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Transformation by Complementation of an Adenine Auxotroph of the Lignin-Degrading Basidiomycete *Phanerochaete chrysosporium*

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Swollen basidiospores of an adenine auxotroph of *Phanerochaete chrysosporium* were protoplasted with Novozyme 234 and transformed to prototrophy by using a plasmid containing the gene for an adenine biosynthetic enzyme from *Schizophyllum commune*. Transformation frequencies of 100 transformants per μg of DNA were obtained. Southern blot analysis of DNA extracted from transformants demonstrated that plasmid DNA was integrated into the chromosomal DNA in multiple tandem copies. Analysis of conidia and basidiospores from transformants demonstrated that the transforming character was mitotically and meiotically stable on both selective and nonselective media. Genetic crosses between double mutants transformed for adenine prototrophy and other auxotrophic strains yielded Ade$^-$ progeny, which indicated that integration occurred at a site(s) other than the resident adenine biosynthetic gene.

The white-rot basidiomycete *Phanerochaete chrysosporium* has been the focus of numerous studies on lignin degradation (6, 24) and cellulose metabolism (11). The purification and characterization of two *P. chrysosporium* extracellular enzymes involved in lignin degradation, lignin peroxidase (20, 25, 41) and manganese peroxidase (14, 15, 31), and elucidation of their catalytic mechanisms (22, 36) have contributed enormously to our understanding of the biochemistry of this process. Sequences for cDNA clones (7, 42) and a genomic clone (39) of lignin peroxidase have recently been published, and a manganese peroxidase cDNA has also been cloned and sequenced in this laboratory (D. Pribrow, M. B. Mayfield, V. J. Nipper, J. A. Brown, and M. H. Gold, J. Biol. Chem., in press). Studies on the regulation and expression of these genes and genetic approaches to structure-function studies of the enzymes would be greatly facilitated by the development of an efficient transformation system for *P. chrysosporium*. In addition, transformation of this fungus would be a major step toward use of this organism for industrial bioprocessing.

Previous genetic studies have included the isolation of auxotrophic marker strains of *P. chrysosporium* (19) and their use in genetic recombination experiments (2) as well as studies indicating that at least some strains of this organism appear to have a primary homothallic mating system (i.e., are self-fertile), producing binucleate homokaryotic basidiospores, and that some wild-type strains of *P. chrysosporium* are heterokaryotic, containing more than one distinct genome (3, 34). We also reported the preparation, fusion, and regeneration of mycelial protoplasts of *P. chrysosporium* (18). In this report, we describe the successful transformation of protoplasted basidiospores of *P. chrysosporium* via complementation of an adenine auxotrophic strain with an adenine biosynthetic gene from the basidiomycete *Schizophyllum commune*.

MATERIALS AND METHODS

Strains. Auxotrophic strains of *P. chrysosporium* OGC107-1 (Ade1), OGC128-2 (Ade2), OGC923-5 (Ade4), OGC128-12 (Nic1), OGC103-1 (Rib1), and OGC923-4 (*leu2*) were obtained as previously described (19). The *leu2* auxotroph deficient in β-isopropylmalate dehydrogenase (28) was crossed with a *ura5* mutant deficient in orotidylic acid pyrophosphorylase (unpublished results) as previously described (3), with subsequent screening for a leucine auxotroph which produced abundant basidiospores. Ade2 *leu2* double mutants were then obtained by crossing the single auxotrophs. Auxotrophic mutants were maintained on slants as previously described (2) and were induced to fruit on one-sixth-strength modified Vogel medium (38) containing 4.5% Walseth cellulose with the appropriate nutritional supplement(s) as previously described (3, 17).

Transforming DNA. Plasmids containing two different adenine biosynthetic genes from *S. commune*, pADE2 and pADE5 (13), were generously provided by R. Ullrich, University of Vermont. These genes were isolated by complementation of *S. commune* adenine auxotrophs by using an *S. commune* genomic library cloned into the *Bam*HI site of plasmid pRK9 (30) and subsequently recovered in *Escherichia coli* (13). Plasmid DNA was prepared by the alkaline lysis method (26) and purified via ethidium bromide-CsCl equilibrium gradient centrifugation.

Chemicals. CsCl was obtained from Var Lac Oid Chemical Co., Bergenfield, N.J. Novozyme 234 was obtained from Novo Industries, Bagsvaerd, Denmark; Cellulase CP was obtained from Sturge Enzymes, Birmingham, United Kingdom; and Cellulase TV was obtained from Miles Laboratories, Inc., Elkhart, Ind. Polyethylene glycol was obtained from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc., Rockville, Md. [^{32}P]dCTP was obtained from Dupont, NEN Research Products, Boston, Mass.

Preparation of protoplasts. Protoplasts were prepared from basidiospores by a modification of the procedure developed for *S. commune* (13, 29). The spores were washed from the lids of fruiting plates in 1 to 2 ml of modified Vogel medium containing 3% malt extract–0.15% yeast extract (MYV), pH 4.8, at concentrations of ~10^7 spores per ml. Spores were swollen at 36°C for 4 to 5 h on a rotary shaker (150 rpm). The suspension was then centrifuged at 400 × *g* for 15 min at room temperature, and the pellet was suspended in 1 ml of

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0.5 M $MgSO_4$–0.05 M maleic acid, pH 5.9 (MgOsm). Novozyme 234 batch 1035 and Cellulase CP (10 mg each) were added, and the sample was incubated at 38°C for 4 h on a rotary shaker (100 rpm). Substitution of Cellulase TV for Cellulase CP had no apparent effect on protoplast formation, regeneration, or transformation frequency. The protoplast suspension was centrifuged for 5 min at 270 × *g* at room temperature to pellet spores and cell wall debris. The supernatant containing the protoplasts was then added to 2 ml of 1 M sorbitol–20 mM MOPS (morpholinepropanesulfonic acid), pH 6.3 (SorbOsm). The pellet was washed in 1 ml of MgOsm and recentrifuged, and the second supernatant was added to the preparation described above. The protoplasts in SorbOsm were pelleted by centrifugation for 15 min at 270 × *g* at room temperature. The pellet was washed with 3 ml of SorbOsm, recentrifuged as described above, and suspended in SorbOsm plus 0.04 M $CaCl_2$, pH 6.3. At this stage, protoplasts were routinely stored at 4°C overnight.

Transformation of protoplasts. DNA (1 μg in 60 μl of 10 mM Tris–1 mM EDTA [TE] plus 0.04 M $CaCl_2$, pH 8) was added to ~2 × 10^6 protoplasts in 100 μl of SorbOsm plus $CaCl_2$, mixed gently, and incubated on ice for 10 min. Control protoplasts were treated with 60 μl of TE plus $CaCl_2$. Samples were underlaid with 160 μl of 44% polyethylene glycol 3350 in 10 mM MES (morpholineethanesulfonic acid), pH 6.75, incubated on ice for 10 min, mixed gently, and incubated for an additional 10 min. Protoplasts were diluted to a concentration of 2.5 × 10^5 to 5.0 × 10^5/ml in asparagine-glucose-salts minimal medium (MM) (35) plus 0.5 M $MgSO_4$, pH 4.8, and 0.1 ml was added to each top agar tube containing 4 ml of MM, 0.4 M $MgSO_4$, and 1% agar at 48°C. The top agar was poured over plates containing MM, 0.5 M $MgSO_4$, and 1.5% agar. Serial dilutions of protoplasts were plated as described above in medium containing 0.001% adenine to determine protoplast regeneration. Plates were incubated at 37°C. Colonies were visible on supplemented plates in 2 to 3 days, whereas transformant colonies first appeared on about day 5 and additional colonies arose over the following 2 weeks.

Analysis of DNA from transformants. Spore suspensions of putative transformants, as well as the wild-type strain OGC101 (3) and the Ade2 auxotrophic strain, were used to inoculate 100 ml of modified Vogel medium containing 2% glucose, 0.04% yeast extract, and 0.001% hypoxanthine for adenine auxotrophs. Cultures were incubated for 24 h at 37°C with shaking (150 rpm). Cells were harvested and the DNA was extracted according to a published procedure (33) except that the RNase A treatment (35 μl at 20 mg/ml) was for 30 min at 37°C. Restriction endonucleases were used according to the recommendations of the manufacturer; gel electrophoresis and Southern transfers to Bio Trace RP nylon filters (Gelman Sciences, Inc., Ann Arbor, Mich.) were done by standard methods (40). Random priming of plasmid DNA with [^{32}P]dCTP (12), Southern and slot blot hybridizations, and autoradiography were performed by standard procedures (26).

Genetic analysis of transformants. Transformants were tested for mitotic stability by plating the conidia on supplemented media and testing the resultant colonies for adenine prototrophy as previously described (19). Transformants were tested for meiotic stability by fruiting on minimal medium, plating basidiospores on supplemented medium, and testing the resultant colonies for adenine prototrophy as previously described (3). To test for the presence of the Ade$^-$ allele in transformants, Ade2 *leu2* double auxotrophs and Ade2 *leu2* strains transformed for adenine prototrophy were crossed with Nic1 and Rib1 auxotrophs. Colonies derived from the resultant basidiospores were picked from triple-supplemented plates to triple-supplemented tubes, and the progeny phenotypes were subsequently determined as previously described (3).

RESULTS

Protoplasting and regeneration of swollen basidiospores. Optimal swelling (approximately 70%) of *P. chrysosporium* basidiospores was obtained in 4 to 5 h at 36°C with concentrations of 10^7 spores per ml in MYV, pH 4.8. Temperatures higher than 36°C resulted in a large decrease in the percentage of swollen spores. Swollen spores could be stored at 4°C for up to 36 h with no loss of viability or protoplasting efficiency. Decreasing the incubation time for protoplasting of swollen basidiospores resulted in a significant decrease in the number of protoplasts. Protoplasts plated on medium supplemented with adenine regenerated at a frequency of ~5%, comparable to the regeneration frequencies obtained with *P. chrysosporium* mycelial protoplasts (18). The use of sorbitol in place of $MgSO_4$ as an osmotic stabilizer in the regeneration medium significantly decreased protoplast regeneration.

Transformation by complementation. Three adenine-requiring auxotrophs of *P. chrysosporium* from different complementation groups (19) were fruited, and the resultant basidiospores were swollen and protoplasted. Attempts were made to transform each of these strains with each of two plasmids containing adenine biosynthetic genes from *S. commune*. Putative transformants were obtained from only one of these experiments: protoplasts of *P. chrysosporium* Ade2, when treated with pADE2, gave rise to colonies when regenerated on minimal medium. Colonies of putative transformants were picked to minimal slants containing modified Vogel medium with 1% glucose to test for prototrophy. Colonies did not arise from control protoplasts that did not receive DNA or from Ade2 protoplasts treated with pRK9 DNA alone or with plasmid pADE5.

Maximum transformation frequency was 100 transformants per μg of pADE2. On the average, approximately 0.04% of viable protoplasts were transformed, with a frequency of 40 transformants per μg. Optimal transformation frequencies were obtained by using approximately 2 × 10^6 protoplasts per μg of DNA. No increase in the frequency of transformation per microgram of DNA was observed with increased amounts of either DNA or protoplasts. However, pADE2 linearized with *Eco*RI resulted in a higher frequency of transformation than did concatemers of circular plasmid. Use of Novozyme 234 alone appeared to decrease the frequency of transformation while not significantly affecting the degree of either protoplast formation or regeneration. Regenerating the protoplasts at 33°C rather than 37°C resulted in slower regeneration of transformants and untransformed controls without affecting the frequency of either. Neither the addition of 1 μg of carrier plasmid DNA (pRK9) nor an increase in the transformation incubation time from 10 to 30 min had a significant effect on transformation frequency.

Analysis of DNA from transformants. Southern blots of DNA extracted from putative transformants were hybridized with ^{32}P-labeled pADE2 (Fig. 1) or pRK9 DNA (Fig. 2). The absence of a rapidly migrating band in samples of uncut transformant DNA suggested that the transforming plasmid was integrated into the chromosome rather than replicated

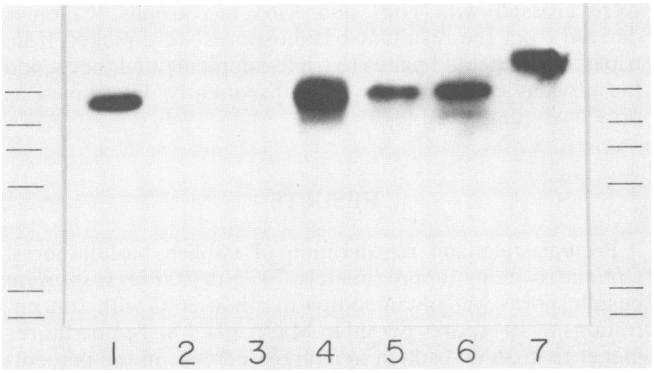

FIG. 1. Southern analysis of DNA from three *P. chrysosporium* transformants, the nontransformed Ade2 auxotrophic strain and the wild-type strain. Samples of total DNA were digested with *Hin*dIII and fractionated by electrophoresis in 0.7% agarose. The blot was hybridized with radiolabeled pADE2 DNA. Lanes: 1, 214 pg of plasmid pADE2 restricted with *Hin*dIII plus 500 ng of DNA from the Ade2 auxotroph; 2 through 6, DNA (500 ng) from the Ade2 auxotroph, wild type, T2, T6, and T8, respectively; 7, 500 ng of undigested DNA from T8. Bars indicate positions of molecular weight standards: from top to bottom, 23, 9.4, 6.6, 4.4, 2.3, and 2.0 kilobases.

as an autonomous plasmid. DNA extracted from *P. chrysosporium* wild-type strain OGC101 or the Ade2 strain did not hybridize either with ^{32}P-labeled pADE2 (Fig. 1) or with ^{32}P-labeled pRK9 (data not shown), even under low-stringency conditions.

In the Southern blot in Fig. 1, approximately two copies per genome of pADE2 were added to 500 ng of DNA extracted from the Ade2 strain. The genome size of *P. chrysosporium* has been determined to be ~4.4×10^7 base pairs (32). In general, the bands obtained with 500 ng of transformant DNA were stronger than that in the pADE2 lane, suggesting the presence of more than two plasmid copies in an equivalent amount of transformant DNA. Similar results were obtained with slot blot hybridizations (data not shown).

*Hin*dIII was used to linearize pADE2 for the Southern blot shown in Fig. 1. *Hin*dIII digests of DNA extracted from various transformants gave one strong band at about 14 kilobases and very faint, slightly smaller bands when probed with ^{32}P-labeled pADE2 (Fig. 1) or pRK9 (data not shown). This result suggests that multiple integration into the *P. chrysosporium* genome is due to tandem duplications or insertions rather than multiple insertions at different sites. The single strong band presumably represents identical multiple copies of the same size as the linearized plasmid, with the faint lower bands corresponding to the two ends of the array. Likewise, the patterns obtained with transformant 2 (T2) DNA digested with various enzymes were similar to those obtained for pADE2 digested with the same enzymes, with the major band from the transformant DNA corresponding to the major plasmid band (Fig. 2).

Mitotic and meiotic stability of transformants. *P. chrysosporium* basidiospores are binucleate, and this organism readily forms heterokaryons (3). In addition, the DNA-treated protoplasts were plated in high concentrations on a medium that was not colony inducing (16), resulting in a faint background regeneration of nontransformed protoplasts. For these reasons, it was found that transformants often contained untransformed nuclei in addition to prototrophic nuclei. Purification of transformants was accomplished by

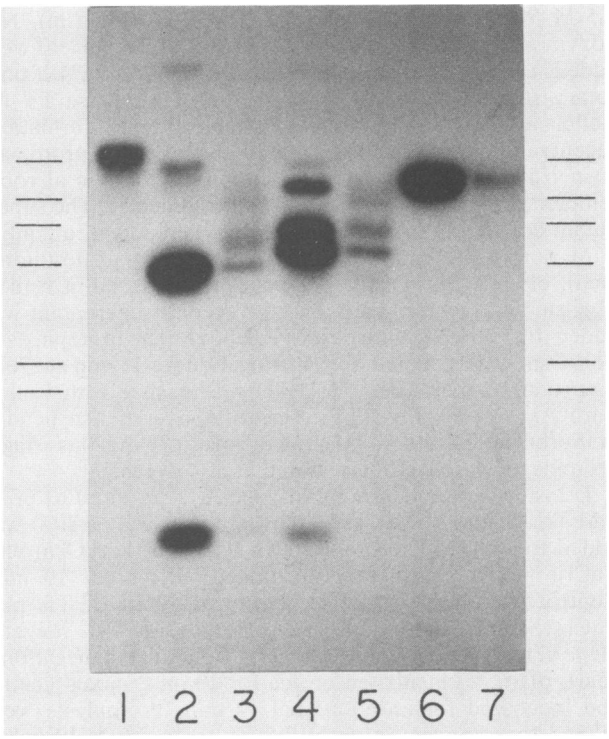

FIG. 2. Southern analysis of DNA from *P. chrysosporium* transformant T2. T2 and pADE2 DNA was digested with various restriction enzymes and fractionated by electrophoresis in 0.7% agarose. The blot was hybridized with radiolabeled pRK9 DNA, and 500 ng of transformant DNA or 10 ng of pADE2 was added to each lane. Lanes: 1, undigested T2; 2 and 3, pADE2 and T2, respectively, digested with *Pst*I; 4 and 5, pADE2 and T2, respectively, digested with *Sal*I; 6 and 7, pADE2 and T2, respectively, digested with *Eco*RI. Bars indicate positions of molecular weight standards: from top to bottom, 23, 9.4, 6.6, 4.4, 2.3, and 2.0 kilobases.

fruiting and selecting prototrophic colonies that arose from single homokaryotic basidiospores (3). Conidia and basidiospores arising from such colonies were all prototrophic, indicating the mitotic and meiotic stability of the transforming character.

Many of the Ade2 *leu2* double auxotrophs transformed to adenine prototrophy did not require purification, probably because of the very low background regeneration on minimal medium observed for double auxotrophs. Basidiospore colonies (27 for T7, 32 for T8, and 34 for T11) and 12 conidial colonies were tested for each of the three Ade$^+$ *leu2* transformants used in the genetic crosses (Table 1). In each case, the transformants proved to be mitotically and meiotically stable.

Genetic analysis of transformants. Ade$^+$ *leu2* transformants were crossed with Rib1 and Nic1 auxotrophs. All such crosses yielded some adenine auxotrophs (Table 1), which suggested that integration of the transforming gene into the recipient genome did not occur homologously with the resultant replacement of the defective adenine biosynthetic gene. Transformants crossed with the Rib1 auxotroph yielded approximately 60% as many Ade$^-$ progeny as did the cross between Rib1 and the untransformed parent, whereas crosses between T8 and T11 and Nic1 yielded 38 and 24% as many Ade$^-$ progeny, respectively, as did the parent cross (Table 1). Crosses between Ade$^+$ *leu2* transformants and arginine and histidine auxotrophs likewise produced some Ade$^-$ progeny (data not shown).

TABLE 1. Genetic recombination of transformed and nontransformed auxotrophs of *P. chrysosporium*[a]

Cross	Progeny							
	Prototrophs	Ade$^-$	Leu$^-$	Rib$^-$ or Nic$^-$	Ade$^-$/Leu$^-$	Ade$^-$/Rib$^-$ or Ade$^-$/Nic$^-$	Leu$^-$/Rib$^-$ or Leu$^-$/Nic$^-$	Ade$^-$/Leu$^-$/Rib$^-$ or Ade$^-$/Leu$^-$/Nic$^-$
Ade2 *leu2* × Rib1	16	16	7	9	5	1	2	2
T7 × Rib1	23	7	15	9	5	5	6	0
T8 × Rib1	9	6	15	1	2	0	2	1
Ade2 *leu2* × Nic1	4	6	2	7	12	3	2	5
T8 × Nic1	11	5	11	20	5	4	3	0
T11 × Nic1	10	1	23	14	2	4	4	2

[a] Crosses were performed and progeny phenotypes were determined as described in the text.

DISCUSSION

Transformation of protoplasts. During the past 2 years there have been reports on the transformation of the basidiomycetes *S. commune* (13, 29) and *Coprinus cinereus* (5, 27). We have adapted the system developed for the transformation of protoplasted basidiospores from *S. commune* (13, 29) and made use of a gene coding for an adenine biosynthetic enzyme in *S. commune* to transform a *P. chrysosporium* adenine auxotroph to prototrophy.

The procedure reported here yields a maximum transformation frequency of ~100 *P. chrysosporium* transformants per μg of pADE2. Although this is low compared with the frequencies now obtainable in other fungal systems (1, 13), it is comparable to those obtained in initial transformation experiments with *Aspergillus nidulans* (43), *Neurospora crassa* (8), and *S. commune* (29). Since pADE2 is a large plasmid (14.0 kilobases), the transformation frequency per microgram of DNA is somewhat misleading. It is also possible that use of a smaller plasmid would increase the frequency of transformation. In addition, since this initial transformation of *P. chrysosporium* used a heterologous gene and promoter, it is possible that subsequent experiments using *P. chrysosporium* DNA sequences will yield higher transformation frequencies.

Our finding that maximum transformation frequencies were obtained with 1.0 μg of DNA per ~2×10^6 protoplasts is comparable to results with *C. cinereus*, in which 3 μg of DNA per 10^7 to 10^8 protoplasts was found to be saturating, although no inhibition of transformation was observed with higher DNA concentrations (5). In that system, about 100 abortive transformants were obtained for every stable transformant, whereas we observed very few unstable transformants and no background of abortive transformants on selection plates, such as are sometimes found in other systems (44).

Analysis of DNA from transformants. The transforming DNA appears to be chromosomally integrated rather than replicated autonomously (Fig. 1 and 2), consistent with results obtained in other filamentous fungi (4, 5, 23, 29). In addition, multiple copies of the plasmid appear to be integrated (Fig. 1). The fact that only one strong band is observed in Southern blots where the DNA has been digested with an enzyme that cuts the plasmid once suggests that the multiple integration is due to tandem duplications or insertions rather than multiple insertions at different sites. Multiple plasmid copies incorporated randomly throughout the genome might be expected to give different-sized bands, corresponding to each randomly inserted copy. In *C. cinereus*, multiple insertions were observed in most of the transformants, and 42.5% of the transformants exhibited tandem duplications of the transforming DNA as well (27).

Genetic analysis of transformants. Although the transforming gene is both mitotically and meiotically stable, as seen by analysis of conidia and of basidiospores from self-crosses, some Ade$^-$ progeny are always obtained from crosses between transformants and other auxotrophic strains (Table 1). These results are indicative of plasmid integration at a site other than the resident Ade2 gene. Such heterologous integration is consistent with results obtained in *S. commune* (29). Heterologous integration of tandem multiple copies of transforming DNA has also been reported in *A. nidulans* (9). Our results do not preclude the possibility that plasmid insertion occasionally occurs at the homologous Ade2 locus. In *C. cinereus* transformed with a cloned tryptophan synthetase gene, insertion occurred at the resident *trp1* locus in ~4% of the transformants (5), although in transformations with the isocitrate lyase gene (*acu7*), no homologous integration of the whole plasmid was observed among the 40 transformants analyzed (27).

Analysis of genetic recombination data with *P. chrysosporium* is complicated by the fact that the organism is homothallic (3). In addition, clumping of basidiospores and low germination frequencies can influence recombination data (2). Nevertheless, the presence of a single randomly integrated copy of the *S. commune* adenine gene in a transformant might be expected to result in one-half as many recombinant Ade$^-$ progeny as in crosses with the untransformed double auxotroph. Thus, the low number of Ade$^-$ progeny in the cross between T11 and Nic1 may represent the integration of multiple copies of the transforming gene. Crosses of *C. cinereus* transformants yielded a preponderance of *acu*$^+$ progeny, presumably because of multiple copies of the transforming DNA (27).

Transforming adenine biosynthetic gene. The DNA used in these experiments complements only those adenine auxotrophs in a single complementation group in *S. commune* and in *P. chrysosporium*. This is strong evidence that it is a structural gene in the adenine biosynthetic pathway. No evidence for extragenic suppression of the Ade2 gene has been observed in *S. commune* (13), nor have we observed any *P. chrysosporium* Ade2 revertants. The biosynthetic pathway leading from phosphoribosyl pyrophosphate to AMP is a 12-step pathway, and many of the enzymes involved have not been well characterized in fungi (10, 21). Since all of our *P. chrysosporium* adenine auxotrophs grow on medium supplemented with hypoxanthine, they cannot be defective in the last two steps of the pathway. In *Saccharomyces cerevisiae* (21) as well as in bacteria (10), steps 8 and 12 (*ade13* in *S. cerevisiae* and *purB* in *E. coli*) are catalyzed by a single bifunctional enzyme, adenylosuccinate lyase. Since step 12 is in the pathway of hypoxanthine conversion to AMP, it is unlikely that Ade2 codes for this

enzyme. Furthermore, the *S. commune* Ade2 gene does not complement either of the pink ade *Schizophyllum* auxotrophs (R. Ullrich, personal communication), which are deficient in steps 6 and 7 of the pathway. Such mutants are visually identifiable due to a build-up of aminoimidazole ribotide, which polymerizes to a dark red pigment on exposure to the air (37). To our knowledge, no such *P. chrysosporium* mutants have been isolated. The Ade2 gene has not been further characterized in either *S. commune* or *P. chrysosporium*. Experiments are planned to determine the genotype of the *P. chrysosporium* Ade2 strain.

Transformation of *P. chrysosporium* with a biosynthetic gene from *S. commune* demonstrates that gene transfer between unrelated basidiomycetes is possible for at least some traits. Further experiments to test the extent of gene transfer between more distantly related filamentous fungi are planned. This transformation protocol should be applicable to other white-rot basidiomycetes which produce basidiospores in culture.

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