Isolation of Expressed Sequence Tags of *Agaricus bisporus* and Their Assignment to Chromosomes

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The genome of the cultivated basidiomycete *Agaricus bisporus* Horst U1 and of its homokaryotic parents has been characterized by using an optimized 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, harboring 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.

The annual world production of the edible mushroom *Agaricus bisporus* (Lange) Imbach amounted to 1,950,000 tonnes in 1995 (2). 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* (28), which renders breeding difficult and rather unprofitable. Most of the basidia produce two spores, which preferentially inherit two nonsister nuclei and retain heterozygosity for most of the parental markers (38). Only approximately 2% of the basidia produce three or four spores, which form predominantly homokaryotic mycelia upon germination (17).

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 (11). By the late 1980s most of the cultivated strains in the world were identical to or derived from those strains (21), leaving the industry with a crop that was genetically very limited and had a high 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 (16), forming a sound basis for further breeding activity. Also, some studies were carried out on the meiotic behavior of *A. bisporus* (18, 39, 41) and on chromosome characterization (29, 36). A few genes of *A. bisporus* have been isolated and were characterized, i.e., the glyceraldehyde-3-phosphate dehydrogenase I and II (13), cellulase 1 (27), cellulase 3 (4), and laccase I and II (25) 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 (3) 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 coworkers (18) 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 (8, 26), were used. We therefore started a genome mapping program in which well-defined genetic markers, preferentially genes, were to be used (34). 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-number cDNAs from a primordial cDNA library and used them as expressed sequence tags (EST) 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 sterilized 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)propanesulfonic acid], pH 7.0}. Single-spore cultures were isolated as described by Fritsche (10), and their homokaryotic nature was identified by the loss of heteroallelism of two markers (*gdhA* and *celI*), both located on the same chromosome.

Escherichia coli LE392 (Promega, Madison, Wis.) was used for phage amplification and λ DNA isolation. *E. coli* DH5 α (GIBCO BRL Life Technology, Gaithersburg, Md.) was used for plasmid transformation and propagation. *E. coli*

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FIG. 1. CHEF analysis of *A. bisporus* H39 and H97. (A) Chromosomes of the parental lines of strain Horst U1 separated by CHEF gel electrophoresis. (B) Diagrammatic interpretation of the karyotypes. Chromosomes are indicated by Roman numerals. Homologous chromosomes are connected with lines. The positions of chromosomal size markers (in megabases) (chromosomes of *S. pombe* and *H. wingei*) are indicated on the left. (C–E) Southern analysis of chromosomes separated by CHEF. Strains are indicated above the panels. Gels were hybridized with the *htbA* gene (C), with the rDNA probe (D), or with the *lcc1* gene. Different CHEF gels were used in various experiments, accounting for the difference in signal strength observed.

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. (31). 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 and Vogelstein (9) or with digoxigenin by using the Dig DNA labelling kit (Boehringer, Mannheim, Germany). Hybridization was carried out overnight at 65°C in standard hybridization buffer (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 100 µg of denatured herring sperm DNA per ml). The filters were washed at 65°C under stringent conditions, dried, and exposed on Kodak X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) at -70°C with intensifying screens. When digoxigenin-labelled probes were used, hybridization and detection of hybrids were carried out according to the conditions recommended in the Dig Chemiluminescent Detection Kit (Boehringer). DNA sequences were determined by 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 λ EMBL4 genomic library of *A. bisporus* H39 by standard methods with cDNA probes.

Generation and identification of EST. EST were generated by random excision of a cDNA library made from primordia and small fruit bodies (5) and were analyzed by sequence analysis combined with a database search by using the program Blast (12). EST 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. (37). Protoplasts were diluted with 2% InCert agarose (FMC Corporation, Rockland, Maine) at 42°C to a final concentration of 0.7% agarose and 1×10^9 to 1.5×10^9 protoplasts per ml. After transfer to a prewarmed 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 of proteinase K per ml 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% Na₃N 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 optimized for the highest resolution of chromosomal bands. The modifications were based on the method previously described (36). Gels were run at 14°C in 0.8% agarose (SeaKem; FMC) in 0.5× TBE (1× TBE is 0.089 M Tris-borate and 0.0025 M EDTA, pH 8.3) with a CHEF-DR II apparatus (Bio-Rad, 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. *SalI* 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 (10). 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 (Fig. 1A). 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 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 1).

Cloning and identification of EST. For rapid isolation of *A. bisporus* EST, 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 analyzed by sequence analysis. A database search using the program Blast (12) identified a number of the EST, and these tags were preferentially used as probes.

Two EST 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

TABLE 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 mobilities 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.

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 (19). Also, as was found for histone genes from other fungi (24), 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 (Fig. 1C). 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 (Fig. 2) were compared with the simple restriction patterns obtained by Southern analysis of an individual lambda clone harboring a single copy of the *hhfA* gene (not shown).

Two EST 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 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 similarity 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 (32).

Two other EST 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 *tpaB*. The other, *tefA*, probably encodes a

FIG. 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 *Hind*III (lanes 4 and 10), *Hind*III (lanes 5 and 11), and *Bam*HI and *Hind*III (lanes 6 and 12). Molecular size markers (in kilobases) are indicated on the left.

translation elongation factor similar to the Efl α 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* (6).

The last EST was identified as encoding a pyrroline 5-carboxylate dehydrogenase and was named *pruA* (for proline utilization) (35). A phosphoglycerate kinase-encoding gene, named *pgkA*, was previously isolated by heterologous hybridization (35).

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 EST 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 hybridization signal was visible for chromosome VIII, indicating that another copy of *htbA*, named *htbB*, is located on the latter chromosome (Fig. 1C). Every other EST probe on a gene gave a clear hybridization signal with only 1 of the 11 chromosome bands for strains H39 and H97 (Table 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 hybridization 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 Fig. 1B. 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 (33) hybridized strongly with chromosome IX of strain H39, indicating that the rDNA repeat is located on that chromosome (Fig.

TABLE 2. Chromosome assignment of *A. bisporus* genes

Gene	Gene product or function	Chromosome	Reference
<i>tefA</i>	Translation elongation factor	I	This article
<i>MAT</i>	Mating type	I	This article, 14
<i>atpD</i>	δ -ATP synthase subunit (mitochondrial)	II	6
<i>pgkA</i>	3-Phosphoglycerate kinase	III	35
<i>rpaB</i>	DNA-directed RNA polymerase I subunit B	IV	This article
<i>pruA</i>	Pyrroline 5-carboxylate dehydrogenase	IV	35
<i>hypA</i>	Hydrophobin A	III/IV	5
<i>htbA</i>	Histone 2B-1	III/IV	This article
EST2	Not identified	V	This article
<i>hhfA</i>	Histone H4-1	VI	This article
<i>hhfB</i>	Histone H4-2	VI	This article
<i>gdhB</i>	NAD ⁺ -glutamate dehydrogenase	VII	35
EST3	Not identified	VII	5
<i>htbB</i>	Histone 2B-2	VIII	This article
<i>rs13A</i>	Ribosomal protein RS13A	VIII	This article
<i>s15a</i>	Ribosomal protein S15a	IX	32
<i>l41A</i>	Ribosomal protein L41	IX	This article
EST1	Not identified	IX	This article
28S rDNA	Large-subunit rRNA	IX	33
<i>gdhA</i>	NADP ⁺ -glutamate dehydrogenase	X	33
<i>cel1</i>	Cellulase	X	27
<i>lcc1</i>	Laccase	XI	25
EST5	Not identified	XI	7
EST4	Not identified	XII	5
<i>gpdA</i>	Glyceraldehyde 3-phosphate	XIII	13
EST6	Not identified	XIII	This article

1D). This chromosome also hybridized with two other EST encoding ribosomal proteins, *l41* and *s15a*, and with the unidentified marker EST1. All four probes hybridized also to a single chromosome in strain H97, and although this chromosome (2.13 Mb) differs considerably in size from its homolog 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 harboring the rDNA cluster are due to differences in copy number of the rDNA unit (15, 22, 23, 30, 40). A difference between the hybridization 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 hybridized to high-molecular-weight DNA on Southern blots of genomic DNA digested with *SalI*, indicating that this restriction enzyme does not cut within the rDNA unit. Total chromosome size DNA of both strains was therefore digested with *SalI* and separated by CHEF electrophoresis, and this revealed a large fragment of approximately 625 kb in strain H97 (Fig. 3A). In this region no band was observed for strain H39. Hybridization of the gel with the partial 28S rDNA probe showed that the large band observed for strain H97 hybridized strongly (Fig. 3B). In addition, a number of weakly hybridizing signals were seen in the lower-molecular-weight region. For strain H39 multiple hybridizing 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

FIG. 3. Analysis of the rDNA repeat on chromosome IX. (A) Chromosomes of strains H39 and H97 digested with *SalI* and separated by CHEF electrophoresis. (B) Hybridization of the gel with the rDNA probe. Molecular size markers consist of concatemers of λ DNA of 48.5 kb (BioRad).

rDNA on chromosome IX of strain H97 is located in one large array. The hybridization signals in the lower-molecular-weight region may indicate the presence of smaller rDNA clusters with a lower copy number. The hybridization 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 (14), 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.

Other currently available *A. bisporus* genes, not previously assigned to a chromosome, were located by CHEF analysis. The NAD⁺-glutamate dehydrogenase-encoding gene (35) was located on chromosome VII. The *cel1* gene, encoding an *A. bisporus* cellulase (27), and the *lcc1* gene, encoding an *A. bisporus* laccase (25), were located on chromosomes X and XI, respectively (Fig. 1E and Table 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 with a set of 86 homokaryotic offspring of Horst U1. Two markers (*pruA* and *rpaB*) showed no recombination and thus are located on the same chromosome. The recombination fraction (recombinant genotypes/parental genotypes) between 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 ex-

TABLE 3. Chromosome assignments of RFLP markers

RFLP marker	Chromosome no. in:	
	This article	Reference 18
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

pected 1:1:1:1 ratio of genotypes. This indicates that p33n5 is located on a different chromosome. Since p33n5 was previously assigned to chromosome III, *pruA* and *rpaB* are assigned to chromosome IV. Similarly, the 3-phosphoglycerate kinase-encoding gene (35) was found to be completely linked to marker p33n5 and therefore located on chromosome III.

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. (41) 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 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 (18), 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. (18) except for p1n37 and p1n55, which were placed on chromosome IX by Kerrigan et al. (18) and on chromosomes VII and VIII, respectively, in the present study (Table 3).

DISCUSSION

In this study we have optimized the separation of the chromosomes of *A. bisporus* 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 EST for the development of a gene-based genetic linkage map. Of the 12 EST 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* (39). EST4 is the same as pDG47 (5) 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 (5).

The optimization 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 (18, 29, 36). 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 reassociation kinetics (1).

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 (42) 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. Hybridization of an rDNA probe to *SalI*-digested chromosomes revealed that the probe does not hybridize 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 hybridization to CHEF blots agrees with the previously published data (18), with one exception. In the first linkage map of *A. bisporus* (18), anonymous RFLP markers p1n37 and p1n55 were placed on the same chromosome (IX), whereas we have placed p1n37 on chromosome VII and p1n55 on chromosome VIII (Table 3). One homokaryon of the parental heterokaryon used by Kerrigan et al. (18) 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 hybridization to a CHEF blot; positioning of the other marker at the end of the same chromosome was based on linkage analysis (18). Since chromosomes VII and VIII are well separated on CHEF gels, an incorrect interpretation of hybridization results is unlikely.

The locations of the rDNA repeat and the genes *cel1* and *lcc1* also differ from those reported by Lodder et al. (20). 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 (Fig. 1). Applying the CHEF technique as described here to protoplasts isolated from a

mycelial culture of the heterokaryotic strain Horst U1 shows that *lccI* does not hybridize 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 that will enable mapping of important traits. At the same time, the characterization of these genes will increase our knowledge of the molecular biology of *A. bisporus*.

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