

Efficient isolation of genes differentially expressed on cellulose by suppression subtractive hybridization in *Agaricus bisporus*

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The production of cellulases on minimal medium in the edible mushroom *Agaricus bisporus* is regulated by the carbon source: induced by cellulose and repressed by glucose. In order to isolate cellulose-growth specific sequences, a cDNA library from *A. bisporus* using suppression subtractive hybridization (SSH) was constructed. Northern blot analysis indicated that a high level of enrichment was achieved; 183 clones were isolated. A preliminary screen with cellulose-specific genes of *A. bisporus* (*cel1*, *cel2*, *cel3* and *cel4*) using Southern hybridization resulted in 28 clones to be *cel3*, and 5 clones were *cel2*. The remaining 144 clones were sequenced. Partial sequences of the following genes were found: a β -glucosidase homologue of the *blvk* gene of *Kluyveromyces marxianus*; a cellulase homologue of an endoglucanase (avicellase III) of *Aspergillus aculeatus*, four different xylanases homologue of the *xyn* genes of different fungi, and one hexose transporter homologue to the *hxtA* gene of *Aspergillus parasiticus*. The apparent full-length of two hydrophobins homologue to the *abh3* gene of *A. bisporus* and one histone homologue to the *h2a* gene of *Aspergillus niger* were also found. The remaining sequences did not have homology to any known genes.

INTRODUCTION

The cultivation of edible mushrooms is a global activity and is a large-scale process utilising microbial technology for the conversion of agricultural by-products into food. Several species of edible mushrooms are cultivated in different regions of the world. *Agaricus bisporus* is the most produced with around 40% of the world production (Miles & Chang 1997). *A. bisporus* is grown on mushroom compost which is composed mainly of lignocellulosic residues and microbial biomass (Wood & Smith 1987). It has been demonstrated that *A. bisporus* produces endoglucanases, cellobiohydrolases, β -glucosidases, cellobiose dehydrogenases, xylanases, mannanases, laccases, manganese peroxidases, which are involved in compost degradation (Wood 1989, Wood & Thurston 1991, Fermor *et al.* 1991, Yagüe *et al.* 1997). At present, four cellulose-growth specific genes have been isolated, *cel1* (endoglucanase), *cel2* (cellobiohydrolase), *cel3* (cellobiohydrolase) and *cel4* (mannanase) (Raguz *et al.* 1992, Chow *et al.* 1994, Yagüe *et al.* 1997). It has been suggested that the cellulolytic system in *A. bisporus*

is similar to that of *Trichoderma reesei* (Whiteford & Thurston 2000). In *A. bisporus* the production of cellulases on minimal medium is regulated by the carbon source: induced by cellulose and repressed by glucose (Manning & Wood 1983). The identification of the genes responsible for these regulations requires methods which can efficiently compare the transcripts expressed in both conditions, and allow the isolation of those transcripts found only in one condition. The suppression subtractive hybridization (SSH) technique is an efficient method for detecting genes which are differentially expressed amongst different cells or among cells under modified conditions (Diatchenko *et al.* 1999). Since genes responsible for or associated with cellulose degradation in *A. bisporus* are regulated by the carbon source, we used SSH in an attempt to identify the genes that are involved in this process.

MATERIALS AND METHODS

Organism and culture conditions

Agaricus bisporus strain D649 was grown and maintained on 50 g l⁻¹ malt extract agar (Oxoid) plates at 25 °C in the dark. Experimental inoculum was grown

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in 250 ml flasks containing 50 ml of Treschow's salt minimal medium with either 0.1% (w/v) D-glucose (Fisons) or 0.05% (w/v) solka floc BW40 (International Filler Corporation, New York) as a carbon source (Manning & Wood 1983). After 14 d of growth, the mycelium from these cultures was fragmented with sterile glass beads, and 1 ml of this suspension was added to fresh sterile Treschow's salt minimal medium with glucose and solka floc as above. All cultures were grown at 25 ° in the dark without shaking. After 14 d of growing, the mycelium was harvested by filtering through a nylon mesh and frozen in liquid nitrogen. The mycelium was kept at -75 ° until use.

The strain used is permanently maintained and preserved at the Horticulture Research International (HRI) collection at Wellesbourne (UK).

RNA extraction, and Northern analysis

Total RNA was extracted from mycelium growing on glucose and solka floc using different phenol:chloroform extractions as previously described (Raguz *et al.* 1992). Poly(A)+ RNA was isolated from the extracted total RNA using the mRNA purification kit (Amersham Pharmacia Biotech, Little Chalfont) according to the manufacturer's instructions. Concentration and purity of both total RNA and Poly(A)+ were determined by their absorbance at 260 and 280 nm. Northern filters were prepared by electrophoresis by glyoxal denatured total RNA (40 µg) from 14 d old glucose and solka floc cultures. The Northern transfer was carried out as described in Sambrook, Fritsch & Maniatis (1989) using a Hybond-N nylon membrane (Amersham Pharmacia Biotech). Fragments were radiolabelled using the High Prime DNA Labeling kit (Boehringer Mannheim), following the manufacturer's instructions. After hybridization, the filters were washed in 2 × SSC, 0.1% (w/v) SDS at room temperature for 15 min, then in 1 × SSC, 0.1% (w/v) SDS at room temperature for 10 min, and finally in 0.1 × SSC, 0.1% (w/v) SDS at 42 ° for 10 min. The filters were wrapped in Saranwrap film and exposed to autoradiography at -70 ° for 3–5 d.

cDNA library construction by suppression subtractive hybridization

SSH was performed between RNA extracted from mycelium growing on solka floc (tester) and RNA extracted from mycelium growing on glucose (driver). All SSH steps were performed according to the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA), except that three hybridizations were performed instead of two.

Cloning and analysis of the subtracted cDNA library

The SSH subtractive cDNA fragments were cloned into the cloning vector PGEM-T (Promega) and

transformed into *Escherichia coli* XL-1 blue competent cells (Stratagene). The transformed bacteria were plated on LB agar plates containing ampicillin (100 µg ml⁻¹), X-gal (40 µl of 20 mg ml⁻¹ stock) and IPTG (40 µl of a 100 mM stock).

Screening the cDNA library by dot blot hybridization

Individual white recombinant clones were randomly picked, dense streaked on LB agar plates containing ampicillin, and left overnight at 37 °. Plasmid DNA was prepared from the recombinant clones using the Wizard Plus SV Minipreps DNA Purification System (Promega). 4 µl of the miniprep from each clone was diluted in 2 × SSC (NaCl/sodium citrate buffer; Sambrook *et al.* 1989) final concentration in a total volume of 200 µl. The samples were heat denatured and applied onto a Hybond-N nylon membrane (Amersham Pharmacia Biotech) using the Bio-Dot SF blotting apparatus (Bio-Rad), following the manufacturer's instructions. The cDNA on the membranes were denatured in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min, neutralization solution (0.5 M Tris-HCl, 1.5 M NaCl) for 5 min, and 2 × SSC for 15 min. The *cel1*, *cel2*, *cel3* and *cel4* genes (Raguz *et al.* 1992, Chow *et al.* 1994, Yagüe *et al.* 1997) were used as probes for hybridization, which were labelled using High Prime DNA Labelling Kit (Boehringer Mannheim). Hybridization was performed at 65 ° overnight in hybridization solution (20 × SSC, 1% SDS, 100 × Denhard's solution). The membranes were washed twice with 2 × SSC, 0.1% SDS at room temperature for 15 min, twice with 0.1 × SSC, 0.1% SDS at 65 ° for 30 min, then exposed to X-ray film at -75 ° in a cassette with intensifying screen.

DNA sequencing

DNA sequencing reactions were carried out using an ABI PRISM 377 DNA Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA) with the ABI Prism Dye Terminator cycle sequencing kit (Perkin Elmer). All sequences were examined using the Mac Vector sequence analysis software program (Oxford Molecular). Sequences described in the present study can be found in the EBI database under accession nos. AJ534338–AJ534359.

RESULTS AND DISCUSSION

Construction of the cDNA library

After three rounds of hybridization, the enriched cDNA fragments were amplified with 35 cycles of PCR. In order to increase the representation of the transcripts present in the sample nine different independent primary PCR were performed (data not shown). The PCR products were pooled and used as a template for a secondary PCR. On agarose gel the subtracted library

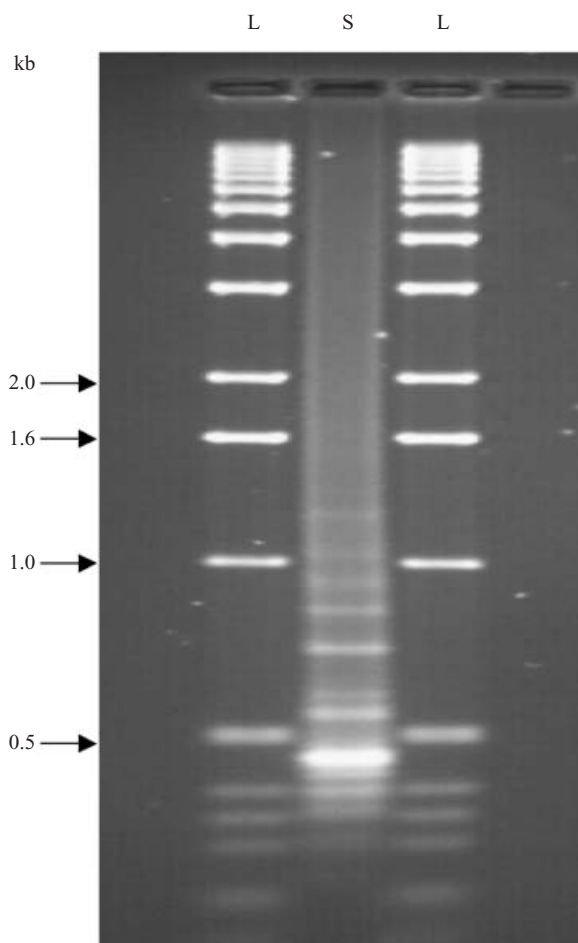


Fig. 1. Secondary PCR product of the *Agaricus bisporus* subtractive cDNA library. After three rounds of hybridization, different independent PCR were performed. The PCR products were pooled together and purified with the Wizard PCR Preps DNA Purification System (Promega). The PCR sample (S) was electrophoresed on a 1.2% (w/v) agarose gel and stained with Et/Br. At both extremes (L) the 1 kb ladder (Gibco, BRL), shows the size markers.

gave 12 well-defined bands with molecular weights ranging from 300 to 1200 base pairs (bp), with a major band at around 450 bp (Fig. 1).

Efficiency of the suppression subtraction hybridization

In order to validate the efficiency of the subtraction, the mixtures of the unsubtracted and subtracted cDNAs were labelled with ^{32}P and hybridized to Northern blots of total RNA from mycelium growing on glucose and solka floc. The unsubtracted cDNA probe (Fig. 2, top left) hybridized strongly to both RNA samples on the blot, possible as a result of common or homologous species of mRNAs among the mycelium. On the other hand, the subtracted cDNA probe (Fig. 2, top right) hybridized strongly to the solka floc RNA, and weakly to the glucose RNA. This analysis indicates that a high level of enrichment of solka floc cDNA has been achieved, and at the same time, a drastic reduction of highly abundant or common cDNAs. The efficiency of

the solka floc specific subtraction was subsequently confirmed after cloning and sequence analysis of the solka flock specific cDNAs. Different approaches for isolation of differentially expressed genes have been used. In our case, we used the SSH technique in order to isolate those sequences which are induced by cellulose on minimal medium in *Agaricus bisporus*. The study showed that the SSH method is an efficient procedure for generating enriched cDNAs for genes differentially expressed of both high and low abundance. Based on Northern blot analysis a high level of enrichment was achieved (Fig. 2).

Screening of the cDNA library

We obtained 183 clones from the enriched cDNA library. In an initial screen of those clones, six did not contain a detectable insert on ethidium bromide stained agarose gel following digestion of the Plasmid DNA with *ApaI* and *SalI*. The remaining 177 clones were screened using the growth-specific genes *cel1*, *cel2*, *cel3* and *cel4* from *A. bisporus* as probes. As a result, it was found that 28 clones correspond to *cel3*, while five clones correspond to *cel2*. Neither *cel1* nor *cel4* were found. The remaining 144 clones (82%) obtained from the cDNA library were sequenced.

Sequence analysis

The cDNA inserts from the 144 clones were partially sequenced using the T7 or SP6 sequencing primers. For those clones that appeared to be full-length, the sequence of both strands were also determined. The sequences were searched by BLASTX analysis (Altschul *et al.* 1997) in the European Bioinformatics Institute (<http://www.ebi.ac.uk/blast2/>) databases. A summary of the sequencing data is shown in Table 1. The 144 clones from the subtracted library for which data sequences were obtained correspond to 60 distinct mRNA species. This amount represents 42.5% from the total number of clones sequenced. 39 clones (27.4%) of these sequences were encountered only once in the set of 142 clones, while 23 species (16.1%) were seen between 2 and 6 times, and only 3 (2.1%) more than 10 times. Among the 60 distinct species, six cDNA (clones 2, 53, 104, 117, 158 and 175) inserts were found to be partial sequences which correspond to *cel3*, a cellobiohydrolase from *A. bisporus* (Yagüe *et al.* 1997); while four were partial sequences (clones 42, 45, 98 and 171) that matched to *cel2*, the other cellobiohydrolase previously found in *A. bisporus* (Yagüe, Wood & Thurston 1994).

The sequences obtained from the cDNA subtracted library can be grouped into two classes. Those which had significant homology with known gene sequences in the databases (EBI), and those which do not have homology with any known sequences in the different databases. In the first group, four clones (87, 88, 89 and 152) were found to match one endoglucanase

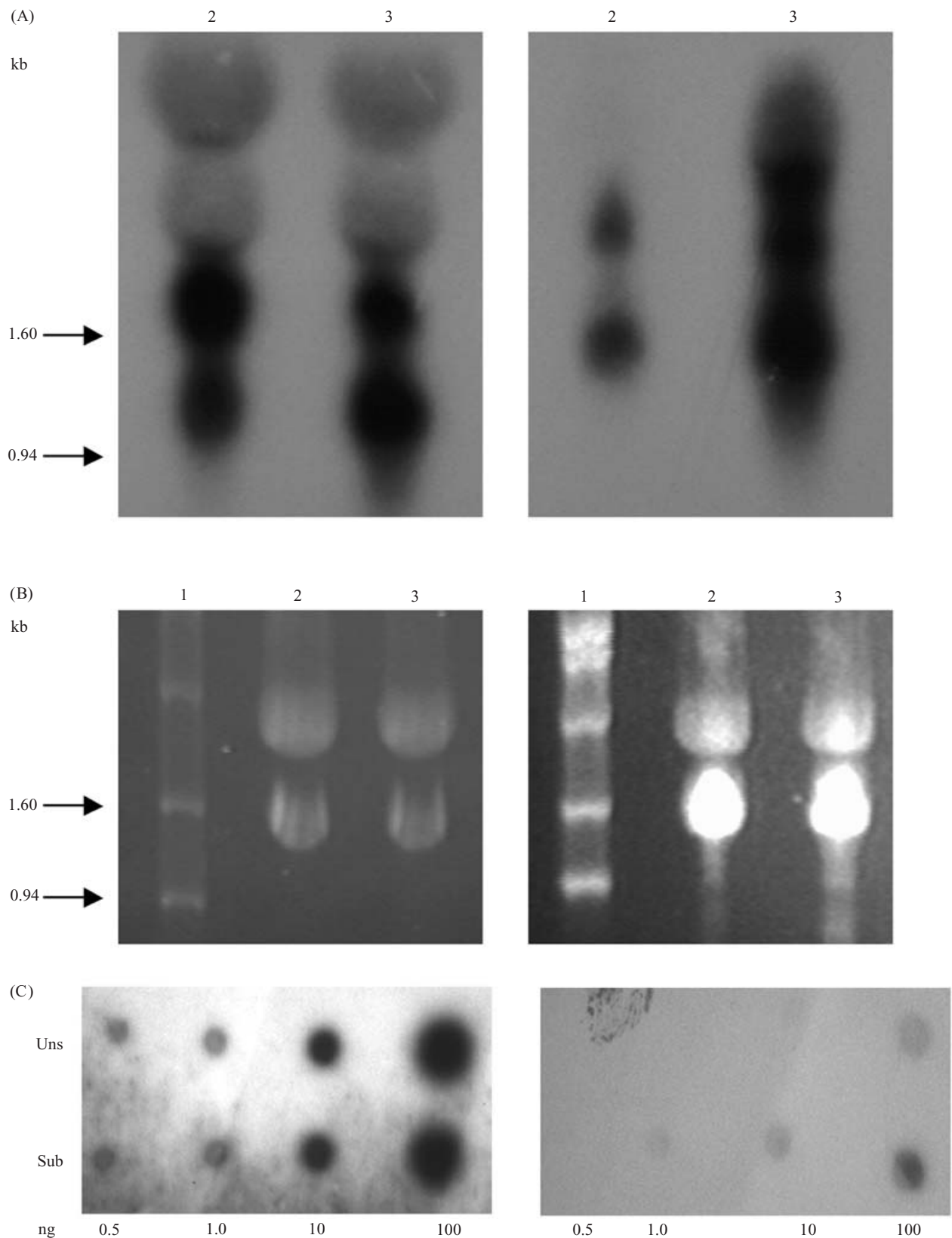


Fig. 2. Efficiency analysis of the *Agaricus bisporus* cDNA subtracted library. (A) After a secondary PCR amplification, 30 ng of the PCR product of the unsubtracted tester (A left) and 30 ng of the PCR product of the subtracted cDNA (A right) were labelled with ^{32}P and used as a probe. (B) The northern blots contained 40 μg of total RNA from mycelium growing on glucose (B, 2) and solka flock (B, 3). The RNA ladder (Gibco, BRL) is at the left hand side (1) of each gel. (C) Southern blot analysis of the unsubtracted library (Uns) and the subtracted cDNA library (Sub) was performed. Different amounts (ng) of the unsubtracted and the subtracted cDNA library were loaded. The *h2a* histone (this study) insert (left) and the *cel3* insert (Chow *et al.* 1994) (right) were radiolabelled and used as a probes.

Table 1. Characteristics of the clones isolated in the subtracted library from *Agaricus bisporus*.

Clone	Redundancy	Gene	Degree of homology	Gene product
81	1	<i>abh3</i>	Full-length	Hydrophobin
146	1	<i>abh3</i>	Full-length	Hydrophobin
96	1	<i>h2a</i>	Full-length	Histone
87	4	<i>cel</i>	Partial	Avicelase III
161	1	<i>bgl</i>	Partial	Beta glucosidase
2	6	<i>cel3</i>	Partial	Cellobiohydrolase
42	4	<i>cel2</i>	Partial	Cellobiohydrolase
17	2	<i>xyn</i>	Partial	Xylanase
149	1	<i>xyn</i>	Partial	Xylanase
38	11	<i>xyn</i>	Partial	Xylanase
13	18	<i>xyn</i>	Partial	Xylanase
55	6	<i>hxtA</i>	Partial	Hexose transporter
25	13	Novel	Unknown	Unknown
33	5	Novel	Unknown	Unknown
114	5	Novel	Unknown	Unknown
28	5	Novel	Unknown	Unknown
72	3	Novel	Unknown	Unknown
109	3	Novel	Unknown	Unknown
62	3	Novel	Unknown	Unknown
18	3	Novel	Unknown	Unknown
23	2	Novel	Unknown	Unknown
79	2	Novel	Unknown	Unknown
31	2	Novel	Unknown	Unknown
71	2	Novel	Unknown	Unknown
10	2	Novel	Unknown	Unknown
*	1	Novel	Unknown	Unknown
32	2	Chimeric		
6	5	Vector	Partial	pGEMT Vector

* 34 clones were found only once.

(avicelase III) from *Aspergillus aculeatus* (Arai *et al.* 1998). Three clones are the same (87, 88, 89) and one is different (152). The two set of sequences match the endoglucanase in different positions. Clone 152 matched the N-terminal region with 157 amino acids (aa), while the other clones at around the middle of the sequence with 231 aa. The amino acid percentage identity between clone 152 and the *A. aculeatus* was 58% of identity, whereas for the other clones (87, 88 and 89) was 53%.

One clone (161) of 166 aa matches with several β -glucosidases from fungi and bacteria. The part of the sequence corresponds to the C-terminal region in those the proteins in the databases. The highest percentage of amino acid identity is with a β -glucosidase from *Kluyveromyces marxianus* (Raynal *et al.* 1987) with an identity 31%, and a β -glucosidase from *Agrobacterium tumefaciens* (Castle, Smith & Morris 1992) with an identity 30%. Four partial cDNAs, 13, 17, 38 and 149 showed sequence similarity at the N-terminal region to xylanases of different fungi. Two of these sequences were highly redundant, clone 13 and 38, which were found 18 and 11 times, respectively (Table 1). Xylanases are involved in xylan degradation. The full-length sequence of the endoglucanasa, β -glucosidase and xylanase *A. bisporus* genes has been determined and will be described elsewhere.

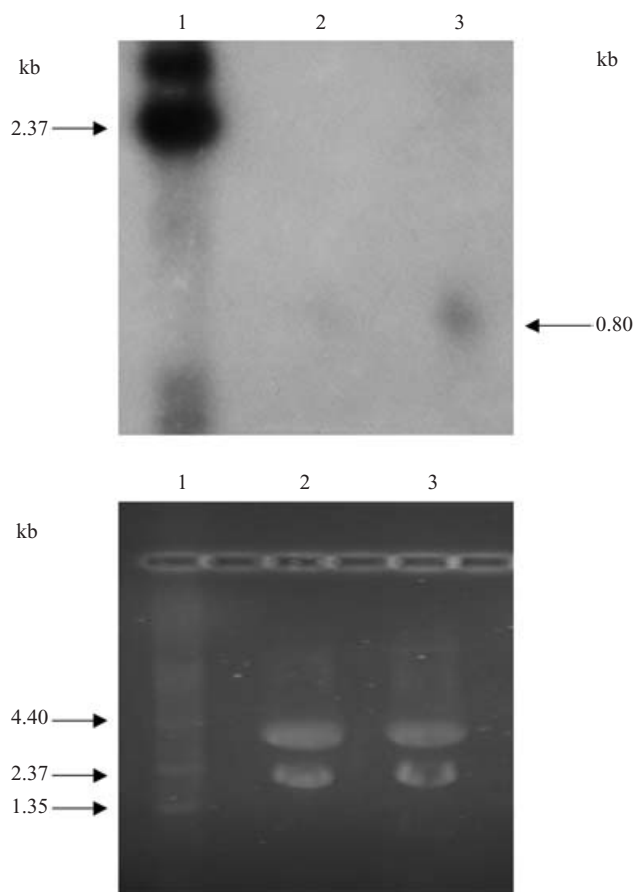


Fig. 3. Northern blot analysis of the hydrophobin from *Agaricus bisporus*. 40 μ g per track of total RNA was run on 1% (w/v) agarose gel and stained with ethidium bromide (bottom). Lane 1, RNA ladder (Gibco BRL); Lane 2, glucose; and Lane 3, Solka Floc.

In cellulolytic fungi, three main enzymes are involved in cellulose degradation: endoglucanases, cellobiohydrolases and β -glucosidases. Of the sequences found in the cDNA subtracted library, 26% of the clones belong to these sequences. At present, in *Agaricus bisporus* four cellulose-specific genes have been found. However, it is likely that, due to the complexity of the substrate there may be more genes involved in cellulose degradation. *Trichoderma reesei*, for instance, produce a wide range of cellulases, including two different cellobiohydrolases, five endoglucanases, and two β -glucosidases (Kubicek & Penttilä 1997).

Cellulases and xylanases are predominantly associated with the degradation of cellulose and hemicellulose degradation. In fungi, the cellulase system is associated with other glycosyl hydrolases, particularly xylanases, because of the close connection of cellulose with hemicelluloses in nature (Tomme, Warren & Gilkes 1995). In the present study, of the total number of sequences found, 43% of the clones correspond to those sequences.

The clones 55 and 172, matched to a hexose transporter gene *hxtA* from *As. parasiticus*. These gene belongs to a large family of related sugar transporter

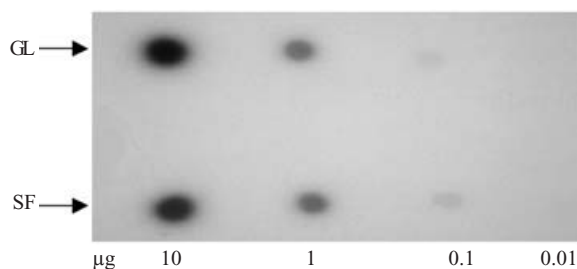


Fig. 4. Blot analysis of the histone *h2a* from *Agaricus bisporus*. Dot blot analysis was performed using different amounts of total RNA (ng). The *h2a* histone full length clone was labeled with [³²P]cDTP and used as a probe. GL, glucose; SF, solka floc.

proteins which are involved in the uptake of glucose (Boles & Hollenberg 1997). Two clones (8 and 61) were homologue to a inorganic phosphate transporter. In addition, homologues to a cytochrome P450, an extensin, a patatin-related protein, a myosin heavy chain kinase, and a stress protein were also found.

From the second group of sequences, there were no homologies found to any known sequences in the public databases. It is interesting to note that despite the whole genome of several fungi has been sequenced, numerous sequences obtained in the present study did not have homology to those organisms. These genes can potentially represent novel genes involved in the cellulolytic system of *A. bisporus*. Further characterisation of these clones needs to be done in order to know the real significance of these genes.

Apart from the sequences described before, the clones 81, 146 and 96 appeared to be full-length (Table 1). The cDNAs were sequenced on both strands. A search in the databases with clones 81 (accession no. AJ293763) and 146 (accession no. AJ293764) identified these cDNAs as hydrophobins with 407 and 399 bp, respectively. Both clones are different from each other in five positions, which led to the substitution of four amino acids. This is probably due to allelism or multiple homologous genes as has been reported for other hydrophobins in *A. bisporus*, where similar results were found using different strains (Lugones, Wösten & Wessels 1998). The deduced *A. bisporus* ABH3-81 amino acid sequence was found to have 96%, 93% and 65% identity with the hydrophobins ABH3-39, ABH3-97 from *A. bisporus*, and PO3 from *Pleurotus ostreatus*, respectively (Lugones *et al.* 1998, Ásgeirsdóttir, de Vries & Wessels 1998). Northern analysis using clone 81 showed that it hybridized to a single transcript of 800 nucleotide (nt), and is only detected in RNA from solka floc cultures but not in glucose cultures (Fig. 3). These results showed that the *abh3-81* hydrophobin might play a role in the utilization of cellulose in *A. bisporus*. It has been suggested that hydrophobins might be involved in the attachment of the hyphae onto hydrophobic surfaces (Wösten *et al.* 1994). The results obtained in the present study are similar to those found with other fungal hydrophobins

like *Qid3* from *Trichoderma harzianum*, and *hfb2* from *T. reesei* (Lora *et al.* 1994, Nakari-Setälä *et al.* 1997). These hydrophobins were only expressed on vegetative mycelium grown on submerged culture media containing cellulose or their derivatives and repressed by glucose. The *A. bisporus* *abh3-81* gene could play a role in the attachment of the fungus onto the substrate.

A search in the databases with clone 96 revealed that the full-length was obtained. The cDNA insert is 596 bp long with an ORF consisting of 420 bp. The cDNA fragment contains a coding region for histone H2A (accession no. AJ293758). The predicted histone H2A protein of *A. bisporus* is 139 amino acid residues in length with a calculated molecular weight of 14732 Da. The sequence homology of the *A. bisporus* histone with other fungal species showed that they are roughly the same. The percentage of amino acid identity of the H2A *A. bisporus* histone compared with other fungal histones is as follows: *Schizosaccharomyces pombe* (76%), *Emericella nidulans* (and *A. nidulans* anamorph; 74%), and *Saccharomyces cerevisiae* (70%). H2A histones belong to core histones which play an important role in processes such as packing, replication, and transcription of DNA (Felsenfeld 1992). In *A. bisporus* two types of histones have been found previously, H2B and H4 (Sonnenberg *et al.* 1996). The *h2a* histone reported in the present study is the first of the type H2A histones in *A. bisporus*. The expression of the H2A histone was investigated using total RNA from cultures growing on minimal medium with glucose and solka floc as a carbon source. Different amounts of total RNA were glyoxal denatured, spotted on a nylon membrane, and hybridized to the histone fragment as above. The results show that the level of transcript were equal in both conditions (Fig. 4, bottom). The Northern blot analysis of the *A. bisporus h2a* gene showed that this is a constitutive gene, which managed to 'escape' the subtraction procedure.

Two clones (32 and 34) which apparently had a great similarity with the C-terminal sequence of the *cel2* gene of *A. bisporus* resulted to be a chimeric composed of sequences from two different gene transcripts. Apparently, the chimeric formation in the SSH method is a frequent event during the hybridization procedure (Zhang, Underwood & D'Ercole 2000). They have estimated that approximately 2% of a cDNA subtracted library can contain chimeric clones. In the present study, the two clones represent 1% of the total number of clones. Although, it cannot be ruled out that other clones could be chimerics.

Further analysis of the sequences obtained will provide important information for understanding the genes involved in the mechanism of cellulose degradation in *A. bisporus*.

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