The *Agaricus bisporus* *pruA* Gene Encodes a Cytosolic Δ^1 -Pyrroline-5-Carboxylate Dehydrogenase Which Is Expressed in Fruit Bodies but Not in Gill Tissue

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A fortuitously cloned 3'-truncated cDNA encoding the *Agaricus bisporus* Δ^1 -pyrroline-5-carboxylate dehydrogenase was used to characterize the complete gene. The gene would encode a cytosolic polypeptide of 546 amino acids, and the basidiomycetous gene was evenly expressed in various parts of the mushroom except for the gills. No expression was detected in compost-grown mycelium. The steady-state mRNA level of the gene in the vegetative phase was determined on simple synthetic media and was two- to threefold higher with ammonium or proline as the sole nitrogen source compared to glutamate as the sole nitrogen source. Moreover, the steady-state mRNA level was not markedly influenced by addition of ammonium phosphate to proline- or glutamate-utilizing cultures. The results suggest that ammonium and the amino acids proline and glutamate are equally preferred nitrogen sources in this organism and are consistent with previous observations of H. M. Kalisz, D. A. Wood, and D. Moore (Trans. Br. Mycol. Soc. 88:221–227, 1987) that *A. bisporus* continues to degrade protein and secrete ammonium even if ammonium and glucose are present in the culture medium.

The edible fungus *Agaricus bisporus* has a great commercial value, and a considerable amount of research has been focused on optimization of large-scale cultivation of this mushroom crop. The crop is often, but not always, cultivated on a horse manure-based compost devoid of readily decomposable compounds. This compost, which is selective for *A. bisporus* contains only complex carbon and nitrogen sources. The ammonium content is very low, and since nitrate is not utilized as a nitrogen source by *A. bisporus* (2), nitrogen is mainly salvaged from a compost fraction known as the N-rich lignin-humus complex which partly consists of the microbial biomass which accumulates during the humification process (18) including symbiotic bacteria (14).

Basic studies on nitrogen metabolism have been focused mainly on ammonium assimilation and the characterization of enzymes primarily involved in this pathway (2, 3, 43). Little is known about the utilization of organic nitrogen sources. Protein can be utilized as the sole source of nitrogen and carbon (28); single amino acids, however, cannot support growth without an additional carbon source (2). Proteinase activity is not repressed by ammonium in the presence of glucose as carbon source (28, 29), but the catabolism of the peptides and amino acids generated by these proteinases is not well known. The fortuitous cloning of a partial cDNA encoding a γ -glutamyl semialdehyde dehydrogenase, better known as Δ^1 -pyrroline-5-carboxylate dehydrogenase (P5C dehydrogenase), gave us the opportunity to monitor proline degradation in *A. bisporus* at the molecular level.

MATERIALS AND METHODS

Fungal strains and culture conditions, bacterial strains, DNA techniques, and chemicals. *A. 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, the strains were cultured on DT80 medium supplemented with glucose as carbon source (45).

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

Plasmid pUC19 (50) was used as vector for (sub)cloning and amplification of obtained DNA fragments. Standard DNA manipulations were carried out essentially as described by Sambrook et al. (41). Restriction enzymes and other enzymes used for DNA manipulations were purchased from BRL and used according to the supplier's instructions. DNA sequences were determined manually with a T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden) or by using an ALFexpress DNA sequencer (Pharmacia Biotech) in combination with a Cy5 AutoRead sequencing kit (Pharmacia Biotech). Synthetic deoxyoligonucleotides used for sequencing were also purchased from Pharmacia Biotech. Probes were labelled by random priming using [α -³²P]dATP (13).

Cloning of the P5C dehydrogenase-encoding gene. A 3'-truncated *pruA* encoding cDNA was isolated from a cDNA library constructed from primordia and small fruit bodies from Horst U1 (11) and identified by sequence analysis in conjunction with a database search using the program Blast (19). This truncated cDNA was subsequently used as a probe for the screening of a λ EMBL4 genomic library of *A. bisporus* H39 using conditions described before (43). Positive plaques were purified and λ DNA was isolated by standard techniques. Hybridizing fragments were cloned and analyzed. An apparent full-length cDNA was obtained by screening the cDNA library with the truncated cDNA as probe.

Isolation of total genomic DNA and Southern blot analysis. Total DNA from fungal strains was isolated according to the method of De Graaff et al. (10), digested with various restriction enzymes, separated on 0.8% agarose gels and transferred onto a Hybond-N hybridization transfer membrane (Amersham International plc., Amersham, United Kingdom). Preparation of *A. bisporus* chromosomal DNA and contour-clamped homogeneous electric field (CHEF) electrophoresis of intact chromosomes were performed as described elsewhere (46) with a Bio-Rad CHEF DrII system (Bio-Rad Laboratories Inc., Richmond, Calif.). The membranes were hybridized overnight in standard hybridization buffer (SHB; 6 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μ g of herring sperm DNA per ml) (1 $\times$ SSC is 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0) at 65°C with a genomic 5.5-kb *Pst*I fragment of *pruA* as a probe and washed at 65°C with a final buffer of 0.5 $\times$ SSC and 0.1% SDS.

Preparation of cell extracts and assay of enzyme activities. Freshly harvested fruit bodies were used for the isolation of mitochondria as described in reference 12 with modifications (15). The content of the isolated mitochondria was released by an osmotic shock by resuspending the mitochondria in 0.1 mM Tris, pH 8.5. DL- Δ^1 -pyrroline-5-carboxylic acid was purchased from Sigma (St. Louis, Mo.) as 2,4-dinitrophenylhydrazine hydrochloride double salt and purified as described elsewhere (38). P5C dehydrogenase activity was measured (27) in 70 mM Tris (pH 8.5)–3 mM NAD⁺–0.6 mM DL-P5C. Glucose-6-phosphate dehydrogenase (G6PD) and citrate synthase (CS) enzyme activities were determined in 50 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 7.5)–5 mM MgCl₂–

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FIG. 4. Northern blot analysis of *pruD* and *gdhA* mycelial transcripts. Total RNA was isolated from *A. bisporus* Horst U1 mycelium grown on proline (Pro), ammonium phosphate (Am), or glutamate (Glu) as sole nitrogen source. To the proline- and glutamate-utilizing cultures, 20 mM ammonium phosphate, pH 6.8, was added and expression of *pruD* and *gdhA* was monitored over time. Pro and Glu lanes 1, total RNA isolated before addition of ammonium phosphate; Pro and Glu lanes 2 to 5, total RNA isolated 2, 4, 8, and 24 h after addition of ammonium phosphate. The blots were rehybridized with a 28S rDNA fragment to provide for a loading control.

peel. In contrast, expression was not detected in gills and in mycelium grown on sterilized compost. Another, otherwise identical membrane was hybridized with a partial cDNA specifying translation elongation factor 1 α . Equal expression of this gene was found in all assayed parts of a fruit body, and the levels were comparable to those in mycelium (Fig. 3), indicating good growth of *A. bisporus* on sterilized compost. Cap, peel, stem, and gill tissue of mature fruit bodies and extracts from mycelium grown on compost plates were assayed for P5C dehydrogenase enzyme activity. The activities that were found were in agreement with the results obtained by Northern analysis. No P5C dehydrogenase activity was found in gill tissue and compost grown mycelium, while activities in cap and peel tissue were 28 and 25 nmol mg of protein⁻¹ min⁻¹, respectively. The P5C dehydrogenase activity measured in stem tissue was 13 nmol mg of protein⁻¹ min⁻¹, which is also in accordance with the lower *pruD* mRNA levels observed in that tissue if the mRNA levels of the upper and lower stem are averaged.

The steady-state *pruD* expression in vegetative mycelium was measured by Northern analysis in buffered liquid cultures with 20 mM proline, 20 mM glutamate, or 20 mM ammonium as phosphate salt as sole nitrogen source and 100 mM glucose as carbon source. In addition, 20 mM ammonium phosphate was added to proline- and glutamate-utilizing cultures, and *pruD* mRNA levels in these cultures were followed for 24 h. We also followed the response of the mycelial mats to addition of ammonium phosphate by hybridizing the same blots with a *gdhA* probe encoding NADP⁺-dependent glutamate dehydrogenase (43). The *pruD* probe again hybridized with a transcript of about 1,700 nt. The results also clearly indicated that the steady-state concentration of the *pruD* transcript was only moderately affected by the nitrogen source involved (Fig. 4). The levels of *pruD* in mycelium with proline and ammonium as sole nitrogen source were similar, and no significant ef-

fects were seen after addition of 20 mM ammonium phosphate to proline-utilizing cultures. Densitometric scanning of the Northern blots revealed a two- to threefold-lower steady-state *pruD* mRNA level in glutamate-utilizing cultures, but this reduced expression level was not significantly changed by ammonium addition. The Northern blots, however, do show an ammonium-induced, 10-fold decrease of *gdhA* steady-state mRNA levels, indicating that the mycelial mats can sense the added ammonium quickly and respond to it (Fig. 4). The proline- and ammonium-utilizing cultures were also assayed for P5C dehydrogenase activity. The activities were 24 and 15 nmol mg of protein⁻¹ min⁻¹, respectively, indicating that growth on ammonium does not lead to a significant reduction of enzyme activity, and this is in agreement with the results obtained by Northern analysis.

DISCUSSION

Systematic breeding of the mushroom crop *A. bisporus* is hampered mainly by the secondarily homothallic life cycle of this fungus, which leads to a very low level of homokaryotic spores (40) and an apparent low frequency of recombination events (32). For an efficient introduction of commercially important traits from the now available diverse germplasm (31) to the present strains, a detailed genomic map is needed that links genomic markers to these traits. For the construction of a gene-based genomic map of the hybrid strain Horst U1 and to increase our knowledge of the molecular biology of *A. bisporus*, we have used a primordial cDNA λ library as a source of gene markers and simply characterized a number of cDNA clones by partial sequence determination in combination with a database search. One of these cDNAs was an apparent 3'-truncated cDNA specifying a protein with significant homology to Δ^1 -pyrroline-5-carboxylate dehydrogenase from the yeast *S. cerevisiae* and humans. The proline utilization pathway has been studied thoroughly in this yeast and in the filamentous fungus *A. nidulans* (reviewed in references 6 and 17). As we already knew that other aspects of nitrogen metabolism were different in *A. bisporus* (3, 29, 43), this was a good opportunity to compare at least one step of the proline utilization pathway with the corresponding one in ascomycetes. The complete gene was therefore cloned from a homokaryotic strain. The amino acid sequences of the *A. bisporus* and the yeast and human P5C dehydrogenases showed a high level of identity with 50 and 46% identical amino acids, respectively. However, no N-terminal mitochondrial import signal was found in the basidiomycetous gene, and this was confirmed by the findings that P5C dehydrogenase was found exclusively in the cytosolic fraction. The respiratory competence of the isolated mitochondrial fraction (12, 15) and the complete absence of citrate synthase in the cytosolic fraction indicated that intact mitochondria were isolated.

P5C is not only a substrate for P5C dehydrogenase but also an intermediate in NADPH-dependent cytosolic proline biosynthesis mediated by P5C reductase. P5C reductases are entirely different enzymes that share no significant sequence identity or similarities with P5C dehydrogenases (9). Therefore, it has been suggested that the separation of catabolic and biosynthetic enzymes in yeast prevents futile cycling (7). The *A. nidulans* P5C dehydrogenase enzyme, however, appears to be cytosolic (1) and futile cycling can be reduced considerably by strict control of gene expression, as is the case for the structural genes of the proline utilization pathways of *S. cerevisiae* and *A. nidulans*. They are induced by the product of the *PUT3* gene (5) and the *prnA* gene (23), respectively, which are activated in some way by an increased intracellular proline concentration.

The characterized general and specific proline transporters of yeast (25, 26) and *A. nidulans* (48) are regulated by the nitrogen source such that proline cannot be imported in the presence of a repressing nitrogen source like ammonium and regulation of the structural genes via inducer exclusion can thus be envisaged. The yeast and *A. nidulans* structural genes are, however, in addition nitrogen repressed by the *GLN3* (8) and *areA* (36) gene products, respectively, and nitrogen repression by itself accounts for a fourfold decrease of expression of the yeast *PUT2* gene (6, 8).

For presently unclear reasons, tissue-specific control of *pruA* expression is seen between gills and other parts of a fruit body of *A. bisporus*. In addition, no detectable *pruA* expression and enzyme activity was found in mycelium grown on sterilized compost, which by its nature is probably devoid of simple inducers for the system. On the other hand, it is also possible that compost contains repressors for the system. Northern analyses of total RNA from *A. bisporus* mycelium utilizing various simple nitrogen sources did show *pruA* expression. However, no ammonium-induced nitrogen metabolite repression of the steady-state mRNA level of *pruA* was observed. Enzyme activities were also not significantly reduced in ammonium-utilizing cultures, whereas the same RNA samples did show a 10-fold-decreased *gdhA* steady-state mRNA level in the presence of ammonium, which also appeared to be quickly sensed by the organism since the reduced *gdhA* steady-state mRNA level was already obtained 2 h after an ammonium shift.

Kalisz et al. (29) found that added protein is degraded and that concomitantly ammonium is secreted by *A. bisporus* even in the presence of ammonium and glucose. We also observed a significant ammonium increase in the medium if casein or albumin was used as sole carbon and nitrogen source (33). The simplest explanation is that this increase results from uptake and subsequent degradation of amino acids by *A. bisporus* which appears to be independent from the presence of ammonium in the medium. This suggests that regulation through inducer exclusion does not work in this system and suggests that at least some of the imported amino acids and peptides are also used and might even be preferred as carbon source.

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REFERENCES

- Arst, H. N., Jr., S. A. Jones, and C. R. Bailey. 1981. A method for selection of deletion mutations in the L-proline catabolism gene cluster of *Aspergillus nidulans*. *Genet. Res. Camb.* **38**:171–195.
- Baars, J. J. P., H. J. M. Op den Camp, J. M. H. Hermans, V. Mikes, C. Van der Drift, L. J. L. D. Van Griensven, and G. D. Vogels. 1994. Nitrogen assimilating enzymes in the white button mushroom *Agaricus bisporus*. *Microbiology* **140**:1161–1168.
- Baars, J. J. P., H. J. M. Op den Camp, A. H. A. M. Van Hoek, C. Van der Drift, L. J. L. D. Van Griensven, J. Visser, and G. D. Vogels. 1995. Purification and characterization of NADP-dependent glutamate dehydrogenase from the commercial mushroom *Agaricus bisporus*. *Curr. Microbiol.* **30**:211–217.
- Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70**:241–250.
- Brandriss, M. C. 1987. Evidence for positive regulation of proline utilization in *Saccharomyces cerevisiae*. *Genetics* **117**:429–435.
- Brandriss, M. C., D. A. Falvey, A. G. des Etages, and S. Xu. 1995. The roles of PUT3, URE2 and GLN3 regulatory proteins in the proline utilization pathway of *Saccharomyces cerevisiae*. *Can. J. Bot.* **73**(Suppl. 1):S153–S159.
- Brandriss, M. C., and B. Magasanik. 1981. Subcellular compartmentation in the control of converging pathways for proline and arginine metabolism in *Saccharomyces cerevisiae*. *J. Bacteriol.* **143**:1359–1364.
- Daugherty, J. R., R. Rai, H. M. El Berry, and T. G. Cooper. 1993. Regulatory circuit for responses of nitrogen catabolic gene expression to the GLN3 and DAL80 proteins and nitrogen catabolite repression in *Saccharomyces cerevisiae*. *J. Bacteriol.* **175**:64–73.
- Davis, C. R., M. A. McPeck, and C. Robertson McClung. 1995. Molecular characterization of the proline-1 (pro-1) locus of *Neurospora crassa*, which encodes Δ^1 -pyrroline-5-carboxylate reductase. *Mol. Gen. Genet.* **248**:341–350.
- De Graaff, L. H., H. Van den Broek, and J. Visser. 1988. Isolation and expression of the *Aspergillus nidulans* pyruvate kinase gene. *Curr. Genet.* **11**:499–503.
- De Groot, P. W. J., P. J. Schaap, A. S. M. Sonnenberg, J. Visser, and L. J. L. D. Van Griensven. 1996. The *Agaricus bisporus* *hypA* gene encodes a hydrophobin and specifically accumulates in peel tissue of mushroom caps during fruit body development. *J. Mol. Biol.* **257**:1008–1018.
- Derikx, P. J. I., H. J. M. Op den Camp, A. M. Wagner, G. Straatsma, L. J. L. D. Van Griensven, and G. D. Vogels. 1990. Respiratory pathways in *Agaricus bisporus* and *Scytalidium thermophilum*. *FEMS Microbiol. Lett.* **66**:307–312.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal. Biochem.* **132**:6–13.
- Fermor, T. R., and D. A. Wood. 1981. Degradation of bacteria by *Agaricus bisporus* and other fungi. *J. Gen. Microbiol.* **126**:377–387.
- Gallinet, J. P., J. Girardot, P. Masson, and C. Lance. 1993. A method for the assessment of the quality of mitochondrial preparations. *J. Theor. Biol.* **161**:431–460.
- Gavel, Y., and G. von Heine. 1990. Cleavage-site motifs in mitochondrial targeting peptides. *Protein Eng.* **4**:33–37.
- Gavrias, V., B. Cubero, B. Cazelle, V. Sophianopoulou, and C. Scazzocchio. 1994. The proline utilisation gene cluster of *Aspergillus nidulans*, p. 225–232. In K. A. Powell, A. Renwick, and J. F. Peberdy (ed.), *The genus Aspergillus*. Plenum Press, New York, N.Y.
- Gerrits, J. P. G. 1988. Nutrition and compost, p. 27–35. In L. J. L. D. Van Griensven (ed.), *The cultivation of mushrooms*. Darlington Mushroom Laboratories Ltd., Rustington, United Kingdom.
- Gish, W., and D. J. States. 1993. Identification of protein coding regions by database similarity search. *Nat. Genet.* **3**:266–272.
- Glaser, P., F. Kunst, M. Arnaud, M.-P. Coudart, W. Gonzalez, M.-F. Hullo, M. Ionescu, B. Lubochinsky, L. Marcelino, I. Moszer, E. Presecan, M. Santana, E. Schneider, J. Schweizer, A. Vertes, G. Rapoport, and A. Danchin. 1993. *Bacillus subtilis* genome project: cloning and sequencing of the 97 kb region from 325° to 333°. *Mol. Microbiol.* **10**:371–384.
- Hempel, J., H. Nicholas, and R. Lindahl. 1993. Aldehyde dehydrogenases: widespread structural and functional diversity within a shared framework. *Protein Sci.* **2**:1890–1900.
- Hu, C. A., W.-W. Lin, and D. Valle. 1996. Cloning, characterization and expression of cDNAs encoding human Δ^1 -pyrroline-5-carboxylate dehydrogenase. *J. Biol. Chem.* **271**:9795–9800.
- Hull, E. P., P. M. Green, H. N. Arst, and C. Scazzocchio. 1989. Cloning and physical characterization of the L-proline catabolism gene cluster of *Aspergillus nidulans*. *Mol. Microbiol.* **3**:553–559.
- Humphrey, T., and N. J. Proudfoot. 1988. A beginning to the biochemistry of polyadenylation. *Trends Genet.* **4**:243–245.
- Jauniaux, J.-C., and M. Grenson. 1990. *GAP1*, the general acid permease gene of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **190**:39–44.
- Jauniaux, J.-C., M. Vandenbol, S. Vissers, K. Broman, and M. Grenson. 1987. Nitrogen catabolite regulation of proline permease in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **164**:601–606.
- Jones, S. A., H. N. Arst, Jr., and D. W. MacDonald. 1981. Gene roles in the *prn* cluster of *Aspergillus nidulans*. *Curr. Genet.* **3**:49–56.
- Kalisz, H. M., D. Moore, and D. A. Wood. 1986. Protein utilization by Basidiomycete fungi. *Trans. Br. Mycol. Soc.* **86**:519–525.
- Kalisz, H. M., D. A. Wood, and D. Moore. 1987. Production, regulation and release of extracellular proteinase activity in Basidiomycete fungi. *Trans. Br. Mycol. Soc.* **88**:221–227.
- Kaput, J., M. C. Brandriss, and T. Prussak-Wieckowsky. 1989. In vitro import of cytochrome c peroxidase into the intermembrane space: release of the processed form by intact mitochondria. *J. Cell Biol.* **109**:101–112.
- Kerrigan, R. W. 1991. What on earth is the *Agaricus* rescue program? *Mycologist* **5**:22.
- Kerrigan, R. W., J. C. Royer, L. M. Baller, Y. Kohli, P. A. Horgen, and J. B. Anderson. 1993. Meiotic behaviour and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. *Genetics* **133**:225–236.
- Kersten, M. A. S. H., and P. J. Schaap. Unpublished data.
- Kozak, M. 1989. The scanning model for translation: an update. *J. Cell Biol.* **108**:229–241.
- Krzywicki, K. A., and M. C. Brandriss. 1984. Primary structure of the nuclear PUT2 gene involved in the mitochondrial pathway for proline utilization in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4**:2837–2842.
- Kudla, B., M. X. Caddick, T. Langdon, N. C. Martinez-Rossi, C. F. Bennet, S. Sibley, R. W. Davies, and H. N. Arst, Jr. 1990. The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. *Mutations*

- affecting specificity of gene activation alter a loop residue in a specific zinc finger. *EMBO J.* **9**:1355–1364.
37. Ling, M., S. W. Allen, and J. M. Wood. 1994 Sequence analysis identifies the proline dehydrogenase and Δ^1 -pyrroline-5-carboxylate dehydrogenase domains of the multifunctional *Escherichia coli* PutA protein. *J. Mol. Biol.* **243**:950–956.
38. Mezl, V. A., and W. E. Knox. 1976. Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. *Anal. Biochem.* **74**:430–440.
39. Nussinov, R. 1990. Sequence signals in eukaryotic upstream elements. *Crit. Rev. Biochem. Mol. Biol.* **25**:185–221.
40. Raper, C. A., J. R. Raper, and R. E. Miller. 1972. Genetic analysis of the life-cycle of *Agaricus bisporus*. *Mycologia* **64**:1088–1117.
41. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. Schaap, P. J., Y. Müller, P. W. J. De Groot, J. Visser, J. J. P. Baars, A. S. M. Sonnenberg, and L. J. L. D. Van Griensven. 1995. Molecular characterization of genes involved in carbon metabolism and ammonium assimilation of the button mushroom *Agaricus bisporus*, p. 103–109. In T. J. Elliott (ed.), Science and cultivation of edible fungi. Balkema, Rotterdam, The Netherlands.
43. Schaap, P. J., Y. Müller, J. J. P. Baars, H. J. M. Op den Camp, A. S. M. Sonnenberg, L. J. L. D. Van Griensven, and J. Visser. 1996. Nucleotide sequence and expression of the gene encoding NADP⁺-dependent glutamate dehydrogenase (*gdhA*) from *Agaricus bisporus*. *Mol. Gen. Genet.* **250**:339–347.
44. Schaap, P. J., Y. Müller, and J. Visser. 1996. EMBL accession no. X97204.
45. Sonnenberg, A. S. M., J. G. H. Wessels, and L. J. L. D. Van Griensven. 1988. An efficient protoplasting/regeneration system for *Agaricus bisporus* and *Agaricus bitorquis*. *Curr. Microbiol.* **17**:285–291.
46. Sonnenberg, A. S. M., K. Den Hollander, A. P. J. Van de Munckhof, and L. J. L. D. Van Griensven. 1991. Chromosome separation and assignment of DNA probes in *Agaricus bisporus*, p. 57–61. In L. J. L. D. Van Griensven (ed.), Genetics and breeding of *Agaricus*. Pudoc, Wageningen, The Netherlands.
47. Sonnenberg, A. S. M., P. W. J. de Groot, P. J. Schaap, J. J. P. Baars, J. Visser, and L. J. L. D. Van Griensven. 1996. Isolation of expressed sequence tags of *Agaricus bisporus* and their assignment to chromosomes. *Appl. Environ. Microbiol.* **62**:4542–4547.
48. Sophianopoulou, V., and C. Scazzocchio. 1989. The proline transport protein of *Aspergillus nidulans* is very similar to amino acid transporters of *Saccharomyces cerevisiae*. *Mol. Microbiol.* **3**:705–714.
49. Umar, M. H., and L. J. L. D. Van Griensven. 1995. Morphology of *Agaricus bisporus* in health and disease, p. 563–570. In T. J. Elliott (ed.), Science and cultivation of edible fungi. Balkema, Rotterdam, The Netherlands.
50. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved phage M13 cloning vectors and host strains; nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**:103–109.