan *et al.*, 1993), 12 anonymous DNA markers used by Kerrigan were assigned to chromosomes of both parental lines by CHEF analysis. The assignment of these RFLP markers agrees with the data presented by Kerrigan *et al.* (1993) except for p1n37 and p1n55, which were placed on chromosome IX by Kerrigan *et al.* (1993) and on chromosomes VII and VIII, respectively, in the present study (Table 8.3).

Table 8.3. Chromosome assignment of RFLP markers

RFLP marker	Chromosome no. in :	
	This article	Kerrigan *et al.* (1993)
p1n17	I	I
p1n31	I	I
p1n148	I	I
p1n150	I	I
p4n6	II	II
p33n5	III	III
p33n25	V	V
p1n125	VI	VI
p1n37	VII	IX
p1n55	VIII	IX
p1n36	X	X
p4n14	XII	XII

Discussion

In this study we have optimised the separation of the chromosomes of *A. bisporus* strains H39 and H97, the constituent homokaryons of strain Horst®U1. In addition, we have shown the usefulness of a cDNA library as a source for well-defined ESTs for the development of a gene-based genetic linkage map. Of the 12 ESTs presented here, we were able to identify 6 by sequence analysis. The functions of the six remaining genes are still unclear. However, EST1 appears to encode repetitive amino acid sequences with considerable similarity to those of the SPT5 general transcription factor of *S. cerevisiae* (Swanson *et al.*, 1991). EST4 is the same as pDG47 (De Groot *et al.*, 1996) and is specifically expressed in fruit bodies. EST3 is the same as pDG71 and appears to be constitutively expressed at high levels in all stages of the life cycle (De Groot *et al.*, 1996).

The optimisation of the electrophoretic separation of chromosomes has led to a highly reproducible resolution of the chromosomes of both parental strains into 11 bands. Ethidium bromide staining of these gels suggests that each karyotype has two double bands. Chromosomes III and IV probably have similar lengths in both strains. The independent segregation of markers assigned to these more intensely stained bands confirmed that they represent two chromosomes. In addition, chromosomes V and VI of strain H39 and chromosomes VI and VII of strain H97 could not be resolved. Since homologous chromosomes V, VI, and VII have different lengths, markers could unambiguously be assigned to these chromosomes. From the results we conclude that the two karyotypes have

13 chromosomes; this agrees with earlier reports (Sonnenberg *et al.*, 1991; Royer *et al.*, 1992; Kerrigan *et al.*, 1993). The estimated genome size for each strain is 31 Mb. This correlates well with the estimated genome size of 34.2 Mb for *A. bisporus*, calculated on the basis of re-association kinetics (Arthur *et al.*, 1982).

To confirm the identification of homologous chromosomes of strains H39 and H97, at least two markers were assigned to each chromosome. All markers assigned to the same chromosome in one parent were also assigned to a single chromosome in the other parent. This indicates that we have identified all homologous chromosomes in both lines and that these lines share a common genetic structure at least for the markers used. The karyotypes show a remarkable chromosome polymorphism. Chromosome length polymorphisms have been observed in many fungi (Zolan, 1995) and can result from mitotic and/or meiotic processes. The difference in length of homologous chromosomes IX is largely due to differences in copy number of the rDNA unit. Hybridisation of a rDNA probe to *Sal*I digested chromosomes revealed that the probe does not hybridise to one single fragment. This means either that more than one rDNA cluster is present on chromosome IX or that within the culture used to isolate intact chromosomes, variation exists in the length of rDNA units. Since the banding pattern seems to vary in different cultures (data not shown), the latter explanation seems to be more likely.

The assignment of RFLP markers to chromosomes by hybridisation to CHEF blots agrees with the previously published data (Kerrigan *et al.*, 1993), with two exceptions. In the first linkage map of *A. bisporus* (Kerrigan *et al.*, 1993), anonymous RFLP markers p1n37 and p1n55 were both placed on chromosome IX, whereas we have placed p1n37 on chromosome VII and p1n55 on chromosome VIII (Table 8.3). One homokaryon of the parental heterokaryon used by Kerrigan *et al.* (1993) was derived from a wild isolate, and the other was from the American Type Culture Collection. The strains used in both studies might, therefore, be unrelated, and this could explain the discrepancy. However, it is possible that the assignment of both markers to the same chromosome in the previous study is due to the limited number of offspring used (52 homokaryons). Only one of the markers was assigned to a chromosome by hybridisation to a CHEF blot; positioning of the other marker at the end of the same chromosome was based on linkage analysis (Kerrigan *et al.*, 1993). Since chromosomes VII and VIII are well separated on CHEF gels, an incorrect interpretation of hybridisation results is unlikely.

The locations of the rDNA repeat and the genes *cel1* and *lcc1* differ from those reported by Lodder *et al.* (1993). In their study, chromosomes were isolated from protoplasts derived from gill tissue of fruit bodies. CHEF gels were therefore prepared from heterokaryotic cells

containing 26 chromosomes which may have led to erroneous assignment of genes to individual chromosomes. The *lcc1* gene, for example, was assigned to the smallest chromosome, whereas in our study *lcc1* was located on chromosome XI (Figure 8.1(e)). Applying the CHEF technique as described here to protoplasts isolated from a mycelial culture of the heterokaryotic strain Horst®U1 shows that *lcc1* does not hybridise to the smallest chromosome (data not shown).

Here we have used the *MAT* locus and 25 genes of which 17 were identified, to mark all individual chromosomes of the parental lines of strain Horst®U1. These results will serve as a firm basis for the development of a linkage map and will enable mapping of important traits. At the same time, the characterisation of these genes will increase our knowledge of the molecular biology of *A. bisporus*.

Acknowledgements

We appreciate the skilful technical assistance of Y. Müller, K. Den Hollander, and A. P. J. Van de Munckhof. We thank C. H. Thurston (King's College, London, United Kingdom) for supplying the *cel1* and *lcc1* probes and P. A. Horgen (University of Toronto, Toronto, Canada) for the anonymous RFLP markers.

This study was financed by Bromyc BV, a subsidiary of the Cooperative Netherlands Mushroom Growers Association (CNC).

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Summary
and
concluding remarks

Summary and concluding remarks

During the last decades, cultivation of edible fungi for the production of mushrooms has developed into a large industry. It is one of the few examples of large scale bioconversion of lignocellulosic waste into a high cost product. Optimal growth of the most popular mushroom *Agaricus bisporus* is obtained on a specifically prepared fermented substrate. During the fermentation process a diverse microflora develops in the compost and managing the succession of these micro-organisms seems to be essential in making the compost selective for growth of the vegetatively slow growing mushroom fungus. However, since *A. bisporus* is very reluctant to fruit under axenic conditions, the microbiota in the compost and the casing layer appear, for currently unknown reasons, also to be relevant for optimal initiation of fruit body formation. Therefore, mushrooms are cultivated under non-sterile conditions which unfortunately introduces a high risk of crop losses caused by bacterial and fungal infections. Nowadays this has become a severe threat to the expanding industry which has only a few, genetically very similar strains available. A classical approach to breeding is hampered by the secondarily homothallic life-cycle of *A. bisporus*. To enable an efficient breeding programme of *A. bisporus*, the Netherlands mushroom industry set up a special programme of which one of the goals was the identification of a large number of genes. Those would provide tools for the construction of a gene-based linkage map of strain Horst®U1 and therefore accellerate the output of breeding programs intended to yield new commercially better mushroom strains.

In order to study the genetic processes that are involved in fruiting of *A. bisporus*, we focused on the cloning and characterisation of genes that are specifically expressed during fruit body development. Additionally, to study genetic aspects of compost utilisation by *A. bisporus*, we envisaged cloning of genes involved in the release of sugars from the plant polysaccharides present in compost. Furthermore, experiments were focused on the cloning of housekeeping genes which would enable us to compare the structure of *A.* bisporus genes with homologous genes of related organisms and would give us the opportunity to monitor, on the RNA level, the activity of basic biochemical routes in different parts of the mushrooms.

In Chapter 1, aspects of commercial mushroom cultivation that directly influence vegetative growth of the *A. bisporus* mycelium and its utilisation of the compost substrate are discussed and an overview is given of presently cloned *A. bisporus* genes whose products are involved in compost utilisation. This chapter further describes mushroom development in morphogenetic, biochemical and molecular terms and explains, with data available from other filamentous fungi, how fungal sporulation might be controlled on the

molecular level. At the same time this explains why the isolation of fruit body specific genes would provide valuable research tools to study fruiting of *A.* bisporus on the molecular level.

The construction of a reliable linkage map of mushroom strain Horst®U1 required the isolation of a large number of well-defined gene markers. In order to clone genes whose products are involved in various aspects of the mushroom metabolism, we applied diverse cloning techniques. The cloning strategies, and preliminary results therefrom, that are used for the cloning of fruit body specific genes, compost utilisation genes and housekeeping genes of carbon and nitrogen metabolism are discussed.

Isolation of fruit body specific genes from *A. bisporus* was performed using differential screening techniques. For this, a cDNA expression library was constructed with RNA isolated from young developing fruit body primordia which appear 5 - 6 days after fruit body initiation using commercial cultivation conditions. This library was expected to be enriched in cDNAs derived from genes involved in the development of the primordium into mature fruit bodies. The first visible sign of fruit body formation, however, is the appearance of mycelial aggregates onto which primordia develop. Construction of a cDNA library from such aggregates was therefore also considered, but unfortunately isolation of sufficient RNA under axenic conditions is hampered by technical difficulties. Screening the primordial cDNA library for genes that are highly expressed and fruit body specific resulted in the isolation of two genes, *hypA* and *hypB*, encoding small cell wall proteins belonging to the fungal family of hydrophobins. Typical features of hydrophobins are the presence of eight cysteine residues in a characteristic pattern and a hydrophobic signal sequence for secretion. Overall, homology between hydrophobins is rather low but the proteins have typical hydrophobicity patterns and based on these patterns they are ordered into two classes. The translated peptide sequences of both *hypA* and *hypB* have characteristics that typify hydrophobins and based on their hydrophobicity patterns they can be classified as class I hydrophobins (Chapters 2 and 4).

A common property of hydrophobins is their tendency to self-assemble at the interface between hydrophobic and hydrophilic environments. Hydrophobin aggregates are insoluble in hot SDS solutions and can be solubilised into monomers with trifluoroacetic acid. These properties have been used to purify hydrophobins from a number of fungi. An enormous accumulation of *hypA* messengers to more than 60% of the total mRNA pool, was found in the outer tissue of mushroom caps. From this peel/pileipellis tissue, using the properties of hydrophobin aggregates, a protein was purified that proved, by peptide sequencing, to be the product of the *hypA* gene. Accumulation and self-assembly of HYPA hydrophobin

monomers therefore seems to be responsible for the formation of a protective hydrophobic layer surrounding the mushroom caps (Chapter 2).

In order to generate additional fruit body specific genes, a second screening was performed, in which strong induction in fruit bodies was the only selection criterion (Chapter 3). From this screening nine unrelated genes were isolated and four of these are apparently only expressed in fruit bodies. These nine genes and the two hydrophobins were all assigned to chromosomes using CHEF-analyses showing that they are scattered over the *A. bisporus* genome. Thus, so far there is no evidence for clustering of developmentally regulated genes in *A. bisporus*. Northern analyses showed that if changes in expression levels of these genes occurred, they correlated well with morphological changes of the fruit bodies observed during fruit body development. Three of the fruit body induced genes, that show low but detectable mRNA levels in vegetative mycelium, were identified by database analysis. One of the genes that was identified, *sepA*, appeared to encode a septin. Septins are proteins involved in the formation of septa during cell division. Highest expression in mature mushrooms for this gene was found in the transitional zone between the cap and the stipe. In agreement with this the upper region of the stipe, as observed by simple light microscopy, consists of short, rapidly dividing cells between which septa are formed.

A second gene, *atpD*, encodes the delta subunit of the F_0-F_1 ATP-synthase complex and as in the *N. crassa* and *S. cerevisiae* homologues, a mitochondrial import signal is proposed. A third gene has significant homology with members of different families of the cytochrome P450 superfamily and was classified as the first member of a new cytochrome P450 family. At present, however, it is unclear what the specific roles of these proteins are, and if they are essential for normal fruit body development to occur. Furthermore, a number of the cloned fruit body specific genes remains unidentified. Analogous genes may be present only in closely related fungi that produce sporulating structures similar to *A. bisporus* fruit bodies.

Chapter 4 reports the identification and characterisation of the *hypB* hydrophobin. Comparison of expression patterns obtained for the two hydrophobin genes, *hypA* and *hypB* showed that the genes are differently regulated. While *hypA* messengers accumulate in the less differentiated hyphae of the partial veil and the pileipellis surrounding the mushroom cap, *hypB* messengers accumulate in the transitional zone in between cap and stipe tissue. Differentiation of primordial hyphae into distinct cap and stipe tissues occurs at stage two of development and during this stage highest *hypB* mRNA levels were found. Together with the observation that bacterial infections in the stipe often do not enter the fruit body caps, this suggests that HYPB could be part of a protective layer in between cap and stipe. Using

in situ hybridisation techniques, highest *hypA* expression was not found in the outer cell layer of the mushroom caps but in the zone connecting the pileipellis and the plectenchyma which indicated that excreted HYPA monomers do not self-assemble before they have reached a hydrophobic/hydrophilic interface.

The ability of the HYPA hydrophobin to form aggregates onto a hydrophobic surface was investigated further using Atomic Force Microscopy (Chapter 5). The use of AFM has allowed the monolayer to be imaged at molecular resolution for the first time. The percentage of HOPG (highly oriented pyrolytic graphite) surface covered with HYPA was dependent on the concentration of the HYPA solutions in the adsorption experiment. Exposure to 2 µg/ml HYPA solutions resulted in a monolayer of highly ordered HYPA molecules and a high (≈89%) surface coverage of the graphite. The observed structures resemble the rodlet structures observed by electron microscopic analysis on the pileipellis of fruit body caps and are also similar to the rodlet structures observed with the well-studied Sc3p protein of *S. commune* attached onto teflon. Exposure of a higher (20 µg/ml) HYPA concentration to the HOPG surface resulted in AFM images that showed less ordered structures and a lower (≈74%) surface coverage. In this experiment occasionally HYPA bilayers were observed and unexpectedly similar bilayers were observed after washing HOPG surface, which was exposed to a high concentration HYPA solution, with subsequently cold and hot SDS solutions. The HYPA layer was only partly removed upon washing with these SDS solutions whereas it was largely (but not completely) removed when washing was performed with TFA. These results show that HYPA behaves very similar to Sc3p upon exposure to a hydrophobic surface and supports the hypothesis that the outer layer of mushroom caps consists mainly of self-assembled HYPA aggregates.

In Chapter 6, we describe the cloning and characterisation of three *A. bisporus* housekeeping genes. The genes coding for 3-phosphoglycerate kinase (*pgkA*) and pyruvate kinase (*pkiA*) were cloned using heterologous hybridisation techniques, and the *aldA* gene coding for aldehyde dehydrogenase resulted from random sequencing of cDNA clones. The three genes are spiked with numerous small intervening sequences whose positions do not coincide with conserved intron positions in ascomycetes. Phylogenetic analysis based on amino acid sequence comparisons also show that *A. bisporus* has diverged early from the group of ascomycete and deuteromycete fungi. Analysis of the codon usage of *A. bisporus* based upon the sequences of the genes isolated thus far, indicates that the fungus has a preference for C or T in the third base position of the codons. The cloned housekeeping genes were also used as probes to monitor different metabolic routes in fruit bodies by

studying their expression levels in various parts of mature mushrooms. These expression studies showed that mature mushrooms have defined regions with different metabolic activity. For the *aldA* gene, expression levels in specific parts of the fruit bodies are strongly elevated compared to vegetatively grown mycelium and the expression pattern mimics that found for the *hypA* gene encoding a fruit body specific hydrophobin.

Cloning and characterisation of the *xlnA* gene encoding an endo-1,4-ß-xylanase gave us a better insight in the regulation of enzymes that can degrade lignocellulosic compounds of compost (Chapter 7). The translated peptide sequence of *xlnA* encodes a protein of 35 kDa belonging to glycosyl hydrolase family 10 which comprises xylanases as well as cellulases. Surprisingly, Northern analysis showed that the *xlnA* gene is much stronger expressed on compost than on its actual substrates xylan and cellulose. Expression patterns of *xlnA* were very similar to those obtained with *cel3* gene encoding a cellobiohydrolase. This indicated that these genes, and probably also other genes encoding enzymes which are necessary to release sugars from the lignocellulosic complex, are co-regulated. The regulation of their transcription seems to be an elegant example of adaptation of a fungus to its natural niche.

The application of several of the isolated *A. bisporus* genes as well-defined markers for linkage mapping purposes is described in Chapter 8. Genes could serve this function when at least an RFLP or sequence mutation was discovered between the homokaryotic constituents of Horst®U1. In order to rapidly increase the number of available marker genes sequencing of random cDNAs was applied which led to the identification of a large number of genes. Among the fortuitously identified genes are *rs13A*, *l41A* and *s15A* all encoding ribosomal proteins, *htbA* and *hhfA* encoding core histone proteins, the *tefA* gene encoding translation elongation factor 1α and *pruA* encoding pyrroline-5-carboxylate (P5C) dehydrogenase. To each linkage group at least two markers were assigned using a set of 86 homokaryotic offspring. In contrast to anonymous DNA markers, allocation results obtained with gene markers were always in agreement with results from CHEF-analyses. The use of marker genes combined with chromosome separation techniques therefore forms a solid base for the development of gene-based linkage map and will make *A. bisporus* more amenable for efficient breeding systems. This improves the prospects of the development of agronomically improved mushroom strains in the near future.

In this thesis, we show that a number of *A. bisporus* genes are only expressed at high levels during fruit body development. Two highly expressed genes encode hydrophobins of which HYPA self-assembles into a protective hydrophobic layer surrounding the mushroom caps, whereas *hypB* mRNA accumulates in the transitional zone between the cap and the stipe. Expression studies of genes involved in compost utilisation showed adaptation of the

fungus towards the use of compost as its natural growth substrate. Furthermore, cloning of housekeeping genes proved to be valuable in studying the architecture of *A. bisporus* genes.

With the isolation of a large number of gene markers for the establishment of a linkage map and the availability of a large collection of wild isolates, systematic breeding of strains with a better agronomic performance and an increase of the genetic diversity among the cultivated mushroom strains may be expected in the near future. Nevertheless, further research is required to elucidate the biology underlying compost utilisation and fruit body development.

Samenvatting
en
conclusies

Samenvatting en conclusies

Door de introductie van nieuwe technisch hoogwaardige teeltmethoden is de productie aan champignons de laatste jaren zeer sterk toegenomen. De champignonteelt is een van de weinige succesvolle voorbeelden waarbij op grote schaal lignocellulose-afval wordt omgezet in een waardevol eindprodukt. Voor de teelt van champignons wordt gebruik gemaakt van een specifiek geprepareerd gefermenteerd substraat. Tijdens deze fermentatie ontwikkelt zich een variatie aan micro-organismen in de compost. Controle over de ontwikkeling van deze micro-organismen lijkt van essentieel belang te zijn voor het verkrijgen van een substraat dat selectief is voor het zich vegetatief langzaam vermeerderende mycelium van *A. bisporus*. De micro-organismen die aanwezig zijn in de compost en de deklaag op de compost zijn tevens belangrijk voor de vruchtlichaamvorming gezien het feit dat dit differentiatieproces bij *A. bisporus* zeer moeizaam verloopt in een steriele omgeving. Commerciële teelt van champignons vindt daarom plaats onder niet-steriele omstandigheden wat wel als nadeel een verhoogd risico op bacteriële- en schimmelinfecties met zich meebrengt. Omdat er slechts enkele, genetisch vrijwel identieke, commerciële *A. bisporus* rassen op de markt zijn, kunnen infectieziekten aanzienlijke schade veroorzaken wat een serieuze bedreiging vormt voor de champignonindustrie. Mede daarom is er dringend behoefte aan nieuwe, genetisch verschillende rassen. Klassieke veredeling van champignons wordt echter bemoeilijkt door de secondair homothallische levenscyclus van *A. bisporus* en het ontbreken van een betrouwbare genetische kaart van het commerciële ras Horst®U1, dat wereldwijd het meest wordt gebruikt. Om het veredelingsproces van champignons door toepassing van moleculaire technieken efficiënter te laten verlopen hebben de Nederlandse champignonkwekers een fonds gevormd om onderzoek te doen verrichten. Het voorliggende proefschrift vormt een weergave van onderzoek naar de identificatie, karakterisering en lokalisatie van een groot aantal gen markers.

Om op genetisch niveau de vruchtlichaamvorming van *A. bisporus* te kunnen bestuderen was een van de hoofddoelen van dit onderzoek de isolatie van genen die betrokken zijn bij de aanleg en verdere ontwikkeling van het vruchtlichaam. Andere kloneringsexperimenten waren gericht op de isolatie van genen coderend voor enzymen die betrokken zijn bij het vrijmaken van suikers uit de van stro afkomstige polysacchariden die nog aanwezig zijn in de compost. Daarnaast is getracht om huishoudgenen coderend voor enzymen die betrokken zijn bij het koolstof- en stikstofmetabolisme te kloneren. Omdat deze genen doorgaans goed geconserveerd zijn, kan de structuur van deze *A. bisporus* genen vergeleken worden met de

gelijksoortige genen uit verwante schimmels. Tevens kan op gen-expressieniveau de aan- of afwezigheid bepaalde metabole routes tijdens de vruchtlichaamvorming worden bestudeerd.

In het inleidende hoofdstuk worden aspecten van de champignonteelt besproken die direct van invloed zijn op kolonisatie door het *A. bisporus* mycelium van het compost substraat en er wordt een overzicht gegeven van de tot nu toe geïdentificeerde *A. bisporus* genen coderend voor enzymen die betrokken zijn bij compost afbraak. Morfogenetische, biochemische en moleculaire aspecten van de vruchtlichaamontwikkeling worden beschreven, de laatstgenoemde noodzakelijkerwijs aan de hand van gegevens die beschikbaar zijn over verwante filamenteuze schimmels.

De constructie van een betrouwbare genetische koppelingskaart voor het ras Horst®U1 vereiste de isolatie van een groot aantal goed gedefinieerde gen markers. De verschillende kloneringsstrategieën die zijn gebruikt voor de isolatie van vruchtlichaamspecifieke genen, genen coderend voor 'compost afbrekende' enzymen en huishoudgenen van het koolstof- en stikstofmetabolisme worden besproken.

Voor de isolatie van vruchtlichaamspecifieke genen is gebruik gemaakt van een differentiële screeningstechniek. Hiertoe werd uit zeer jonge vruchtlichamen (primordia), die onder commerciële teeltomstandigheden 5 - 6 dagen na de initiatie van de vruchtlichaamvorming zichtbaar zijn, RNA geïsoleerd en een cDNA expressiebank geconstrueerd. Verondersteld werd dat deze cDNA bank een relatief hoog gehalte aan cDNAs bevat die gerelateerd zijn aan genen die betrokken zijn bij de ontwikkeling van primordium naar volwassen champignon. Omdat de werkelijke initiatie van de vruchtlichaamvorming al in een eerder stadium plaatsvindt is ook gekeken of het mogelijk was om van geaggregeerde hyfedraden, wat algemeen wordt beschouwd als het eerste stadium van de vruchtlichaamontwikkeling, een cDNA bank te construeren. Echter, onder de door ons gewenste steriele omstandigheden worden maar zeer weinig van deze aggregaten gevormd en het bleek technisch niet haalbaar om hieruit voldoende RNA te isoleren.

In eerste instantie zijn uit de primordium cDNA bank genen geïsoleerd die specifiek tijdens de vruchtlichaamontwikkeling op zeer hoog niveau tot expressie komen. Dit resulteerde in de isolatie van twee genen, *hypA* en *hypB*, beide coderend voor een hydrofobine eiwit. Hydrofobines zijn kleine celwandeiwitten die tot nu toe alleen in filamenteuze schimmels zijn gevonden. Kenmerkend voor hydrofobines is de aanwezigheid van acht cysteine residuen in een kenmerkend patroon en een hydrofobe N-terminale sequentie coderend voor een secretiesignaal. Ondanks het feit dat de aminozuurvolgorden tussen de verschillende hydrofobines in het algemeen niet sterk geconserveerd zijn, komen

de hydrofobiciteitspatronen van deze eiwitten vaak zeer sterk overeen. De hydrofobiciteitspatronen van HYPA en HYPB lijken sterk op die van de veel gevonden type I hydrofobines (Hoofdstukken 2 en 4).

Ook kenmerkend voor hydrofobines is dat ze, via een proces van zelf-assemblage aggregeren op de grensvlakken van hydrofiele en hydrofobe oppervlakken. De aggregaten van type I hydrofobines zijn doorgaans bestand tegen kokende 2% SDS oplossingen maar door een behandeling met trifluor azijnzuur kunnen deze hydrofobine complexen uiteenvallen in monomeren. Deze typische hydrofobine eigenschap is met succes gebruikt om hydrofobines uit diverse schimmels te isoleren. Omdat Northern analyse van het *hypA* gen aantoonde dat zeer hoge *hypA* messenger concentraties aanwezig zijn in de buitenste laag (vel) van de hoed van de champignon, is dit weefsel gebruikt voor isolatie van hydrofobines. Na TFA extractie van dit weefsel werd een eiwit geïsoleerd van ±9 kDa dat na bepaling van de N-terminale aminozuurvolgorde geïdentificeerd werd als het HYPA eiwit. De aanwezigheid en zelf-assemblage van het HYPA eiwit in het geschubde vel van de champignonnenhoed lijkt daarom in belangrijke mate bij te dragen aan het hydrofobe waterafstotende karakter van champignons en biedt de champignon daarmee waarschijnlijk ook bescherming tegen sommige infecties van buitenaf (Hoofstuk 2).

Additionele vruchtlichaamspecifieke genen zijn geïsoleerd door iets andere selectiecriteria te gebruiken. Door alleen te selecteren voor genen die sterk verhoogde expressie vertonen gedurende de vruchtlichaamontwikkeling werden negen nieuwe genen geïsoleerd. Met Northern analyse werd aangetoond dat vier van deze genen vrijwel alleen tot expressie komen in champignons, en dus waarschijnlijk alleen betrokken zijn bij de ontwikkeling van de vruchtlichamen. De andere genen kwamen zeer laag maar niettemin aantoonbaar tot expressie in vegetatief mycelium. Door chromosoomscheidingstechnieken (CHEF) te combineren met Southern analyses is aangetoond dat de gekloneerde vruchtlichaamspecifieke genen op verschillende chromosomen gelokaliseerd zijn en dus zijn er vooralsnog geen aanwijzingen dat dergelijke genen in *Agaricus* geclusterd voorkomen, dit in tegenstelling tot bijvoorbeeld de ascomyceet *A. nidulans*. Analyse van de relatieve expressieniveaus van de geïsoleerde vruchtlichaamspecifieke genen tijdens de vruchtlichaamvorming toonde aan, dat wanneer er veranderingen in expressieniveaus plaatsvinden, deze overeen lijken te komen met de morfologische veranderingen tijdens de ontwikkeling van de champignons. Door middel van sequentie- en databank analyse konden slechts drie genen worden geïdentificeerd. Eén gen, later *sepA* genoemd, codeert voor een eiwit dat homologie vertoont met eiwitten die betrokken zijn bij de vorming van septa tussen delende cellen, zogenaamde septines. Accumulatie van *sepA* mRNA werd

waargenomen in het bovenste gedeelte van de steel van de champignon. In overeenstemming hiermee was door microscopische studies aangetoond dat dit gedeelte van de champignon bestaat uit snel delende, korte cellen. Een tweede gen (*atpD*) dat kon worden geïdentificeerd codeert voor de delta subunit van het F_0-F_1 ATP synthase complex en evenals voor de homologe eiwitten van *Neurospora crassa* en *Saccharomyces cerevisiae* kon aan de hand van de N-terminale aminozuursamenstelling worden voorspeld dat het eiwit is gelokaliseerd in het mitochondrion. De aminozuursequentie van een derde gen dat werd geïdentificeerd (*cypA*) heeft significante homologie met cytochroom P450 eiwitten uit verschillende P450 subfamilies, daarom is CYPA voorlopig geclassificeerd als het eerste geïdentificeerde eiwit van een nieuwe cytochroom P450 subfamilie. Vooralsnog blijft het onduidelijk wat de specifieke rol van deze eiwitten is en of ze van essentieel belang zijn voor een normale vruchtlichaamontwikkeling. Alledrie deze genen komen in ieder geval aantoonbaar tot expressie in de vegetatieve fase. De andere geïsoleerde vruchtlichaamspecifieke genen konden niet door middel van database analyse worden geïdentificeerd. Mogelijk is de verspreiding van dergelijke genen beperkt tot schimmels met een vergelijkbaar complex vruchtlichaam en die komen in de databases (nog) nauwelijks voor.

In Hoofdstuk 4 wordt de identificatie en karakterisering van het *hypB* hydrofobine gen besproken. Vergelijking van de expressieniveaus van *hypA* en *hypB* gedurende de ontwikkeling van het vruchtlichaam en in de verschillende weefseltypes van volwassen champignons toonden aan dat de beide genen verschillend gereguleerd worden. Waar *hypA* messengers in hoge concentraties accumuleren in velum- en velweefsel, komt het *hypB* gen in volwassen champignons voornamelijk tot expressie in de zone waar hoed en steel weefsel in elkaar over gaan. Al in een vroeg stadium van de vruchtlichaamontwikkeling wordt het hoogste mRNA niveau van *hypB* bereikt en dit lijkt overeen te komen met het tijdstip waarop het jonge primordium differentieert in dit zeer verschillende hoed- en steelweefsel. Door middel van Northern analyse waarbij gebruik werd gemaakt van *in situ* hybridisatie technieken is aangetoond dat de hoogste *hypA* mRNA concentratie niet in de buitenste cellaag aanwezig is, maar in een meer naar binnen gerichte cellaag. Het HYPA eiwit dat verantwoordelijk is voor het hydrofobe karakter van het champignonoppervlak wordt dus waarschijnlijk na uitscheiding door deze cellaag eerst naar buiten getransporteerd alvorens te assembleren tot een groot eiwitcomplex.

De kenmerkende eigenschap van hydrofobines om, via een proces van zelf-assemblage, zich te hechten aan hydrofobe oppervlakten is met het gezuiverde HYPA eiwit bestudeerd met behulp van Atomic Force Microscopy (Hoofdstuk 5). Met deze gevoelige techniek is

voor het eerst moleculaire resolutie verkregen van de eiwitten in een hydrofobinelaag. Het percentage van het HOPG (highly oriented pyrolytic graphite) oppervlak dat gecoat werd met HYPA bleek afhankelijk te zijn van de concentratie van de HYPA oplossing waarmee het adsorptie experiment werd uitgevoerd. Incubatie met een 2 µg/ml HYPA oplossing resulteerde in adsorptie van een geordende eiwitlaag en een hoge (89%) bedekkingsgraad van het HOPG oppervlak. De HYPA structuren komen sterk overeen met de rodlet structuren die met electronenmicroscopische technieken in velweefsel van champignons zijn waargenomen en lijken ook sterk op de rodlet structuren van het uitvoerig bestudeerde Sc3p hydrofobine van *Schizophyllum commune*. Wanneer HOPG werd blootgesteld aan hogere concentraties (20 µg/ml) HYPA, werd een lagere bedekkingsgraad (74%) verkregen en bovendien was de geadsorbeerde eiwitlaag minder geordend. Bij deze hogere concentraties werden soms geadsorbeerde HYPA moleculen bovenop de eerste laag waargenomen en dit was ook het geval na het wassen van het HOPG oppervlak met 2% SDS oplossingen. Door het wassen met SDS werd maar een gedeelte van het geadsorbeerde HYPA losgeweekt terwijl na een TFA wasstap de HYPA hydrofobine laag voor het grootste gedeelte was verwijderd. De met de AFM techniek verkregen resultaten tonen aan dat HYPA, net als Sc3p, bij blootstelling aan een hydrofoob oppervlak, de neiging heeft te aggregeren en zich te hechten aan dit oppervlak. Dit versterkt het idee dat de hydrofobe laag waarmee champignons zijn omgeven bestaat uit geassembleerde HYPA moleculen.

In Hoofdstuk 6 wordt de isolatie en structuur van drie huishoudgenen van *A. bisporus* beschreven. De genen coderend voor 3-fosfoglyceraat kinase (*pgkA*) en pyruvaat kinase (*pkiA*) werden gekloneerd met behulp van heterologe hybridisatietechnieken terwijl het aldehyde dehydrogenase (*aldA*) gen werd geïdentificeerd door het sequencen van een aantal random gekozen cDNA kloons. De coderende sequenties van *A. bisporus* genen worden veelvuldig onderbroken door kleine intronen. De posities van de intronen in deze drie huishoudgenen komen niet overeen met tussen ascomyceten geconserveerde intronposities. Ook uit fylogenetische analyses gebaseerd op de mate van conservering van aminozuurvolgorden blijkt dat *A. bisporus* al vroeg in de evolutie is afgesplitst van deze groep schimmels. Analyse van het codongebruik van de tot nu toe gekloneerde *A. bisporus* genen geeft aan dat *A. bisporus* bij voorkeur codons gebruikt met een C of T op de derde base-positie. Om de aanwezigheid van bepaalde metabole routes in champignons te bestuderen zijn de drie beschreven huishoudgenen ook gebruikt als probes voor Northern analyses. In de verschillende weefsels van een volwassen champignon werden duidelijke verschillen in expressieniveaus van deze genen waargenomen. Het mRNA niveau van zowel *pgkA* als *aldA* in champignons is sterk verhoogd ten opzichte van vegetatief mycelium, en

het expressiepatroon van *aldA* in de verschillende onderdelen van de champignon lijkt zeer sterk op dat van het *hypA* hydrofobine gen.

Klonering en karakterisering van het *xlnA* gen dat codeert voor een endo-1,4-β-xylanase is beschreven in Hoofdstuk 7. Het *xlnA* gen codeert voor een eiwit van 35 kDa dat op basis van sequentie homologie kon worden geclassificeerd als een zogenaamde familie 10 glycosyl hydrolase. Tot deze familie van glycosyl hydrolases behoren zowel cellulose als xylan afbrekende enzymen. Northern analyse toonde aan dat *xlnA* een veel hoger expressieniveau bereikt bij groei op compost dan wanneer de compost componenten xylan en cellulose als substraat voor *A. bisporus* worden gebruikt. Voor het *A. bisporus cel3* gen dat codeert voor een cellobiohydrolase werden zeer overeenkomstige expressiepatronen gevonden. Dit geeft aan dat transcriptie van *xlnA* en *cel3* en mogelijk andere genen die betrokken zijn bij compost degradatie op een zelfde wijze wordt gereguleerd. Deze vorm van transcriptieregulatie van *A. bisporus* lijkt een mooi voorbeeld van adaptatie van een schimmel aan zijn natuurlijke groeisubstraat.

Het gebruik van gekloneerde genen als goed gedefinieerde markers voor karteringsdoeleinden is beschreven in Hoofdstuk 8. Genen waarvoor een RFLP of een sequentie mutatie is gevonden tussen de beide homokaryotische ouders van de gekweekte champignon Horst®U1, kunnen worden gebruikt voor koppelingsanalyses. Om snel een aantal gekloneerde genen te genereren werd de DNA volgorde van een aantal random gekozen cDNAs uit de primordium cDNA bank bepaald. Door database analyse kon een aantal van deze genen worden geïdentificeerd waarvan *rs13A*, *l41A* en *s15A* coderen voor ribosomale eiwitten, *htbA* en *hhfA* coderen voor histon eiwitten, het *tefA* gen codeert voor translatie elongatie factor 1α en *pruA* codeert voor pyrroline-5-carboxylaat (P5C) dehydrogenase. Door middel van koppelingsanalyse kon met een set van 86 homokaryotische nakomelingen aan iedere koppelingsgroep tenminste twee markers worden toegekend. De resultaten hiervan kwamen, in tegenstelling tot localisatiestudies met anonieme DNA markers, goed overeen met CHEF-analyses. De combinatie van geïdentificeerde markergenen en geoptimaliseerde chromosoom-scheidingstechnieken vormt daarom een goede basis voor de ontwikkeling van een betrouwbare genetische kaart en biedt perspectieven voor een versnelde klassieke veredeling van de champignon.

De resultaten beschreven in dit proefschrift laten zien dat een aantal *A. bisporus* genen tijdens de ontwikkeling van het vruchtlichaam zeer sterk verhoogde expressieniveaus vertonen. Twee van de geïsoleerde vruchtlichaamspecifieke genen, waarvan expressie in de vegetatieve fase van de levenscyclus niet waarneembaar is, coderen voor hydrofobines. Het HYPA hydrofobine is in hoge concentraties aanwezig in de hydrofobe laag die de hoed van

de champignon omgeeft terwijl *hypB* voornamelijk tot expressie komt in een overgangszone tussen hoed en steel. Daarnaast toonden expressiestudies met twee genen die betrokken zijn bij de afbraak van polysacchariden uit stro die in compost aanwezig zijn aan dat *A. bisporus* zich op transcriptie-regulatieniveau heeft aangepast aan het groeien op compost. De algemene opbouw van *A. bisporus* genen werd bestudeerd door bepaling van de genstructuur van een aantal genen en de isolatie van een aantal huishoudgenen maakte hierbij een vergelijking mogelijk met analoge genen van andere filamenteuze schimmels.

Met de identificatie en localisatie van een groot aantal genen is een goede basis gecreëerd voor het ontwikkelen van een koppelingskaart voor de gekweekte champignon, gebaseerd op goed gedefinieerde gen markers. In de nabije toekomst moet dit met behulp van moderne moleculaire technieken en de beschikbaarheid over een collectie wild-isolaten kunnen leiden tot een efficiënt veredelingsprogramma waarin nieuwe rassen met een grotere genetische diversiteit en met specifiek gewenste eigenschappen waaronder resistenties, gegenereerd kunnen worden. Voor een betere sturing van de processen die plaatsvinden tijdens de teelt van champignons is verder moleculair onderzoek niettemin onontbeerlijk.

Curriculum vitae

Petrus Wilhelmus Johannes de Groot werd op 12 april 1968 in Oldeholtpade geboren. In 1986 behaalde hij het VWO diploma aan de Rijksscholengemeenschap te Steenwijk en begon hij aan de Landbouwuniversiteit Wageningen aan een studie Moleculaire Wetenschappen. Met hoofdvakken in de Moleculaire genetica en de Microbiologie (bacteriële genetica) werd deze studie in 1992 afgerond. Het in dit proefschrift beschreven onderzoek maakte deel uit van een samenwerkingsproject van het Proefstation voor de Champignoncultuur en de sectie Moleculaire Genetica van Industriële Micro-organismen van de Landbouwuniversiteit Wageningen (MGIM) en werd uitgevoerd als assistent in opleiding van de vakgroep Microbiologie en Evolutiebiologie van de Katholieke Universiteit Nijmegen. Binnen dit project was hij van 1992-1993 gedetacheerd op het Proefstation en vanaf 1993 bij de sectie MGIM. Sinds 1 maart 1998 is hij als post-doc werkzaam bij de gistgroep van het Instituut voor Moleculaire Celbiologie van de Universiteit van Amsterdam.

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Nawoord

Nu het schrijven van dit proefschrift eindelijk is afgerond wordt het tijd eens stil te staan bij alle mensen die, ieder op hun eigen manier, een bijdrage hebben geleverd aan de totstandkoming ervan.

Op de eerste plaats wil ik mijn promotor Leo van Griensven bedanken. Gedurende mijn gehele promotieperiode heeft hij een belangrijke sturende rol vervuld. De wekelijkse fax was daarbij ook voor mij nuttig om dingen op een rijtje te zetten. Jaap Visser, jij hebt het mogelijk gemaakt dat ik als gastmedewerker bij de sectie werkzaam kon zijn, wat voor de voltooiing van dit proefschrift van essentieel belang is geweest. Daarvoor hartelijk dank. Peter Schaap, jij bent van onschatbare waarde geweest voor mijn onderzoek. Jouw steun, interesse en uitstekende ideeën werkten zeer motiverend. Het meest echter zal ik de, in mijn ogen soms eindeloze, discussies missen die we hebben gevoerd over conceptversies van de diverse manuscripten.

Het eerste jaar van mijn AIO-schap ben ik werkzaam geweest op het Proefstation voor de Champignoncultuur. De mensen van het proefstation waarmee binnen het genoomkarteringsproject nauw werd samengewerkt zijn: Anton Sonnenberg, Johan Baars, Angela van de Munckhof en René van der Vlugt. In het hele proefschrift is, met name in de vorm van diverse CHEF-blots, deze samenwerking tussen beide groepen terug te vinden en vooral hoofdstuk 8 is een mooi voorbeeld hiervan. Verder wil ik Robert Roeven vermelden aan wie ik de *in situ* hybridisatie plaatjes van Hoofdstuk 4 en de omslag te danken heb. Verder is de collegialiteit en gezellige sfeer binnen de afdeling Rassen en Veredeling één van de dingen die me van de periode Horst is bijgebleven, waarvoor iedereen hartelijk dank.

Het grootste gedeelte van mijn promotietijd heb ik doorgebracht bij de sectie MGIM van de Landbouwuniversiteit Wageningen. In het champignonnenlab zwaaide Yvonne Rusch de scepter. Yvonne, bedankt voor al je handige tips en goede adviezen. Daniëlle Basten, jouw bijdrage aan het xylanasewerk is zeer waardevol geweest, succes met je eigen promotieonderzoek. Verder wil ik iedereen van MGIM die mij heeft geholpen of van adviezen heeft voorzien daarvoor bij deze bedanken. In lab 1004 maakten gedurende de *Agaricus* periode achtereenvolgens Bernd, Michel, Patrick, Daniëlle en Monique voor korte of minder korte tijd hun opwachting. Al deze mensen en alle andere medewerkers en studenten van de sectie bedankt voor de leuke sfeer op het lab.

I would also like to thank Patrick Gunning of the Institute of Food Research Norwich Laboratory, UK for his valuable contribution on the Atomic Force Microscopy of the

HYPA protein. Patrick, I enjoyed writing an article together and I hope to hear more from you in the near future.

Natuurlijk gaat het leven van een promovendus niet altijd over rozen, maar gelukkig zijn er dan altijd weer je vrienden en familie om voor wat afleiding te zorgen. Dat gebeurde dan bijvoorbeeld door te gaan sporten, wandelen, kaarten, naar de kroeg te gaan of een HGA uitreiking bij te wonen. Alle Henken, oftewel Ben, Linda, Maurits, Boukje, Gerard, Flix, Petra, Jeanine, Jip, Marlies, Paul en Simon, bedankt. Jeanine, jouw wezenlijke bijdrage wil ik zeker niet onvermeld laten en komt duidelijk tot uiting in de Freelance figuren die in diverse hoofdstukken te vinden zijn. Tenslotte wil ik mijn familie bedanken voor de interesse die ze de afgelopen jaren hebben getoond. Lieve Pa en Moe, bedankt voor alles.

Bedankt!

Piet.