MATING SYSTEM AND DNA TRANSFORMATION OF THE LIGNIN-DEGRADING BASIDIOMYCETE *PHANEROCHAETE CHRYSOSPORIUM*

Margaret Mary Alic
B.A., Reed College, 1972

A dissertation submitted to the faculty of the

Oregon Graduate Institute
of
Science and Technology

in partial fulfillment of the
requirements for the degree
Doctor of Philosophy
in
Molecular Biology

February, 1990

The dissertation "Mating System and DNA Transformation of the Lignin-Degrading Basidiomycete Phanerochaete chrysosporium" by Margaret Mary Alic has been examined and approved by the following Examination Committee:

Michael H. Gold, Thesis Advisor
Professor

James M. Cregg
Associate Professor

David R. Corbin
Assistant Professor

Robert C. Ullrich
Professor, University of Vermont

ACKNOWLEDGMENTS

Many people have contributed to the work presented in this dissertation. Mary Mayfield and Therese Cheng were responsible for the isolation of most of the auxotrophic strains of Phanerochaete chrysosporium used in this study. Mary Mayfield patiently taught me general microbiological techniques and the particular idiosyncrasies of Phanerochaete, and I thank her for her support. Ted Molskness initiated the biochemical studies of the leucine auxotrophs presented in Chapter 3. Celia Letzring, as an American Heart Association (Oregon affiliate) Summer Research Fellow, assisted with the crosses described in Chapter 4. Donna Walker contributed her expertise to the scanning electron microscopy studies described in Chapter 4. Janet Kornegay, as an American Heart Association (Oregon affiliate) Summer Research Fellow, contributed to the initial development of the transformation system. Janet carried out many of the transformations described in Chapter 5, as well as some of the Neurospora transformations described in Chapter 6. With unfailing good humor and high standards of orderliness, Edie Clark applied her expert technical skills to the experiments described in Chapter 6. I am grateful to each of these individuals, and to all of my colleagues in the lignin degradation lab, for their friendship, assistance and support.

G. B. Kohlhaw, Purdue University, generously provided the β-isopropylmalate used in the leucine assays. R. C. Ullrich, University of Vermont, graciously provided S. commune plasmids and auxotrophic strains, and C. Yanofsky, Stanford University, supplied N. crassa plasmids.

I am very grateful to Bob Ullrich for the useful advice he has provided throughout the course of this research. I am also grateful to Charlie Specht, University of Vermont, Erik Selker, University of Oregon, and Patricia Pukkila, University of North Carolina, for helpful discussions and advice. I would like to thank the members of my thesis committee for their careful reading of this manuscript and Nancy Christie and Darcy Mills for their help in preparing the manuscript.

Finally, I would like to gratefully acknowledge the contributions made by my research advisor, Michael Gold, to the successful completion of this dissertation. Not only has this research benefited from his broadly-based knowledge and his insightful advice, but Mike personally counted every transformant obtained in the experiments in Chapters 5 and 6. I am deeply indebted to my advisor for his continuous concern for this research and for his unfailing support of my graduate education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PUBLISHED CHAPTERS

Chapter 2:

Alic, M., and M. H. Gold. 1985. Genetic recombination in the lignin-degrading basidiomycete Phanerochaete chrysosporium. Appl. Environ. Microbiol. 50:27-30.

Chapter 3:

Molskness, T. A., M. Alic, and M. H. Gold. 1986. Characterization of leucine auxotrophs of the white rot basidiomycete Phanerochaete chrysosporium. Appl. Environ. Microbiol. 51:1170-1173.

Chapter 4:

Alic, M., C. Letzring, and M. H. Gold. 1987. Mating system and basidiospore formation in the lignin-degrading basidiomycete Phanerochaete chrysosporium. Appl. Environ. Microbiol. 53:1464-1469.

Chapter 5:

Alic, M., J. R. Kornegay, D. Pribnow, and M. H. Gold. 1989. Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete Phanerochaete chrysosporium. Appl. Environ. Microbiol. 55:406-411.

Chapter 6:

Alic, M., E. K. Clark, J. R. Kornegay, and M. H. Gold. 1990. Transformation of Phanerochaete chrysosporium and Neurospora crassa with adenine biosynthetic genes from Schizophyllum commune. Curr. Genet. In press in a slightly abridged form.

ABSTRACT

Genetic recombination occurred between auxotrophic strains of the lignin-degrading basidiomycete Phanerochaete chrysosporium. Cytological studies demonstrated that the basidiospores are binucleate.

Six leucine auxotrophic strains of P. chrysosporium were characterized genetically and biochemically.

The mating system and formation of basidiospores in P. chrysosporium were studied using wild-type and auxotrophic strains. The prototrophic basidiospore progeny of crosses between auxotrophic strains were shown to be homokaryotic recombinants rather than complementary heterokaryons. Various wild-type strains were shown to have multinucleate cells lacking clamp connections and to possess a variable number of sterigmata per basidium. Single basidiospores from three wild-type strains all produced fruit bodies and basidiospores. Nonfruiting as well as fruiting isolates were obtained from single basidiospores of five other wild-type strains. Basidiospores from fruiting isolates always yielded colonies that fruited, again indicating that the spores are homokaryotic. Nonfruiting isolates from the same strain did not produce basidiospores when paired, indicating that these isolates do not represent mating types. Basidiospores from strain OGC101 also gave rise to colonies which did not grow on cellulose (Cel^{-}). This evidence suggests that at least some strains of P. chrysosporium are heterokaryons with primary homothallic mating systems.

A DNA transformation system was developed for P. chrysosporium. Swollen basidiospores of an adenine auxotroph were protoplasted and transformed to prototrophy using a plasmid containing the gene for an adenine biosynthetic enzyme from Schizophyllum commune. Southern blot analysis demonstrated that plasmid DNA was integrated into the chromosomal DNA in multiple copies. Recombination experiments demonstrated that integration occurred at site(s) other than the resident adenine biosynthetic gene.

A second P. chrysosporium adenine auxotroph was transformed to prototrophy with a second adenine biosynthetic gene from S. commune. Fragments containing both adenine biosynthetic genes were subcloned into the plasmid pUC18 and transformants obtained with these subclones were analyzed. The subclones were mapped for restriction sites and the approximate locations of the complementing genes were determined. One of these plasmids was used to transform the Neurospora crassa ade2 strain, thereby identifying the complementing adenine biosynthetic gene as encoding phosphoribosylaminoimidazole synthetase.

CHAPTER 1: INTRODUCTION

Lignin is a complex, optically inactive phenylpropanoid polymer that constitutes 20-30% of extracted wood (1). It is the second most abundant natural polymer, after cellulose; and since its presence in plant cell walls retards the breakdown of cellulose, lignin degradation is a key biochemical process in the cycling of biospheric carbon (2). White rot basidiomycetous fungi are primarily responsible for the initiation of the decomposition of lignin and appear to be the only organisms that are capable of degrading lignin completely to carbon dioxide and water (3-5). Most studies on lignin degradation have focused on the hymenomycete Phanerochaete chrysosporium and there is a rapidly accumulating body of knowledge concerning the physiology and biochemistry of this organism (3-5).

During secondary metabolism, P. chrysosporium secretes two extracellular heme peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), which, together with an H_2O_2-generating system, appear to be the major components of its lignin degradative system (LDS) (3,4). This degradative system is quite nonspecific and has potential biotechnological applications, not only for the transformation of lignocellulose into useful pulps, chemicals and fuels (4), but also for the degradation of a variety of environmentally-persistant pollutants (6).

Classical Genetic Analysis

For many years research emphasized the physiology of lignin degradation by P. chrysosporium and, with the discoveries of MnP and LiP, the characterization and mechanisms of these enzymes. However it became apparent that to understand the process of lignin degradation in its totality, as well as to utilize P. chrysosporium for bioprocessing applications, required both an understanding of the life cycle and genetics of this organism and the ability to genetically manipulate its lignin degradative system. Since almost all of fungal genetics is based on the study of mutations at specific chromosomal loci (7), early genetic studies with P. chrysosporium were directed toward the isolation of mutants, both auxotrophic mutations in biosynthetic pathways and mutations in expression of the LDS.

The isolation of such mutations in P. chrysosporium was facilitated by the observations that colonial growth could be induced on medium containing L-sorbose and sodium deoxycholate and that conidiospores (asexual spores) from these colonies could be replica plated to selective media (8), as first described for selection of mutants in bacteria by Lederberg & Lederberg (9).

Auxotrophic mutants. Fungal genetics was developed using biochemical mutants, first utilized in Neurospora crassa by Beadle and Tatum (10). Auxotrophs, mutants which fail to grow on minimal medium without the addition of one or more specific supplements, are often the most useful biochemical mutants. The isolation of P. chrysosporium auxotrophic mutants (11) demonstrated for the first time that this

organism could be manipulated by classical genetic techniques. These auxotrophs have proved useful in studies designed to elucidate the life cycle of P. chrysosporium, as well as in the development of a DNA transformation system for this organism (see below).

In initial studies using UV and X-ray mutagenesis of conidiospores, Gold and coworkers (11) isolated 33 amino acid, vitamin and adenine-requiring auxotrophs and assigned them to complementation groups via the formation of heterokaryotic mycelia. Liwicki et al. (12) later isolated several auxotrophic mutants using gamma irradiation. Although fluorescent staining with mithramycin A has shown the conidia to be predominately multinucleate (Alic and Gold; unpublished), the development of a medium that promoted production of uninucleate spores (11) and the use of sonication to unclump the conidia (Chapter 2) facilitated the isolation of additional mutants.

The toxic analog 5-fluoro-orotate was used to select ura5 mutants (13) which were shown by biochemical assays to be deficient in orotate phosphoribosyl transferase (Alic, Letzring and Gold; unpublished). Growth tests on various substrates led to the tentative identification of several other P. chrysosporium auxotrophs: The histidine auxotrophs, His3 and His4, appear to be deficient in histidinol dehydrogenase; and the arginine auxotroph, Arg2 (11), appears to be deficient in ornithine carbamoyltransferase (Alic and Gold; unpublished). The tryptophan auxotroph, Trp1, which is unable to grow on indole (Alic and Gold; unpublished), is presumably deficient in the β reaction of tryptophan synthetase (14). The biochemical characterization of the P. chrysosporium leucine auxotrophs and identification of the Ade1

auxotroph via transformation of a characterized N. crassa mutant are described in Chapters 3 and 6 respectively.

Genetic manipulations, such as cell fusions or uptake of nucleic acids, require the removal of the fungal cell wall and regeneration of the resulting protoplasts (15). Gold and coworkers (16) used Novozym 234 and Cellulase CP to produce mycelial protoplasts of wild-type and auxotrophic strains of P. chrysosporium. Protoplast fusion in the presence of polyethylene glycol (PEG) and $CaCl_2$, as well as resolution of heterokaryons into their constituent homokaryotic strains via protoplast formation, were demonstrated utilizing auxotrophs (16).

P. chrysosporium auxotrophs isolated in our laboratory (11) have proved invaluable for the genetic studies described in this thesis. Chapter 2 describes studies on genetic recombination utilizing various auxotrophic strains. A subsequent report utilizing independently-isolated auxotrophic strains has appeared elsewhere (17). The use of auxotrophic strains to demonstrate the predominately heterokaryotic nature of conidiospores is described in Chapter 2; whereas the homokaryotic nature of the binucleate basidiospores (sexual spores) of P. chrysosporium is demonstrated utilizing auxotrophic recombinants in Chapter 4.

Phenol Oxidase Mutants. Some of the earliest studies aimed at elucidating the mechanisms of lignin degradation by P. chrysosporium involved the isolation of phenol oxidase negative (PO^-) mutants, selected for by the absence of a color reaction on medium containing o-anisidine. Many such mutants were unable to degrade lignin or lignin model compounds, whereas revertants regained the wild-type degradative

abilities (12,18,19). Most of these mutants exhibited varying degrees of pleiotropy. Although Ander and Eriksson (18) attributed these to a regulatory effect of phenol oxidase, Gold and coworkers (19) showed that their mutant, 104-2, was clearly pleiotropic and suggested that it was defective in the onset of secondary metabolism. Liwicki et al. (12) claimed to have isolated PO^- mutants with enhanced lignin degradation as compared to the wild type, as well as PO^- mutants with delayed or unaffected lignin degradative abilities. However the subsequent recognition of the heterokaryotic nature of P. chrysosporium wild-type strains (Chapter 4) and the fact that the mutation rates for these characters (0.05% - 0.7% of survivors) are several orders of magnitude higher than expected (7) suggests that at least some of these presumptive mutants may actually have been wild-type homokaryons. Eriksson et al. (20) isolated cellulase negative (Cel^-) mutants of P. chrysosporium that over-produced phenol oxidase. However in this study, a "spontaneous mutation" rate of 0.15% was obtained by screening conidia of a Cel^- mutant for PO^+ (20).

Nitrogen-Deregulated Mutants. Ligninolytic activity in cultures of P. chrysosporium is a secondary metabolic function that is derepressed by nitrogen depletion. Addition of NH_4 or various nitrogenous compounds to ligninolytic cultures suppress the degradation of lignin as well as production of the secondary metabolite veratryl (3,4-dimethoxybenzyl) alcohol (4,21). Thus, there has been an ongoing interest in the isolation of mutants deregulated for nitrogen and thus capable of degrading lignin during primary metabolism. The discovery that several polymeric dyes, including Poly B-411, Poly R-481 and Poly Y-606, could

serve as substrates for the ligninolytic system of P. chrysosporium, and that the pleiotropic mutant 104-2 was unable to decolorize these dyes, suggested a simple new selection, not only for ligninolytic mutants, but also for mutants capable of expressing the LDS under nitrogen-sufficient conditions (22,23). Utilizing this protocol, Kuwahara and Asada (24) isolated mutant strains of P. chrysosporium that decolorized the dye rhemazol brilliant blue under high nitrogen, low oxygen conditions. Interestingly, one of these mutants was shown to exhibit high performance liquid chromatographic (HPLC) and isoelectric focusing profiles of extracellular proteins under high nitrogen conditions that were distinct from those of the wild-type strain (24).

Other Mutant Strains. There are reports of various other mutant strains derived from P. chrysosporium. These include strains lacking cellulases (20,25-27); glucose oxidase-negative strains (arising with a "mutation rate" of 3%) (28,29); and strains with LiP activity exceeding wild-type levels (30,31). However as indicated above, work in this laboratory (Chapter 4) and elsewhere (32) has demonstrated that wild-type strains of P. chrysosporium are heterokaryotic and that homokaryotic basidiospore-derived isolates can vary tremendously in expression of the LDS, as well as in cellulolytic functions. Therefore the isolation of mutants other than auxotrophs must be regarded with some skepticism.

Mating System in P. chrysosporium

As with most organisms, sexual reproduction is an important mechanism for maintaining genetic diversity within fungal populations and an understanding of the mechanisms for sexual reproduction is

essential for experiments involving genetic recombination and strain development. In addition, the various mating systems found in basidiomycetous fungi are both complex and unique, and systems such as that of Schizophyllum commune have become models for the study of eukaryotic gene regulation.

The subdivision Basidiomycotina are higher fungi which form external sexual spores, the basidiospores, on the basidium, a swollen club-shaped cell. Basidiomycete taxonomy is based on the morphology of the basidia (33). P. chrysosporium belongs to the class Hymenomycetes, in which the basidia are formed on a hymenium, rather than as single dispersed cells; to the subclass Holobasidiomycetidae, in which the basidia are single-celled; and to the order Aphyllophorales (bracket fungi or polypores) (34).

The basidiomycete life cycle is almost entirely haploid, with karyogamy followed immediately by meiosis within the basidia of the fruit body or mushroom (basidiocarp). In most gasteromycetes and hymenomycetes the dominant vegetative form is the dikaryon with two compatible paired nuclei per cell (34). However some hymenomycetes, including P. chrysosporium, are coenocytic with numerous nuclei per cell (Chapter 4). As a result of hyphal anastomosis and mutation, coenocytic mycelia may be heterokaryotic, containing a variety of nuclear genomes in varying proportions (see below).

There are three basic types of mating systems in basidiomycetes. Primary homothallic organisms are self-fertile. Heterothallism, simultaneously discovered in 1917 by Marie Bensaude in Coprinus lagopus

and by Hans Kniep in S. commune, can be either bipolar, with one mating type factor, or tetrapolar, as in C. lagopus and S. commune, with two mating type factors, A and B (35). In both types of heterothallism, there are multiple alleles for each factor (36). The formation of a fertile dikaryon via hyphal anastomosis in a heterothallic organism requires the presence of different ("compatible") mating type factors in the haploid monokaryons. During vegetative growth, the dikaryotic nuclei undergo synchronous division, often with one nucleus migrating through the cell septum and the other nucleus migrating through a structure called a clamp connection. Tetrapolar heterothallism is by far the best-studied of basidiomycete mating systems (7,35,37), and two Aα mating type alleles have recently been cloned from S. commune (38). In contrast, only about 10% of hymenomycetes have been identified as homothallic (35) and the genetic mechanisms for self-fertility and homothallism are not well-understood in higher fungi (36).

Phanerochaete is a broadly-based genus without well-defined limits (39). Although all species have multinucleate cells with few septa (40,41), some members of the subgenus Phanerochaete, which includes P. chrysosporium, may have scattered single or multiple clamps (42,43), or verticillate (whorled) clamps (40,41), even on primary mycelium (hyphae arising from a single basidiospore) (44). Clamps, opposite or whorled, are also sometimes found on the hyphae of the basidiocarp, a character associated with homothallism (39). Boidin (40) drew a strong correlation between holocoenocytic behavior (mycelia always multinucleate), rare or verticillate clamps, binucleate basidiospores and homothallism. Thus, Phanerochaete has always been considered to be

a genus of homothallic fungi (39). It has been suggested that the problems associated with species delineation within this genus may be related to their homothallism (45).

The species Phanerochaete chrysosporium was first described by Burdsall and Eslyn (46) and shown to be conspecific with other previously identified species including Sporotrichum pulverulentum (47). The species was distinguished by its large cystidia (sterile cells interspersed among the basidia) and the complete absence of clamps (41,46). The hyphal system of P. chrysosporium is monomitic (43); ie. there are no distinguishable secondary (dikaryotic) mycelia (Chapter 4; ref. 48).

The demonstration that P. chrysosporium could be induced to form fruiting bodies and eject viable basidiospores under conditions of nitrogen limitation opened the way for detailed examination of mating in this organism. Fruit body formation was found to be subject to glucose catabolite repression which could be partially relieved by exogenous adenosine 3',5'-monophosphate (cAMP). Walseth cellulose and xylose were found to be the best carbon sources for fruit body formation and light and thiamine were shown to be necessary (49).

The studies in Chapters 2 and 4 indicate that, as suggested by early work on the genus, at least some strains of P. chrysosporium are indeed primary homothallic. The evidence for homothallism can be briefly summarized as follows: 1) Studies using the fluorescent dye mithramycin A have shown all mycelia, including fruiting mycelia, to be coenocytic rather than dikaryotic. 2) In all strains tested, every monosporous conidiospore colony fruits as proficiently as the parent wild type. 3) In all strains tested, either some or all of the

monosporous basidiospore isolates fruit. Conidia arising from basidiospore isolates always exhibit the parental homokaryotic phenotype. The only exception found to 2) and 3) above is the P. chrysosporium type culture HHB-6251. With this strain, colonies arising from either single basidiospores or sonicated conidia never fruit (Alic and Gold; unpublished). This strain has not been studied further. 4) Since the binucleate basidiospores are homokaryotic, with the two nuclei arising via a post-packaging mitotic division (see Chapter 4), this cannot not be a case of secondary homothallism, wherein two nuclei of compatible mating types migrate into each basidiospore (36). 5) Nonfruiting basidiospore (homokaryotic) isolates from the wild-type strains ME-446 and OGC101 (a derivative of ME-446), as well as from several other strains, do not undergo normal fruiting cycles in pairwise combinations, indicating that these are not conventional mating type segregants. 6) Nonfruiting basidiospore isolates vary in phenotype, from a complete lack of fruit body formation to the production of small numbers of basidiospores, suggesting that these isolates are exhibiting different blocks in the sexual developmental pathway. In addition, pairwise combinations of nonfruiting isolates from different strains, as well as nonfruiting and Cel^- isolates from OGC101, can occasionally form viable fruit bodies, indicating that complementation can occur between mutants blocked at different steps in the developmental cycle. 7) Further evidence for homothallism comes from studies of auxotrophic mutants derived from ME-446 and OGC101 (Chapter 2; ref. 17). These auxotrophs exhibit the same variety of developmental phenotypes as the parent wild-type strains. However, no blocks exist to nutritionally

forced heterokaryosis among these auxotrophs, and the vast majority of such heterokaryons can undergo karyogamy, as evidenced by genetic recombination, indicating that no mating type incompatibilities exist among these strains (50). Such crosses are usually not possible in heterothallic basidiomycetes such as S. commune (51).

In contrast to these studies, Thompson and Broda (48) have reported that P. chrysosporium strain ME-446 has a bipolar heterothallic mating system, apparently with only two mating type alleles (32). However their conclusions are based solely on the mycelial interactions observed between single basidiospore isolates, without regard to sexual development or basidiospore formation (48). Although we have observed some of the same morphological phenomenona (Alic and Gold; unpublished), it has not been possible to correlate these with conventional mating type reactions (Chapter 4). Thus, we have interpreted the successful fruiting by homokaryotic isolates, with the production of large quantities of viable basidiospores, as a valid test for homothallism (52). The fact that Thompson and Broda (48) have been unable to obtain fruiting of P. chrysosporium strains which fruit well in our laboratory (Chapter 4) suggests that significant differences exist in either specific isolates or in experimental methods.

It is conceivable that different laboratory isolates of P. chrysosporium may have different mating systems. It has recently been suggested that fungal mating systems may be more flexible than previously thought and that heterothallism may be easily masked except under conditions favoring outbreeding (53). Homothallism in the genus Phanerochaete was first called into question when the presumed

homothallism of a related holocoenocytic fungus, Stereum hirsutum, was challenged (54). This work was extended to Phanerochaete velutina, with the suggestion of a multi-allelic bipolar compatability system controlling production of secondary mycelium (55). It now appears that populations of Stereum species have both outcrossing (multi-allelic unifactorial [bipolar] heterothallism) and non-outcrossing (homothallic) breeding strategies (56) and that the species may in fact be in the process of evolving toward homothallism (44). Several other species of basidiomycetes are known to contain both homothallic and heterothallic races (55). These include the coenocytic white rot polypore Heterobasidion annosum (52), and two species which are closely-related to the genus Phanerochaete within the family Corticiaceae (57), Gleocystidium tenue and the dikaryotic species Sistotrema brinkmannii (58,59). Nutritionally-forced heterokaryosis has been observed between primary homothallic and bipolar heterothallic strains of H. annosum (60) which, like P. chrysosporium, always have binucleate basidiospores (52). Genetic recombination has been shown to occur between bipolar and homothallic strains of S. brinkmannii (58,61). Certain matings between homothallic stocks in this species also exhibit incompatibility reactions (62).

In some strains of P. chrysosporium, only a fraction of the monosporous basidiospore isolates are capable of fruiting; whereas all monosporous conidiospore isolates fruit (Chapter 4). Johnsrud and Eriksson (27) obtained identical results with strain P-1271. These results are not without precedent. Some primary (homokaryotic) mycelia of Phanerochaete laevis fruit and the resulting identical basidiospore

progeny do not exhibit incompatibility reactions (44). Some, but not all, monosporous isolates of the homothallic strain of H. annosum fruit in the laboratory, producing large numbers of viable basidiospores (60). It has been suggested that the homothallic isolates of S. brinkmannii may be analogous to laboratory mutants of S. commune and C. lagopus which are constitutive for those functions controlled by mating type genes ($A_{mut}B_{mut}$) (61,62). It is theoretically possible that such a system could be operational in those strains of P. chrysosporium in which only some of the basidiospore isolates fruit (OGC101, ME-446, ML-21, BKM-F-1767, P-1271). In such strains, the homokaryotic fruiters could be constitutive mutants for mating type loci and the nonfruiters could be wild type for mating type with compatible mating type alleles being so rare as to be undetectable in the population. However the variable phenotypes of the nonfruiting isolates argue against this hypothesis. In any case, the strains of P. chrysosporium in which all basidiospore isolates fruit (ME-416, FP-104297, P-2843f) (Chapter 4) must at the present time be considered to be primary homothallic.

Although the basidia of the P. chrysosporium type culture, HHB-6251, were originally reported to be 4-sterigmate (46), Stalpers (41) found that the basidia from one isolate had a variable number of sterigmata, ranging in number from two to eight, with the majority having five or six. The length of the sterigmata varied and occasionally the basidiospores on a single basidium matured in two groups rather than simultaneously (41). This result has since been confirmed for strain P-1271 by Johnsrud and Eriksson (27), for strain ME-446 by Thompson and Broda (48), and for a number of P. chrysosporium strains in our

laboratory (Chapter 4). Likewise, the basidia of S. brinkmannii bear between four and eight basidiospores (58) and the basidia of both strains of H. annosum, although predominately four-spored, include some two-spored basidia (52). It is possible that this variability in sterigmata is related to the unusual mating behavior of these species.

There obviously remain unanswered questions about the mating system of Phanerochaete chrysosporium. However the fact that homokaryotic isolates are either intrinsically capable of fruiting (Chapter 4), or can acquire this ability via genetic recombination (Alic and Gold; unpublished), and the absence of apparent barriers to karyogamy among auxotrophic strains (Chapter 2) have important implications for the development of DNA transformation in this organism (Chapters 5 and 6), as well as for strain development and bioprocessing applications utilizing P. chrysosporium.

The P. chrysosporium Genome

Genome Content. Fungal taxonomy has always been fraught with difficulties, in part due to the complexities of sexuality alluded to in the previous section. Thus in recent years, DNA base composition and hybridization studies have been used to analyze genetic relationships among species and strains (63). The first analysis of the P. chrysosporium genome was performed by Raeder and Broda (64): "Gradient stretching" in CsCl gradients containing bisbenzimide, which binds to adenine residues thereby reducing their bouyant density in CsCl (65), was used to separate out the mitochondrial DNA and 10 kilobase (kb) tandemly-arranged repeats of ribosomal RNA (rRNA) genes, as AT-rich

satellites. By comparing such CsCl-bisbenzimide gradients with marker DNAs of known GC content, strains ME-446 and BKM-F-1767 were shown to have chromosomal GC contents of 59%, with 51% GC satellites strongly enriched for ribosomal DNA, and mitochondrial GC contents of 33% (64). These are similar to values reported for other basidiomycetes (63).

Labeled clones from λ libraries of P. chrysosporium strains ME-446 and BKM-F-1767 were hybridized under stringent conditions to EcoRI-restricted total DNA from these two strains as well as strains from France and Papua New Guinea. From the relative strengths of the hybridization signals, Raeder and Broda (64) concluded that ME-446 and BKM-F-1767 were closely related to each other, but much less closely related to the other two strains. Restriction fragment patterns and cross-hybridization of mitochondrial DNA from the four strains corroborated this conclusion and suggested that the strains from France and Papua New Guinea might be more closely related to each other (64). ME-446 and BKM-F-1767 were found to differ in the proportion of methylated bases in their respective genomes (66).

Knowledge of genome size is important for construction of genomic libraries, estimating gene copy number, etc. Labeled single-copy clones from a partial EcoRI λ genomic library of ME-446, with known distribution of label between the fungal and vector components, were hybridized with fungal genomic DNA alone, and with mixtures of fungal and vector DNA. From the equimolar ratios of the two DNAs, the haploid genome size of P. chrysosporium was estimated to be 4.4×10^7 base pairs (bp) (64, 67), of which between 20 and 30% is mitochondrial and ribosomal DNA

(67). This value is similar to those obtained for other filamentous fungi (68).

Heterokaryosis. As pointed out above, heterokaryosis — the existence of genetically-different nuclei within the same mycelium — is not uncommon among coenocytic fungi (7). Heterokaryosis demands only that the different genomes are similar enough to survive in the same cytoplasm. Mycelial anastomosis and spontaneous mutation among the 10^6 to 10^9 nuclei present in a typical fungal mycelium would be expected to lead to heterokaryosis in those organisms lacking mechanisms for preventing it (34). In heterothallic basidiomycetes, anastomosis between mycelia of compatible mating types results in fertile dikaryon formation. In ascomycetes such as N. crassa, vegetative incompatibility factors, which regulate mycelial anastomosis, are well known (69). Whether similar factors for preventing hyphal fusion and resulting heterokaryosis can operate in homothallic basidiomycetes is unclear. However heterokaryosis obviously provides a mechanism for maintaining genetic diversity in homothallic organisms which lack barriers to inbreeding.

Raeder and Broda (64) noticed that several of their single-copy λ genomic clones (see above) hybridized to more EcoRI fragments in the total genomic DNA of ME-446 and BKM-F-1767 than were present in the original clones, indicating that the genome contained more than one allele for that sequence. This observation led to the suggestion that these strains of P. chrysosporium might be diploid (64). However, diploidy in basidiomycetes is normally restricted to the basidia (7,60), with only one known exceptional species (69a).

Chapter 4 details our discovery of various phenotypic differences among homokaryotic basidiospore isolates of strains OGC101, ME-446 and others, demonstrating the heterokaryotic nature of at least some wild-type strains of P. chrysosporium. Although Johnsrud and Eriksson (27) asserted that the basidiospores of strain P-1271 could be either homokaryotic or heterokaryotic, the methods they used to collect basidiospore progeny were never published, and it is possible that their heterokaryotic isolates did not arise from single basidiospores. The difficultes of isolating single basidiospores from this organism are discussed in Chapter 4. Raeder and Broda (70) have confirmed the heterokaryotic nature of the mycelia and conidia and the homokaryotic nature of the basidiospores for strain ME-446 in their analysis of restriction site polymorphisms.

Raeder and Broda (70) suggest that ME-446 is a dikaryon with two parental types and, for at least some of their clones, there appear to be only two parental types; ie. the wild type exhibits a restriction pattern which is a combination of the two patterns exhibited by individual basidiospore isolates. However, additional parental types with changes in restriction sites other than those tested, or allelic changes in genes other than those probed, would go unnoticed in such an analysis. In addition, in heterokaryons the percentage of nuclear types may vary greatly (71), so that less common parental types may go unnoticed in an RFLP (restriction fragment length polymorphism) analysis. In these studies the parental homokaryons were not isolated; nor is cytological evidence for dikaryosis in ME-446 presented (70,72). In our laboratory, fluorescent microscopy of ME-446 failed to reveal any

dinucleate mycelia (Chapter 4) even though, in most heterothallic basidiomycetes, dikaryons constitute the predominant vegetative phase (34,71). The wide range of morphological responses observed in pairings between basidiospore isolates of ME-446 (48) might also be taken as suggestive of more than two parental types. The segregation of fruiting, nonfruiting and Cel^- basidiospore isolates in OGC101 suggests that this strain is comprised of at least three genotypes. However, our data for ME-446 and several other strains indicates only that there are at least two parental types (Chapter 4).

RFLP Mapping. The contruction of genetic linkage maps, using sequence (restriction fragment length) polymorphisms detected with random single-copy probes hybridized to restriction digests of genomic DNA, was first proposed by Botstein et al. (73) for use in mapping the human genome. RFLP mapping was adapted for use in N. crassa by Metzenberg et al. (74). In these experiments linkages were scored by the meiotic segregation of probed RFLPs from classical genetic markers (75,76). Segregation of RFLPs can also be used to construct genomic maps in fungi for which no genetic markers exist and Raeder and Broda (70) suggested that RFLPs be used to map the genome of P. chrysosporium strain ME-446.

Haylock et al. (77) first isolated mRNA from P. chrysosporium. In vitro translation demonstrated different protein products from primary (3-day high nitrogen cultures) and idiophasic (6-day low nitrogen cultures) mRNA (77,78). An 8000-clone λ genomic library was probed with cDNA probes derived from these mRNAs and 38 clones expressed only during secondary metabolism were identified (72,79). However, these clones are

likely to represent only a small proportion of idiophase-specific genes. Since most clones in a library of 15-23 kb inserts will carry more than one gene, hybridization signals will be additive and signals from differentially-expressed genes might be obscured, while weakly-expressed genes might not be detected at all. In addition, mRNA expressed only at the onset of secondary metabolism would have been missed in this screen (79). Raeder et al. (72) also suggest that there may be changes in size and/or stability of mRNA species that are expressed during both primary and secondary metabolism.

By probing the SalI-digested genomic DNA of 53 single basidiospore progeny with 20 of these idiophase-specific cloned sequences, Raeder and Broda (70) were able to demonstrate segregration of 14 restriction site polymorphisms and several instances of genetic linkage. Five of the 20 sequences cross-hybridized and four of these showed 100% linkage, indicating that they arose from the same locus (70).

By increasing the number of RFLP markers to 38, and probing PstI as well as SalI digests of the 53 recombinant homokaryons, Raeder et al. (72) developed a preliminary genetic map of ME-446. Eighty probes of 15-23 kb inserts from λ EMBL3 and λ EMBL4 genomic libraries were hybridized to digested DNA from homokaryotic progeny. The probes included: 27 genes specifically transcribed during secondary metabolism (11 LiP clones and 16 unique sequences in two unlinked clusters); a cellulase gene; and 52 randomly-chosen clones which did not hybridize to ribosomal or mitochondrial DNA (72). One of these clones was identified as a presumptive mating type locus, based on incompatibility behavior in pairings of 12 of the 53 strains (48). Forty-three of the 80 clones

showed segregation of RFLPs, but 5 gave complex and multiple segregation patterns. Suggested explanations for these complex patterns included the presence of dispersed repetitive sequences, multiple inserts or multiple clones in the original plaques (72). However the authors have not ruled out the possibility that the wild type ME-446 may be comprised of more than two genomes, since the parental types have yet to be identified. The remaining 38 sequences used to construct their genomic map each segregated two patterns, 37 of them in approximately equal proportions.

Using a cosegregation frequency of ~70% as the criterion for linkage, 34 of the markers showed linkage to one or more other markers. This resulted in delineation of 6 linkage groups plus 4 unlinked markers, the latter including a LiP gene cluster (LC1) and the single marker exhibiting unequal allele segregation. From these data, the authors concluded that there are less than 10 chromosomes with a genetic map length of no more than 1000 centimorgans (72). This is in agreement with cytological observations that P. chrysosporium OGC101 has 6-7 chromosomes (Letzring et al.; unpublished results) and is similar to N. crassa which has seven chromosomes (80) and S. commune which is thought to have eleven (81). N. crassa also has a map length of about 1000 centimorgans (82). The number of centimorgans per kb of DNA is much higher in filamentous fungi than in higher eukaryotes, suggesting that the frequency of meiotic crossing over is very high and that only genes in very close proximity will remain linked (83).

Genes Encoding Extracellular Peroxidases

Fungal genes were first cloned in Saccharomyces cerevisiae (84) and N. crassa (85) in 1976-1977. Since that time, a large number of genes have been cloned from filamentous fungi and many have been sequenced. These nuclear genes appear to be intermediate in structure between the genes of S. cerevisiae and those of higher eukaryotes. For example, unlike yeast, filamentous fungal genes usually have introns, but they tend to be quite short (50-250 bp). Intron/exon junction sequences and an internal splicing signal for lariat formation appear to be conserved among these genes. In some respects, the 5' non-coding regions of filamentous fungal genes appear more variable than in higher eukaryotes. Whereas some fungal genes have the canonical TATA sequence in their promoter regions, others simply have an AT-rich region 30-100 bases upstream of the ATG translation start site. Likewise, fungal genes may or may not have a CAAT box. The typical fungal gene has several apparent polyadenylation sites, but the polyadenylation signal of higher eukaryotic genes, AATAAA, is only rarely present (86).

With the discovery of extracellular peroxidases involved in the lignin degradative system of P. chrysosporium (87-89), and the recognition that these enzymes are each expressed as a series of isozymes (90-92), researchers in the field of lignin biodegradation became interested in cloning the genes encoding these enzymes. Studies of the structure and regulation of these genes have numerous potential applications including: elucidation of enzyme mechanisms and structure-function relations; site-directed mutagenesis for enzyme modification;

expression of these enzymes in industrial microorganisms; and manipulation of the lignin degradative system in P. chrysosporium.

Lignin Peroxidase. The first cDNA clones for lignin peroxidase were isolated by Zhang et al. (93). Differential hybridization of an idiophasic cDNA library of strain BKM-F-1767 with cDNA probes made from 2-day and 6-day poly(A) RNA, led to the isolation of ~850 clones specific to secondary metabolism. Screening of these clones with synthetic ogligonucleotide probes based on partial amino acid sequences of LiP isozyme H8, led to the isolation of four LiP cDNA clones (94).

In 1987, Tien and Tu reported the first LiP cDNA sequence. Partial sequencing of positive clones obtained from an idiophasic cDNA library of BKM-F-1767 screened with a polyclonal antibody to H8, revealed two similar but not identical clones. One clone was sequenced completely and shown to encode a mature protein product of 345 amino acids with a 28-residue leader sequence. One potential N-glycosylation site and 43 potential O-glycosylation sites were identified. Residues thought to be essential for peroxidase activity (distal and proximal histidines and the distal arginine) were identified, and the sequences surrounding these residues were shown to be homologous with other peroxidases (95).

The sequences of four other LiP cDNAs from BKM-F-1767 have been published (96,97). Two of these clones have a nucleotide sequence homology of 71.5% and an amino acid homology of 68.5% (96), and one of these (CLG5) appears to encode LiP isozyme H10 (98). A third clone seems to be an allelic variant of the original Tien and Tu cDNA, encoding H8 (97). This is consistent with BKM-F-1767 being a

heterokaryotic wild-type strain (Chapter 4). The fourth cDNA encodes a homologous isozyme, perhaps H6 (97).

Sequencing of a LiP cDNA encoding the most abundant LiP isozyme from P. chrysosporium strain OCG101 has shown the mature protein product to be composed of 343 amino acids, preceded by a 21-amino acid signal peptide and a 7-amino acid propeptide. The N-terminal, hydrophobic and C-terminal segments of the signal peptide are typical of those for secreted proteins and S-factor analysis suggests that the signal peptide is cleaved after the alanine at position 21. The cleavage site for the propeptide follows the sequence -Lys-Arg- (Ritch, Nipper, Akileswaran, Pribnow and Gold; manuscript in preparation), suggesting that its removal involves an enzyme similar to the yeast Kex2 endoprotease (99).

Genomic sequences of H8 alleles (97,100-102) and two closely-related genes from BKM-F-1767 (102), and two LiP isozymes from ME-446 (103,104) have also been published. In addition, Brown et al. (104) sequenced the 5' end of three other very similar LiP genes from ME-446. The LiP genes all have eight small (49-69 bp) introns interspersed throughout, with intron/exon junction sequences that conform to those in other filamentous fungi, and internal splice sequences that are not as strictly conserved (86,101). Intron 1 splits the putative signal sequence from the propeptide and intron 8 is adjacent to the last, proline-rich exon (102). The 5' non-coding region includes a CAAT sequence at about position -110 and a TATA box at about -81 relative to the translation initiation codon (100). S1 nuclease mapping has confirmed that transcription initiates downstream of these sequences (105). The sequences surrounding the ATG initiation codon, as well as

those surrounding the peroxidase-essential residues (see above), are conserved.

It is apparent that the LiP isozymes are encoded by a family of closely-related genes. All of the LiP genes cross-hybridize to varying extents (104), as do polyclonal antibodies raised against specific isozymes (92,95) (Ritch, Nipper, Akileswaran, Pribnow and Gold; manuscript in preparation). In vitro translation of poly(A) RNAs from 1 through 6-day cultures and immunoprecipitation with polyclonal antibodies to H8, revealed multiple protein bands with Mr in the range 36,000-42,000, first appearing on day 2 and increasing each day, indicating LiP isozymes of varying molecular weights even in the absence of glycosylation, and regulated at the mRNA level (95).

There is also evidence that at least some of these genes may be clustered in the P. chrysosporium genome. Walther et al. (101) found one λ EMBL3 clone which carried two LiP gene copies with as little as three kb separation. Of the 11 LiP clones (shown by oligonucleotide hybridizations to contain at least eight different LiP sequences) used in RFLP mapping of the P. chrysosporium genome, at least six mapped to one cluster, LC1 (72). Four of these were shown by sequence analysis to be related but different LiP genes (104). Two other LiP genes, located on a single clone, defined a second cluster, LC2, mapping to linkage group III. The remainder of the idiophase-specific genes were dispersed throughout the genome (72). Raeder et al. (32) found LiP activity, as measured by veratryl alcohol oxidation, to be independent of the alleles present in LC1 and LC2.

Manganese Peroxidase. The first MnP cDNA, from strain OGC101, was cloned using a polyclonal antibody raised against purified MnP isozyme 1 and sequenced by Pribnow et al. (106). The coding region of this clone has a 68% GC content, with a high proportion of G+C in degenerate codons (106). In contrast, the GC content of LiP genes is similar to the organism as a whole (104). This bias may reflect the high rate of MnP synthesis and is similar to that found in highly-expressed yeast genes (86). The deduced mature protein consists of 357 amino acids preceded by a 21-amino acid leader sequence, with N-terminal, hydrophobic and C-terminal domains characteristic of signal peptides (106,107). The deduced mature N-terminal sequence corresponds to the experimentally-determined N-terminal sequence of purified MnP isozyme 1. A single N-glycosylation site, 49 potential O-glycosylation sites and the proximal His, distal His and distal Arg required for the peroxidase mechanism were identified. The sequences flanking these residues are homologous with those of other peroxidases, including LiP. A putative polyadenylation signal, AATACA (106), matches that in the LiP cDNA CLG5 (96). MnP and LiP cDNAs exhibit about 50% identity on the amino acid level and about 60% identity at the nucleotide level. Both enzymes have an abundance of acidic residues, consistent with their low pIs. Southern blot hybridization analysis suggested the presence of several different MnP genes. A cDNA clone for MnP isozyme 2 was isolated and weakly cross-hybridizes with the MnP-1 probe (106).

A second MnP cDNA has been isolated from strain BKM-F-1767. It encodes a deduced protein of 358 amino acids with a 24-amino acid leader sequence (108).

Recently, a genomic clone encoding MnP-1 was isolated and sequenced. The gene contains six introns ranging in size from 57 to 72 bps and intron splice junction sequences are conserved. However there is little similarity to the intron positions in LiP genes. The distal His and distal Arg are located on the same exon in the MnP gene, whereas they are split by an intron in the LiP genes, even though in both proteins the two residues are separated by only three amino acids. The MnP-1 gene lacks both the propeptide and intron of the LiP leader sequences. MnP also lacks the short C-terminal proline-rich sequence of LiP. In contrast to the extreme GC bias of the MnP coding regions, the introns have a GC content of 56%, with 59% G+C in the 5' upstream region and 41% in the 3' downstream region. The 5' non-coding region of the MnP gene contains a TATAA element at position -81 and three inverted CCAAT elements at -181, -195 and -304 relative to the translation initiation codon (Godfrey, Mayfield, Brown and Gold; manuscript submitted). It also contains a putative SP-1 site (GGGCGG) with the preferred flanking bases (109) at -446 and an AP-2 site (TGGGGA) (110) at -285 relative to the initiation codon (Godfrey, Mayfield, Brown and Gold; manuscript submitted).

Regulation of LiP and MnP. Tien and Tu (95) used *in vitro* translation of poly(A) RNAs and Northern blot analysis to demonstrate that LiP is regulated at the level of mRNA transcription. Northern blot hybridizations of RNA extracted from 5-day nitrogen-sufficient and nitrogen-deficient cultures indicate that MnP is also regulated by nutrient nitrogen at the level of gene transcription (106). mRNA levels correlate with MnP protein activity (108). In addition, MnP enzyme

assays, immunoblot and Northern blot analyses indicate that MnP gene transcription is induced under nitrogen-limiting conditions only in the presence of manganese (Mn) II, the substrate for the enzyme. Addition of Mn to 4-day nitrogen-limited Mn-deficient cultures results in extracellular MnP activity within 6 hours and this induction is inhibited by actinomycin D and cycloheximide. In contrast, Mn has no significant effect on primary growth or on synthesis of the secondary metabolite, veratryl alcohol (Brown, Glenn and Gold; manuscript submitted).

The MnP-1 gene contains three putative heat shock elements similar to the concensus C--GAA--TTC--G within 275 bp upstream of the translation initiation codon (Godfrey, Mayfield, Brown and Gold; manuscript submitted). Although the MnP gene has introns and lacks a long untranslated leader sequence, both of which are uncharacteristic of heat shock genes (111), heat shock will induce transient MnP gene transcription even in the absence of MnII (Brown and Gold; manuscript in preparation).

There is some evidence that intracellular cAMP may be involved in the regulation of the LDS of P. chrysosporium. A large increase in intracellular cAMP precedes the production of veratryl alcohol and the onset of ligninolytic activity (112). Addition of glutamate, which suppresses ligninolytic activity after its onset (21), produces a rapid decrease in intracellular cAMP (112). In high-nitrogen (24mM $NH_4H_2PO_4$) cultures, extracellular cAMP increases substantially with time, while adenylate cyclase activity remains constant. However in low nitrogen (2.4mM $NH_4H_2PO_4$), extracellular cAMP activity remains constant and

adenylate cyclase activity increases with time. Since no cAMP-phosphodiesterase activity could be detected, it was suggested that P. chrysosporium might regulate its internal cAMP levels by adenylate cyclase and by extracellular secretion. The adenylate cyclase of P. chrysosporium requires Mn for maximal activity (113).

Other P. chrysosporium genes. A cellulase gene from P. chrysosporium has been cloned by hybridization of a λ EMBL3 genomic library with a fragment of the exo-cellobiohydrolase I (CBHI) gene from Trichoderma reesei. The P. chrysosporium gene encodes a deduced protein of 516 residues and contains two introns. The transcript is induced by cellulose but not by glucose (114). The P. chrysosporiumg gene encoding phosphoribosylanthranilate isomerase (PRAI) has been isolated by complementation of an E. coli trpC mutant with a P. chrysosporium EMBL3 genomic library. Sequencing data suggests that, as in other filamentous fungi, the gene encodes a trifunctional protein with glutamine amido transferase, indoleglycerolphosphate synthase and PRAI activities (115). A β-tubulin-encoding gene and the ade1 gene encoding phosphoribosylaminoimidazole synthetase (see below) from P. chrysosporium have recently been cloned from a λ EMBL3 library of OGC101, using heterologous probes from N. crassa amd S. commune, respectively (Mayfield, Nipper, Alic and Gold; unpublished). Several other nucleotide biosynthetic genes, as well as genes encoding glycolytic enzymes, are currently being cloned in our laboratory.

Transformation of P. chrysosporium

The first report of DNA-mediated transformation of a fungal species appeared in 1973. Growing cultures of an inositol-requiring mutant of N. crassa were treated with calcium and DNA extracted from the wild type, and inositol-independent conidia were isolated (116). However it was not until 1978, when protoplasts of a leu2 mutant of S. cerevisiae were transformed to prototrophy with a plasmid containing yeast DNA that complemented E. coli leuB mutants (117), that fungal transformation was widely accepted. The following year, the transformation of N. crassa protoplasts was reported (118), and soon the technique was being used with a wide variety of ascomycetes (119). However obtaining stable transformants in basidiomycetes proved more difficult (120), and it was not until 1986 that transformation of S. commune was achieved (121). Transformation of C. cinereus was reported the following year (122).

DNA transformation of P. chrysosporium has a number of potential applications: It would facilitate analysis of LiP and MnP regulatory sequences and genomic position effects, as well as the study of enzyme structure-function relationships via site-directed mutagenesis. It is also possible that high level expression of these enzymes could be achieved by increasing gene copy number or modifying promoter sequences. In addition, transformation-mediated gene disruption experiments could be used to analyze the roles of LiP and MnP isozymes in lignin degradation.

Chapter 5 details the first successful transformation of P. chrysosporium, wherein protoplasts of an adenine auxotroph (Ade2) were

transformed with a plasmid (pADE2) containing an adenine biosynthetic gene from S. commune (123). Chapter 6 describes the transformation of a second P. chrysosporium adenine-requiring auxotroph (Ade1) with a plasmid containing a different adenine biosynthetic gene from S. commune (pADE5) (123). The subcloning and restriction mapping of both of the S. commune genes, and subsequent transformations of P. chrysosporium are also described in Chapter 6. The chapter concludes with the identification of the S. commune Ade5 gene, via transformation of an N. crassa **ade2** strain deficient in phosphoribosylaminoimidazole synthetase.

With transformation systems now available for a large number of fungal species (119), heterologous gene expression among fungi has become an important issue for the development of broad spectrum vectors, for isolation of genes in genetically-uncharacterized fungi, and for evolutionary considerations (124,125). Chapters 5 and 6 describe the successful expression in P. chrysosporium of biosynthetic genes from another basidiomycete, S. commune. Chapter 6 also describes the expression of the S. commune Ade5 gene in the ascomycete N. crassa. In addition, we have successfully transformed a P. chrysosporium **ura5** auxotroph with a plasmid containing the **ura5** gene from the ascomycete Podospora anserina (126) (Alic, Kornegay and Gold; unpublished). Hynes (127) has recently reported the successful transformation of the ascomycete Aspergillus nidulans with the **acu7** gene, encoding isocitrate lyase, from C. cinereus. However the analogous A. nidulans gene, **acuD**, was not expressed in C. cinereus. Likewise a C. cinereus **trp2** mutant could be transformed to prototrophy with either the S. commune **trp1** or the P. chrysosporium **trpC** gene, but not with the A. nidulans **trpC** gene,

although cotransformations revealed that the plasmid containing the latter gene was integrated into the C. cinereus genome (125). In addition, a contruct containing the bacterial hygromycin phosphotransferase gene, conferring resistance to the antibiotic hygromycin B (128), fused to a promoter from the hemibasidiomycete Ustilago maydis (129), was not expressed in either C. cinereus (125) or in P. chrysosporium (Alic and Gold; unpublished results).

In fungi such as P. chrysosporium, in which auxotrophic genetic markers are available, transformation systems based on complementation can take advantage of the variety of metabolic genes that have been cloned in other organisms, as well as providing a method for cloning homologous genes from a genomic library (123). However dominant selective markers, such as hygromycin B or bleomycin (phleomycin) (130-132), are useful for the transformation of wild-type strains. Randall et al. (133) reported low-frequency transformation of P. chrysosporium to G418 resistance, with the bacterial kanamycin-resistant determinant of Tn903 (134) and a P. chrysosporium sequence that apparently supports autonomous replication in yeast (135-137).

Summary of Research

The research described in this thesis builds directly on the early genetic studies of Phanerochaete chrysosporium conducted in this laboratory, including induction of colonial growth and replica plating of colonies (8), and fruit body formation and basidiospore production (49). A large number of auxotrophic mutants of P. chrysosporium had been isolated and assigned to complementation groups via heterokaryon

formation (11). Finally, the formation, fusion and regeneration of mycelial protoplasts of wild-type and auxotrophic strains had been accomplished (16).

Chapter 2 describes systematic studies of genetic recombination in P. chrysosporium, utilizing a variety of auxotrophic strains. No evidence was found for parasexual (somatic) recombination (138-140) in this organism.

Cytological studies in Chapter 2 demonstrate that the basidiospores of P. chrysosporium are binucleate. The frequent occurrence of approximately 1:1:1:1 ratios of parental and recombinant phenotypes suggested that the binucleate basidiospores were homokaryotic, with the two nuclei arising via a postmeiotic mitotic event. In constrast, the conidia arising from heterokaryotic mycelia were >85% heterokaryotic.

Several observations, detailed in Chapter 2, led to the suggestion that P. chrysosporium might be a homothallic (self-fertile) organism: 1) Clamp connections were never observed. 2) >200 isolates of single wild-type conidiospores were all capable of forming fruit bodies and producing copious numbers of viable basidiospores. 3) Many wild-type basidiospore isolates and >70% of the auxotrophic strains fruited. 4) Fruiting heterokaryons were obtained with every attempted combination of complementary auxotrophs and in most cases, genetic recombination was detected.

The results in Chapter 2 suggest that recombination between auxotrophic strains can be used for constructing strains for the study of the lignin degradative system of P. chrysosporium.

The enzymes, gene loci and regulation involved in the biosynthesis of leucine had been studied in detail in several prokaryotic and eukaryotic organisms (141-145) and the leu2 gene encoding β-isopropylmalate dehydrogenase had been cloned in Saccharomyces cerevisiae (117). Thus, it seemed likely that P. chrysosporium leucine auxotrophs would prove useful for future genetic and molecular biology research.

Chapter 3 describes the genetic and biochemical characterization of six leucine auxotrophic strains of P. chrysosporium. This work was initiated by Dr. T. A. Molskness, who successfully applied the assays for leucine biosynthetic enzymes to P. chrysosporium strains. Five of the auxotrophs had been previously described (11) and the sixth was isolated for this study by X-ray mutagenesis. The auxotrophs were divided into three complementation groups via heterokaryon formation. Since all six auxotrophs grew on minimal medium supplemented with α-ketoisocaproate as well as with leucine, the transaminase catalyzing the last step in the leucine biosynthetic pathway was apparently normal in all strains. Therefore the wild-type, auxotrophic and heterokaryotic strains were assayed for the activities of the other enzymes specific to leucine biosynthesis. It was shown that Leu2 and Leu4 (complementation group I) lacked only α-isopropylmalate synthase activity; Leu3 and Leu6 (group III) lacked isopropylmalate isomerase activity; and Leu1 and Leu5 (group II) lacked β-isopropylmalate dehydrogenase activity. Heterokaryons formed from leucine auxotrophs of different complementation groups had levels of activity for all three enzymes that were similar to those found in the wild-type strain OGC101.

Chapter 4 describes our studies of the mating system and basidiospore formation in P. chrysosporium. In experiments performed in collaboration with C. Letzring, prototrophs recovered from crosses between auxotrophic strains were fruited and the progeny of most of these self-crosses were shown to be prototrophic. This indicated that the original prototrophs were wild-type recombinants rather than complementary heterokaryons and that the binucleate basidiospores were homokaryotic.

Cytological studies of various wild-type strains demonstrated that the mycelia were all multinucleate and that basidia bore a variable number of sterigmata. Several other results also indicated that P. chrysosporium has a primary homothallic mating system: 1) Colonies arising from single conidia of various wild-type strains all produced fruit bodies and viable basidiospores. 2) Likewise, single basidiospores from three wild-type strains all produced fruit bodies and basidiospores. 3) Single basidiospores from five other wild-type strains gave rise to isolates that fruited normally and isolates that displayed a range of non-fruiting phenotypes. Pairs of nonfruiting isolates from the same strain did not form sexually-competent dikaryons, indicating that these represented mutations in the sexual developmental pathway rather than mating types. Occasionally, nonfruiting isolates from different strains, or nonfruiting isolates and Cel^- isolates from strain OGC101 did fruit in combination, suggesting that complementation could occur between mutants blocked at different steps in the developmental cycle. Basidiospores derived from such crosses segregated out various phenotypes, including fruiters, indicating that recombination

had taken place. Basidiospores derived from fruiting isolates gave rise to colonies which always fruited, again indicating that the basidiospores are homokaryotic and that the organism is homothallic. However colonies arising from conidia, produced basidiospores that displayed fruiting, nonfruiting, and in the case of OGC101, Cel^-, phenotypes, indicating that the conidia are heterokaryotic.

The homothallism of P. chrysosporium suggests that any two strains of this organism are capable of undergoing recombination and has important implications for strain construction. In addition, homothallism simplifies the production of basidiospores for transformation experiments and means that transformants can be tested directly for meiotic stability.

Basidiospores from strain OGC101 gave rise to fruiting, nonfruiting and Cel^- phenotypes and these basidiospore-derived isolates all differed from each other and from the parental wild type in a variety of characteristics including growth, conidiation and evolution of $^{14}CO_2$ from ^{14}C-side chain-labeled lignin. From these results, it was concluded that OGC101, ME-446, and various other P. chrysosporium strains are heterokaryotic. These observations, which have since been confirmed for ME-446 (32) and BKM-F-1767 (97), helps to explain earlier observations of heterogeneity among mutants (12) (Alic and Gold; unpublished). It also has important implications, both for physiological studies of lignin degradation, and for the construction of strains with desirable phenotypes. In addition, it suggests that some of the heterogeneity observed among isolated LiP and MnP genes may be due to allelic differences.

Chapter 5 describes the first transformation system for P. chrysosporium, in which protoplasted basidiospores of an adenine-requiring auxotroph, Ade2, were transformed to prototrophy using a plasmid containing an adenine biosynthetic gene from Schizophyllum commune (pADE2) (123). In these initial transformations, frequencies of approximately 100 transformants per μg of DNA were obtained. Southern blot analysis of DNA extracted from transformants demonstrated that the 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, indicating that integration occurred at a site or sites other than the resident adenine biosynthetic gene. These results are similar to those reported in transformations of other filamentous fungi (119).

Chapter 6 describes additional transformation experiments with P. chrysosporium. In these experiments, a second P. chrysosporium adenine auxotroph, Ade1, was transformed with a plasmid containing a different adenine biosynthetic gene from S. commune, pADE5 (123). Fragments containing the Ade2 and Ade5 genes from S. commune were subcloned into pUC18. Between 0.05% and 1.5% of viable P. chrysosporium protoplasts were transformed with these subclones. Again, the transforming DNA was shown to be mitotically and meiotically stable. Southern analyses of transformants showed that the plasmids were chromosomally-integrated in

multiple copies at various sites in the P. chrysosporium genome. There were also indications of plasmid rearrangements or deletions.

The subclones, pADE2-3b and pADE5-2g, were mapped for restriction sites and the approximate locations of the complementing genes were determined by transformation experiments with plasmid digests. In many cases, such digests reduced but did not eliminate transformation, suggesting that the fragments were incorporating into the genome in tandem, or that endogenous DNA ligase activity was causing the fragments to religate.

pADE5 and pADE5-2g were used to transform the Neurospora crassa **ade2** strain to prototrophy, thereby identifying the S. commune Ade5 biosynthetic gene as encoding phosphoribosylaminoimidazole synthetase. These experiments also demonstrated the expression of a basidiomycete gene in an ascomycetous fungus.

The plasmid pADE5-2g has been used in the construction of expression vectors for P. chrysosporium. Currently we are comparing the transformation efficiencies obtained using S. commune DNA, with transformation utilizing homologous genes and promoters from P. chrysosporium. Ultimately, transformation of P. chrysosporium should assist in elucidation of regulatory mechanisms for lignin biodegradation.

CHAPTER 2: GENETIC RECOMBINATION IN THE LIGNIN-DEGRADING BASIDIOMYCETE PHANEROCHAETE CHRYSOSPORIUM

The basidiomycete Phanerochaete chrysosporium and other white-rot fungi have potential applications in a variety of schemes for the industrial processing of lignocellulose. These organisms have already been used in numerous studies concerned with lignin degradation (2), cellulose metabolism (146), and lignocellulose bioprocessing (147). The recent isolation of extracellular enzymes involved in lignin degradation (87,88,148-150) has increased our understanding of the biochemistry of this process. The development of genetic methods for producing strains with enhanced lignin-degrading capacities would be a significant step toward the utilization of these organisms for lignocellulose bioprocessing.

In earlier studies we described methods for inducing colonial growth and for replica plating with P. chrysosporium (8). We also determined the physiological conditions required for fruit body formation (49). Subsequently we described methods for the mutagenesis of P. chrysosporium conidia and for the isolation of auxotrophic marker strains (11). Most recently we reported on the preparation, regeneration, and fusion of P. chrysosporium protoplasts (16).

In this report we present cytological studies of basidiospore nuclei and recombination analysis of crosses between auxotrophic strains

of this organism. Marker strains carrying multiple mutations can be readily recovered by fruiting heterokaryons of P. chrysosporium.

Materials and Methods

Organism. Cultures of P. chrysosporium ME-446 were maintained on slants of modified Vogel medium (151) supplemented with 3% malt extract and 0.15% yeast extract (8). Mutant strains were maintained on the same medium supplemented with a vitamin mixture (152) and 0.5% tryptone. Heterokaryons were forced on minimal medium consisting of modified Vogel medium supplemented with 1% glucose (11).

Fruit bodies were produced at 28°C as previously described (49), with a sixfold dilution of modified Vogel medium and 4.5% Walseth cellulose as the carbon source. For auxotrophic strains, the appropriate supplement was added to the fruiting medium (0.01 to 0.1% amino acids or 0.001% vitamins or adenine) (11). The basidiospores, which collected on the lids of inverted plates, were suspended in distilled water (49).

Wild Type Fruiting. The wild-type strain was grown on MGPT slants (2% malt extract with 2% glucose, 0.1% Bacto-Peptone (Difco), 0.001% thiamine, and 1.5% agar) as previously described (11). Conidia were washed from the slants, diluted to 10^6 spores per ml, and sonicated for 3 min at approximately 60 W in a model W 185 F cell disrupter (Heat Systems-Ultrasonics) to unclump the conidia. The conidia were then plated on solid medium (50 to 100 spores per plate), and resultant individual colonies were transferred to fruiting medium.

Germination of Basidiospores and Conidia. Basidiospores were routinely plated in two types of solid media. Medium A consisted of 10% sorbose, 1% yeast nitrogen base, and 3% agar (pH 4.8) (16). Medium B consisted of 2% glucose, 0.1% K_2HPO_4, 0.05% KH_2PO_4, 0.2% Bacto-Peptone, and 0.05% $MgSO_4$ (153) and was supplemented with 0.2% yeast extract. Agar was added to 1.5%, and the pH was adjusted to 5.8. Conidia were routinely plated on modified Vogel medium (151) with 10% sorbose and 2% agar (pH 4.8). The above media were supplemented with 0.01% amino acids, 0.001% vitamins, or adenine as appropriate.

Nuclear Staining. Basidiospore nuclei from wild-type and auxotrophic strains and from heterokaryons were stained and counted as previously described for conidial nuclei (11).

Genetic Analysis of Recombination. Colonies derived from basidiospores or conidia and plated in or on supplemented medium were transferred to minimal and supplemented slants to determine phenotypes (11).

Results

Wild Type Fruiting. Conidia from MGPT slants, which have previously been shown to be about 62% uninucleate (11), were sonicated. Sonication of a conidial spore suspension, under the conditions used, resulted in the separation of all clumps of conidia into individual spores. The germination frequency of these sonicated spores equaled that of unsonicated conidia, indicating that the procedure did not markedly affect viability. All of the colonies thus obtained from single, predominantly uninucleate, wild-type conidia produced fruit bodies and basidiospores on fruiting medium. About the same number of

basidiospores were released (approximately 10^8 spores per plate [60 by 15 mm]), regardless of whether the inoculum consisted of mycelial fragments or a colony arising from a single spore. Of these basidiospores, 20 to 40% germinated when plated in medium B. However, only about 50% of colonies obtained from individual basidiospores produced fruit bodies when transferred to fruiting medium. Of these, 74% released detectable numbers of basidiospores.

Fruiting by Auxotrophic Strains. Under the conditions used, 70% of the auxotrophic strains tested produced basidiospores. However, with certain of the amino acid auxotrophs, 10 times the usual amount of supplement was required for fruiting. Several mutant strains produced fruiting bodies but did not release detectable quantities of basidiospores. Most auxotrophs produced between 10 and 100 times fewer basidiospores per plate as compared with the wild type.

Basidiospore Germination. Germination frequencies of 15 to 40% were obtained with basidiospores from wild-type and auxotrophic strains plated in medium B. However, since medium B does not induce colony formation, sorbose-containing medium was used for recombination analysis. The highest germination with a colony-inducing medium (5 to 15%) was obtained when basidiospores were plated in medium A; this medium was therefore routinely used for recombination analysis. The addition of supplements, including yeast extract (0.01%), did not significantly increase the germination frequency in medium A.

Nuclei per Basidiospore. More than 90% of the basidiospores obtained from the wild-type and auxotrophic strains, as well as those obtained from crosses between auxotrophs, were binucleate (Table 2-1).

Some spores scored as uninucleate may have been poorly stained or positioned in such a way that only one nucleus was visible. It is most probable, however, that a small percentage of basidiospores are uninucleate. The Nic1 x His2 cross produced as many as 8% uninucleate spores.

Recombination Analysis. The results of a variety of crosses between auxotrophic strains are shown in Table 2-2. Of 12 crosses, 11 resulted in the recovery of significant numbers of double-mutant basidiospores, indicating that recombination had indeed taken place. Basidiospores from a Nic1 x Rib1 cross were plated at concentrations of five spores per plate (15 by 100 mm), thus ensuring that each resulting colony arose from a single spore. The distribution of phenotypes obtained in this way did not differ significantly from the distribution obtained when spores were plated at substantially higher concentrations, indicating that the presence of ungerminated spores was not affecting the results. The conidia from heterokaryons did not yield double-mutant recombinants (Table 2-3), indicating that the double mutants isolated from basidiospores did not arise through parasexual recombination (7,138).

Table 2-1. Number of nuclei per basidiospore[a]

Source of basidiospores	% of basidiospores that were:			No. of spores counted
	Binucleate	Uninucleate	Multinucleate	
Wild type	97	3	0	114
Arg4	97	1	2	164
Leu1	96	4	<1	363
Nic1	96	2	2	471
Met1 x Ade1	99	1	0	557
Ade1 x Rib1	98	1	1	424
His1 x Leu1	94	6	0	231
Nic1 x Rib1	99	<1	<1	504
Nic1 x His2	91	8	1	87

[a] Basidiospores from the wild-type and auxotrophic strains and from crosses of pairs of auxotrophic strains were stained with Giemsa hydrochloride and counted as previously described (11).

TABLE 2-2. Recombination analysis of crosses between various auxotrophic strains of *P. chrysosporium*[a]

Cross	Phenotypes and no. of colonies derived from basidiospores			No. of colonies examined
	Prototrophs	Auxotrophs (parental types)	Double mutants (recombinants)	
Ade1 × Rib1	26	19 Ade, 16 Rib	10	71
Nic1 × Rib1	19	18 Nic, 19 Rib	16	72
Nic1 × Rib1[b]	15	8 Nic, 5 Rib	8	36
Ade1 × Nic1	12	20 Ade, 31 Nic	5	68
Met1 × Arg1	21	10 Met, 34 Arg	7	72
Met1 × Ade1	25	18 Met, 21 Ade	7	71
Nic1 × His2	15	40 Nic, 9 His	8	72
Met1 × Ade2	19	14 Met, 21 Ade	18	72
Ade3 × Arg1	13	10 Ade, 42 Arg	6	71
Ade3 × His2	23	11 Ade, 17 His	19	70
Met1 × His1	18	19 Met, 19 His	13	69
Arg3 × Rib1	14	5 Arg, 9 Rib	4	32
His2 × Arg1	41	30 His, 63 Arg	1	135

[a] Parental strains were fruited in pairs as described in the text. The phenotypes of resultant basidiospores were analyzed as described in the text. Abbreviations refer to the phenotype of the auxotroph (11).

[b] Single spores were isolated before germination.

Table 2-3. Phenotypes of basidiospores and conidia produced by several heterokaryons[a]

Heterokaryon	Phenotypes			No. of colonies examined
	Wild-type recombinants + heterokaryons	Auxotrophs (parental types)	Double auxotrophs (recombinants)	
Met1 + Ade2				
Basidiospores	19	14 Met 21 Ade	18	72
Conidia	58	2 Met 8 Ade	0	68
Nic1 + Rib1				
Basidiospores	19	18 Nic 19 Rib	16	72
Conidia	36	0 Nic 0 Rib	0	36

[a] Heterokaryons were forced and basidiospores and conidia were produced, collected, and analyzed for phenotype as described in the text.

Discussion

In a previous report (49), we elucidated the physiological conditions required for fruit body formation and subsequent basidiospore production in P. chrysosporium. In a subsequent study (11), we described the isolation and complementation of auxotrophic mutants in this organism. In this report, we describe the use of these mutants and the technology for fruit body production for the study of genetic recombination in P. chrysosporium.

Our cytological results demonstrate that the basiospores of P. chrysosporium are binucleate (Table 2-1). Genetic recombination accompanies fruit body and basidiospore formation (Table 2-2). The conidia of heterokaryons made from two of our auxotrophic strains did not yield double-mutant recombinants (Table 2-3), indicating that the double mutants isolated from basidiospores did not arise through parasexual recombination (138).

The early observation that P. chrysosporium does not produce clamp connections (46) suggests that this organism is homothallic. Although the absence of clamp connections was confirmed in the present study, some heterothallic basidiomycetes do not produce clamp connections or do so only under particular conditions (59). That more than 200 isolates of unclumped and predominantly uninucleate wild-type conidia were all able to produce fruiting bodies yielding approximately 10^8 basidiospores per plate indicates that this organism has a primary homothallic mating system (7,35,36). Since germination rates for these basidiospores were comparable to those obtained with basidiospores produced by fruiting a

multicelled inoculum, it is unlikely that the single conidia were fruiting homokaryotically (36,154). The existence of homothallism in this organism is further supported by the fact that 70% of the auxotrophic strains produce basidiospores, since it is almost certain that each auxotroph arose from a single viable nucleus. The remaining auxotrophs may be prevented from fruiting by a physiological block(s). Further experiments are currently under way to determine whether the infertility exhibited by more than half of the colonies arising from individual basidiospores is due to a mutation(s) or some physiological or environmental factor.

The recombination analyses shown in Table 2-2 vary somewhat among the crosses between different auxotrophs. However, every cross produced single auxotrophs, double-mutant recombinants, and prototrophs. At least four of the crosses resulted in progeny with phenotypes approaching a ratio of 1:1:1:1. Although homothallism might be expected to give rise to significantly more parental types (single auxotrophs) than wild-type or double-mutant recombinants, there are several possible explanations for the observed ratios. Although preferential fusion of genetically dissimilar nuclei has been described (155), it is more likely that karyogamy is random but that heterokaryotic hyphae have a great advantage in a nutritionally forced heterokaryon. The nuclei of complementary auxotrophs would thus be in close proximity and more likely to fuse and recombine during basidiospore formation. As a result, recombination analysis of nutritionally forced heterokaryons would tend to mimic heterothallism, even in a homothallic organism. Results with conidia support this hypothesis: conidia from

heterokaryons are predominantly (>90%) binucleate (11), and >85% of the conidia from heterokaryons were themselves heterokaryotic (Table 2-3), suggesting that the hyphae from which the conidia arose contain nuclei of complementary genotypes in close proximity. Similar results have been found in the self-fertile Aspergillus nidulans (138).

The ratio of recombinants to parental types of approximately 1:1:1:1 also suggests that the binucleate basidiospores are homokaryotic and that the two nuclei arise from a postmeiotic mitotic event. This is a common occurrence among basidiomycetes (36,139). Furthermore, the existence of postmeiotic mitotic division has been confirmed in P. chrysosporium by electron microscopy (E. C. Setliff; personal communication).

Several of the crosses shown in Table 2-2 show a preponderance of wild-type over double-mutant recombinants, probably owing to contamination by heterokaryotic conidia. It is also possible that particular phenotypes were being selected for either at the level of basidiospore formation or during germination in supplemented media. Additional cytological and genetic studies are being planned to further elucidate the morphogenesis of fruit body and basidiospore formation as well as the mating system of P. chrysosporium.

The use of genetic recombination for obtaining multiple-mutant strains should have application to the construction of complex strains with superior capacities for lignocellulose bioprocessing. In this regard, the construction of mutant strains containing markers for both auxotrophy and ligninolytic defects (19) will be of particular interest.

CHAPTER 3: CHARACTERIZATION OF LEUCINE AUXOTORPHS OF THE WHITE ROT BASIDIOMYCETE PHANEROCHAETE CHRYSOSPORIUM

The basidiomycete Phanerochaete chrysosporium and other white rot fungi have potential applications in a variety of schemes for the bioprocessing of lignocellulose. Numerous studies have focused on lignin degradation (2) and cellulose metabolism (146) in P. chrysosporium, as well as on the bioprocessing of lignocellulose (147). The purification and characterization of two extracellular enzymes involved in lignin degradation (87-90) have increased our understanding of the biochemistry of this process. The elucidation of the genetic system in this fungus and comparison with analogous systems that have been thoroughly studied in other organisms could lead to the development of genetic methods for producing strains with enhanced lignin-degrading capacities. Such methods would represent a significant step toward the utilization of this organism in bioprocessing applications.

In earlier studies we described methods for inducing colonial growth and for replica plating with P. chrysosporium (8) and determined the physiological conditions required for fruiting body formation (49). Subsequently we described the mutagenesis of conidia, the isolation of auxotrophic marker strains, and complementation studies with these various auxotrophs (11). We also reported on the preparation, fusion, and regeneration of P. chrysosporium protoplasts (16) from auxotrophic

strains. Most recently, we reported on the use of these auxotrophs for studies of genetic recombination (156).

The enzymes and the gene loci and regulation involved in the biosynthesis of leucine have been studied in detail in several procaryotic and eucaryotic organisms, including Escherichia coli (144), Neurospora crassa (141,142), and Saccharomyces cerevisiae (143,145,157). In this report we present the results of biochemical genetic studies on various enzymes involved in the biosynthesis of leucine in P. chrysosporium. Our wild-type strain, the five leucine auxotrophs described previously (11), and a newly isolated leucine auxotroph were used in the studies. Heterokaryons made from leucine auxotrophs of different complementation groups were also used. We demonstrate that our six P. chrysosporium leucine auxotrophs correspond to mutations in three different genes, each leading to a deficiency of one of three enzymes specific to leucine biosynthesis. These mutations are analogous to those identified in other microorganisms (141-143,145,157).

Materials and Methods

Organism and Growth Media. Cultures of P. chrysosporium ME-446 were maintained on slants of Vogel medium N (158), with thiamine replacing biotin (modified Vogel medium) (151), supplemented with 2% glucose. Auxotrophic strains were maintained on slants of double-strength modified Vogel medium supplemented with 3% malt extract, 0.5% tryptone, 0.5% yeast extract, and a vitamin mixture (152). Heterokaryons were forced on minimal medium consisting of modified Vogel medium supplemented with 1% glucose (11).

Fresh cultures for dry-weight determination were grown in liquid modified Vogel medium containing 2% glucose, with or without 3 mM leucine. Fresh cultures for crude cell extracts were grown in the above medium supplemented with 1.5 mM leucine for growth of the auxotrophic strains. The auxotrophs were also grown in the above medium supplemented with 1.5 mM α-ketoisocaproate.

Chemicals. β-Isopropylmalate (β-IPM) for the isomerase and dehydrogenase assays was kindly provided by G. B. Kohlhaw, Purdue University. All other chemicals were purchased from Sigma Chemical Co.

Isolation of the Leucine Auxotroph. In addition to the five previously isolated leucine auxotrophs, a newly isolated strain (Leu6) was obtained through X-ray mutagenesis and tested for complementation with the other leucine auxotrophs as previously described (11).

Dry-Weight Determinations. Spore suspensions (0.25 ml of suspensions of approximately 10^6 spores per ml) were inoculated into 250-ml flasks containing 20 ml of liquid medium as described above. Stationary cultures were incubated at 37°C. After 3 days, the mycelial mats were harvested by suction filtration, dried at 45°C for 48 h in tared dishes, and weighed.

Preparation of Crude Cell Extracts. Stationary cultures were inoculated as described above and incubated at 37°C for 3 to 4 days. The mycelial mats from two such cultures were homogenized in a Waring blender, inoculated into a 2-liter flask containing 1 liter of medium, and incubated at 28°C at 150 rpm on a rotary shaker for 3 days. The mycelia were then harvested, washed by suction filtration, and ground with sand in approximately 25 ml of cold 50 mM Tris hydrochloride (pH

7.1) in a chilled mortar. The resultant suspension was centrifuged at 11,000 x g (4°C), and the supernatant was assayed for enzyme activity.

Enzyme Assays. α-Isopropylmalate (α-IPM) synthase activity was determined by the method of Ulm et al. (143), with 5,5'-dithiobis-(2-nitrobenzoate) to measure the liberation of coenzyme A from acetyl coenzyme A. Reactions were run for 20 min at 30°C and stopped with 95% ethanol. ΔA_{412} was measured with a Shimadzu UV-260 spectrophotometer; cuvettes with a 1-cm light path were used. The value was corrected with a blank lacking the substrate α-ketoisovalerate.

IPM isomerase activity was determined by monitoring the rate of formation of dimethylcitraconate from β-IPM at 234 nm by the method of Gross (141).

β-IPM dehydrogenase activity was determined by monitoring the reduction of NAD at 340 nm in the presence of β-IPM (159). The initial rate of NAD reduction was determined by using a reaction mixture containing 100 mM potassium phosphate (pH 8.0), 0.7 mM $MgCl_2$, 66 mM KCl, 1.1 mM NAD, 4.2 mM pyrazole, and 30 μl of cell extract. β-IPM was then added to a final concentration of 1 mM in a total volume of 0.2 ml, and the new rate of NAD reduction was measured.

Protein concentration in the crude extracts was determined by the method of Lowry et al. (160), with bovine serum albumin as the standard.

Results

In an earlier report we determined the complementation groups for our five leucine auxotrophs (11). The newly isolated leucine auxotroph (Leu6; OGC 1121-3) formed a heterokaryon on minimal medium with all

other leucine auxotrophs except Leu3. Taken with our previous results (11), which we recently confirmed, these results show that these six auxotrophs form three complementation groups consisting of (i) Leu1 and Leu5; (ii) Leu2 and Leu4; and (iii) Leu3 and Leu6.

The dry-weight determinations for 3-day-old stationary cultures of the P. chrysosporium wild type, leucine auxotrophs, and forced heterokaryons of leucine auxotrophs are shown in Table 3-1. When medium lacking leucine was inoculated with conidia from the leucine auxotrophs, there was no detectable growth. When the leucine auxotrophs were grown in media containing 3 mM leucine, their dry weights were similar to the dry weights of the wild type and the heterokaryons. All leucine auxotrophs grew well when the medium was supplemented with α-ketoisocaproate rather than leucine.

The specific activities of the three leucine biiosynthetic enzymes in each of the strain are shown in Table 3-2. The crude extract of Leu2 lacked detectable α-IPM synthase activity, while that of Leu4 exhibited low levels of synthase activity in one assay and no detectable activity in a second assay. The crude extracts of Leu3 and Leu6 lacked detectable isomerase activity. The extracts of Leu1 and Leu5 lacked detectable β-IPM dehydrogenase activity. In contrast, all three enzyme activites were present in the wild type and in the heterokaryotic strains.

In several cases the isomerase and dehydrogenase activities of the auxotrophs were significantly higher than in the wild-type strain (Table 3-2). For example, the specific activities of the isomerase in Leu1 and Leu5 were 10.4- and 9.5-fold higher, respectively, than those of the

TABLE 3-1. Growth of wild-type, leucine auxotrophic, and heterokaryotic strains[a]

Strains	Dry wt (g)[b] when grown in:	
	2% glucose + Vogel medium	2% glucose + Vogel medium + 3 mM leucine
Wild type	0.141	0.123
Leu1	—[c]	0.061
Leu2	—	0.089
Leu3	—	0.105
Leu4	—	0.118
Leu5	—	0.116
Leu6	—	0.109
Leu1 + Leu4	0.106	
Leu5 + Leu6	0.130	
Leu2 + Leu3	0.107	

[a] Culture conditions are described in the text.

[b] Average of two cultures.

[c] —, Negligible.

TABLE 3-2. Specific activity of leucine biosynthetic enzymes in various strains of P. chrysosporium[a]

Strains	Specific activity of:		
	α-IPM synthase	IPM isomerase (10^3 U/mg of protein)[b]	β-IPM dehydrogenase
Wild type	0.50	17.5	3.3
Leu1	0.61	182.0	<0.01
Leu2	<0.001	51.0	13.3
Leu3	1.12	<0.01	3.3
Leu4	0.16, <0.001[c]	63.7	11.3
Leu5	0.62	166.5	<0.01
Leu6	1.07	<0.01	2.0
Leu1 + Leu4	1.22	48.9	3.6
Leu5 + Leu6	0.40	50.7	2.4
Leu2 + Leu3	0.79	25.5	5.8

[a] Cells were grown, broken, and extracted, and enzymes were assayed as decribed in the text.

[b] 10^3 micromoles per minute per milligram of protein or nanomoles per minute per milligram of protein.

[c] Values from two separate experiments.

wild-type strain. In Leu2 and Leu4, the isomerase specific activity was approximately threefold higher. Likewise, the dehydrogenase activities of Leu2 and Leu4 were 4.0- and 3.4-fold higher, respectively, than those of the wild type.

Each of the heterokaryons exhibited all three enzyme activities. In the case of the Leu5-Leu6 heterokaryon, each component had α-IPM synthase activity when grown alone, and synthase activity in the heterokaryon was approximately equal to that in the wild type. However, with heterokaryons in which one component lacked synthase activity, the heterokaryotic strain exhibited higher synthase activity than did the wild type. All three heterokaryons exhibited slightly higher IPM isomerase activity than did the wild type, but in each case the values were lower than the enzyme activity found in the individual auxotrophs that possessed IPM isomerase. The levels of α-IPM dehydrogenase activity in all three heterokaryons were similar to that of the wild type.

Discussion

The leucine biosynthetic pathway has been elucidated in *E. coli*, *S. cerevisiae*, and *N. crassa* (141-145,157) (Table 3-3). The steps in this pathway are catalyzed by the specific enzymes α-IPM synthase, IPM isomerase, and β-IPM dehydrogenase (Fig. 3-1). No mutants completely blocked in the final step of the pathway have been isolated. Therefore, it has been inferred that more than one enzyme can catalyze the transamination of α-ketoisocaproate to leucine (161). We showed previously (11) that our *P. chrysosporium* leucine auxotrophs form three different complementation groups: group I (Leu2 and Leu4), group II

TABLE 3-3. Biochemical genetic characterization of the leucine pathway in *P. chrysosporium*, *S. cerevisiae*, *E. coli*, and *N. crassa*

Organism	Mutant genes			Reference
P. chrysosporium[a]	*leu3* (Leu2, Leu4)[b]	*leu1* (Leu3, Leu6)[c]	*leu2* (Leu1, Leu5)[d]	This work
S. cerevisiae	*leu3*	*leu1*	*leu2*	145
E. coli	*leuA*		*leuB*	144
N. crassa	*leu4*	*leu2*	*leu1*	141

[a] Mutants were characterized as described in Materials and Methods.

[b] (α-IPM synthase).

[c] (IPM isomerase).

[d] (β-IPM dehydrogenase).

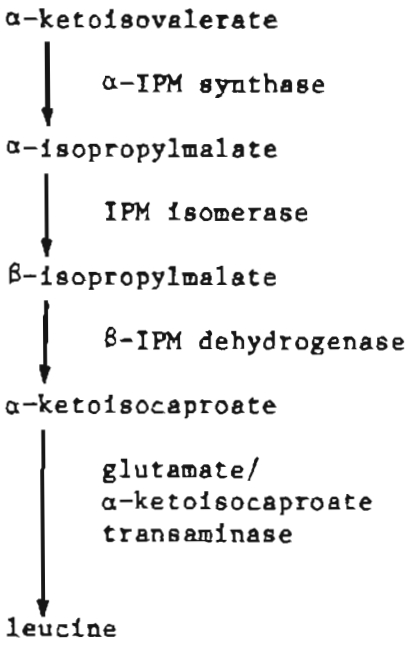

FIGURE 3-1. Leucine biosynthetic pathway in microorganisms (from Ref. 145).

(Leu1 and Leu5), and group III (Leu3). Complementation studies with our newly isolated leucine auxotroph, Leu6, indicate that it belongs with Leu 3 in group III.

Although in the absence of leucine none of the leucine auxotrophs grew in liquid culture, in the presence of leucine they grew approximately as well as the wild type and heterokaryons made from leucine auxotrophs. The poor growth exhibited by Leu1 may be due to poor uptake of leucine or inhibition of growth by leucine in the medium (Table 3-1). Since all of the auxotrophs grew well in liquid culture supplemented with α-ketoisocaproate, none of these strains are deficient in glutamate-α-ketoisocaproate transaminase, the final enzyme in the pathway (162). The results of assays for the other three enzymes in the pathway (Table 3-2) confirm our complementation studies with heterokaryons. Fungal mutations in a single complementation group probably reflect different lesions in the same gene (7). While it is not certain that each of these mutations is in a structural gene, this is the most probable explanation, since each mutation negatively affects only one of the enzymes.

Although Leu4 does not grow in liquid minimal medium, it does occasionally grow at a very slow rate on minimal slants and plates. The low level of α-IPM synthase activity detected in one assay (Table 3-2) suggests that Leu4 is leaky. In several instances, the specific activities of each of the other two leucine biosynthetic enzymes are considerably higher in the auxotrophs than in the wild type (Table 3-2). Similar results have been obtained with N. crassa (141). The high levels of IPM isomerase activity in Leu1 and Leu5 may reflect different

levels of α-IPM. α-IPM induces the IPM isomerase in N. crassa (141). Although enzyme levels varied considerably among the three groups of auxotrophs, those within a group exhibited remarkably similar levels of enzyme activity. This suggests that the high enzyme levels in the auxotrophs as compared with those in the wild type, as well as the variation in activities among the three groups, probably reflect regulation of the pathway.

Heterokaryons made from leucine auxotrophs in different complementation groups exhibited all three enzyme activities. The specific activity of IPM isomerase was higher in the heterokaryons than in the wild type, but lower than in the individual auxotrophs that possess the isomerase activity (Table 3-2).

The leucine genes in yeasts, E. coli, and N. crassa that are analogous to the leucine auxotrophs isolated from P. chrysosporium are shown in Table 3-3. The P. chrysosporium genes have been classified according to the nomenclature system for Saccharomyces cerevisiae. Thus, P. chrysosporium Leu2 and Leu4 lack α-IPM synthase, corresponding to the yeast leu3 gene; Leu3 and Leu6 lack IPM isomerase, corresponding to the yeast leu1 gene; and Leu1 and Leu5 lack β-IPM dehydrogenase, corresponding to the yeast leu2 gene. With this biochemical characterization of the leucine auxotrophs of P. chrysosporium, we are now prepared to utilize these mutants in additional studies designed to further elucidate the genetics and molecular genetics of this organism.

CHAPTER 4: MATING SYSTEM AND BASIDIOSPORE FORMATION IN THE LIGNIN-DEGRADING BASIDIOMYCETE PHANEROCHAETE CHRYSOSPORIUM

The basidiomycete Phanerochaete chrysosporium has been the focus of numerous studies on lignin degradation (2), cellulose metabolism (146), and the bioprocessing lignocellulose (147). The purification and characterization of two P. chrysosporium extracellular peroxidases involved in lignin degradation (87-90,149) have greatly increased our understanding of the biochemistry of this process. Elucidation of the life cycle and genetic system of P. chrysosporium should lead to the development of genetic methods for producing strains with enhanced lignin-degrading properties. Such methods would represent a significant step toward the use of this organism for industrial bioprocessing of lignin.

In earlier studies we described methods for inducing colonial growth in P. chrysosporium and for replica plating colonies (8). We also determined the physiological conditions required for fruit body formation (49) and described the mutagenesis of conidia, isolation of auxotrophic marker strains, and complementation studies with these various auxotrophs (11). In addition, we reported on the preparation, fusion, and regeneration of protoplasts from wild-type and auxotrophic strains (16). Most recently we reported on genetic recombination utilizing auxotrophic strains (156) and on the biochemical characterization of leucine auxotrophs from this organism (163).

In this report, we present genetic evidence from crosses between auxotrophic mutants which indicates that the binucleate basidiospores are homokaryotic. We also present cytological and genetic evidence indicating that P. chrysosporium has a primary homothallic mating system (i.e., it is self-fertile).

Materials and Methods

Organism. The following strains of P. chrysosporium, obtained from either the U.S. Department of Agriculture Northern Regional Research Center or the U.S. Forest Products Laboratory, were maintained on slants as previously described (156): ME-446, ML-21, ME-416, FP-104297, P-2843f, P-1271, and BKM-F-1767. A strain of P. chrysosporium isolated in our laboratory, OGC101, was obtained from ME-446 by plating conidia on medium containing sorbose and selecting individual colonies. This newly isolated strain has somewhat different properties from ME-446. Auxotrophic mutants obtained from either ME-446 or OGC101 were maintained on slants as previously described (156).

Fruiting. Fruit bodies were produced on plates of one-sixth strength modified Vogel Medium (151) containing 4.5% Walseth cellulose as previously described (49,156). Plates were inverted over beakers immediately before ejection of basidiospores to minimize conidial contamination. Spores were collected in 5 ml of 0.1 N HCl containing 0.1% Tween 80. The detergent maximized basidiospore germination in plates, and the acid minimized clumping of the spores without affecting viability. Basidiospores were diluted promptly in 0.1% Tween 80 and plated by overlaying with modified Vogel medium containing 2% sorbose

and 3% agar (pH 4.8) (medium A). Colonies derived from individual basidiospores were either transferred directly to fruiting medium or were first grown on slants and then transferred.

Conidia from mycelia grown on slants containing malt extract, glucose, Bacto-Peptone (Difco Laboratories, Detroit, Mich.), and thiamine (MGPT) (11), previously shown to be approximately 62% uninucleate (11), were sonicated as previously described to unclump the spores (156). The conidia were then diluted and plated on modified Vogel medium with 4% sorbose, 0.02% yeast extract, and 2% agar (pH 5.8) (medium B). Individual colonies were either transferred directly to fruiting medium or grown on slants before transfer.

Heterokaryons between auxotrophic stains were forced on unsupplemented fruiting medium, and the resultant basidiospores were plated in medium A supplemented with 0.01% amino acids or 0.001% vitamins or adenine, as appropriate. Individual colonies were then transferred to minimal and supplemented slants to determine phenotypes (11). The prototrophs so obtained were fruited again, and the resultant basidiospores were plated and examined for phenotype as above (Fig. 4-1).

Cytology. Nuclei in conidia, basidiospores, and mycelia were stained with mithramycin A (Sigma Chemical Co., St. Louis, Mo.) (0.012%) in 30 mM $MgCl_2$-50% ethanol (164) and examined with a Zeiss P7082 fluorescence microscope. The number of sterigmata per basidium was determined either by light microscopy or with a JEOL JSM-35 scanning electron microscope after fixing scrapes from fruiting plates (1.8% formaldehyde, 1.5% glutaraldehyde, 1% acrolein in 0.1 M phosphate buffer

[pH 7] containing 4% sucrose and 0.1 mM $CaCl_2$), dehydrating in acetone, and sputter coating with gold-palladium.

Growth Experiments. Stationary flasks (250 ml) containing 20 ml of liquid medium (2% glucose, 12 mM ammonium tartrate, trace elements) as previously described (165) in 20 mM sodium 2,2-dimethylsuccinate (pH 4.5) were inoculated with 2.5×10^5 spores. Dry weights were then determined as described previously (163) after 3 days of growth at 38°C. Linear growth rates were measured in growth tubes containing 3% malt extract and 0.15% yeast extract in modified Vogel medium N (151) by a previously described method (166). Conidiation was measured after 9 days of growth on slants at 38°C. Using a spatula, the spores were washed into 10 ml of water and counted in a hemacytometer.

Assay of $^{14}CO_2$ Evolution. $^{14}CO_2$ evolution from ^{14}C-side chain-radiolabeled lignin (DHP) was measured as previously described (19), except that the labeled lignin was added to 3-day-old stationary cultures.

Results

Basidiospore Formation. Since all strains of P. chrysosporium which we examined had binucleate basidiospores, it was of interest to determine whether the spores were heterokaryotic (i.e., two nuclei migrating into each spore) or homokaryotic, with the two nuclei arising via a postpackaging mitotic event. Five different pairs of auxotrophic strains of P. chrysosporium were crossed as shown in Table 4-1. In every case except His3 x Arg3, all four phenotypes were recovered from the original crosses, indicating that recombination had occurred.

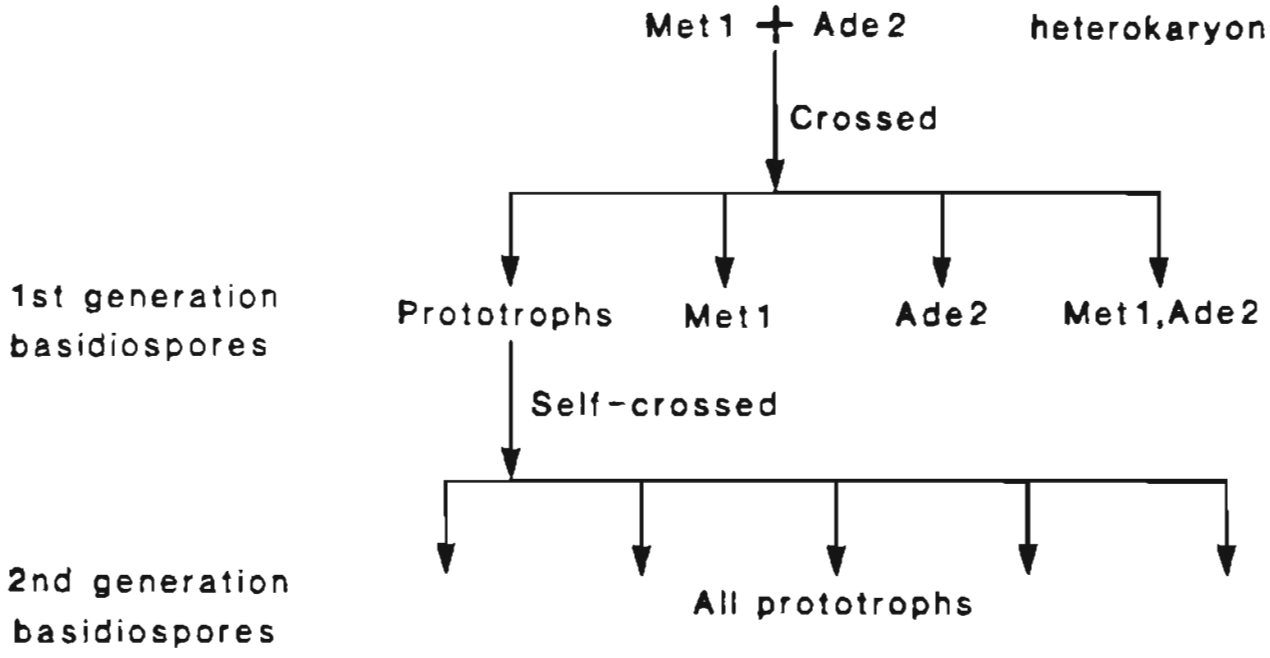

FIGURE 4-1. Prototrophs recovered from basidiospores of nutritionally forced heterokaryons were fruited and the progeny phenotypes determined as described in the text.

TABLE 4-1. Self-crosses of prototrophs recovered from crosses between auxotrophic mutants of *P. chrysosporium*[a]

Original cross (prototrophs)	Prototrophs	Auxotrophs[b]
Nic1 x Rib1		
1	35	0
2	30	0
3	27	0
4	34	0
5	33	0
6	36	0
7	16	0
8	18	0
9	36	35 Rib^-
10	25	1 Rib^-
11	34	1 Nic^-
12	24	3 Nic^-
13	13	5 Nic^-
14	7	10 Nic^-
15	10	5 Nic^-
Met1 x His1 (1)	24	0

(continued)

TABLE 4-1, continued:

Original cross (prototrophs)	Prototrophs	Auxotrophs[b]
Met1 × Ade2		
1	35	0
2	35	0
3	49	0
4	41	0
5	60	0
6	16	0
7	18	0
His3 × Arg3		
1	36	0
2	34	0
3	34	0
4	32	0
His2 × Ade3		
1	33	0
2	35	0
3	26	0

[a] Crosses were performed as described in the text.

[b] No double auxotrophic recombinants were recovered.

Prototrophs recovered from these crosses, which theoretically could be either wild-type recombinants or heterokaryons, were then transferred to fruiting medium (Fig. 4-1). Basidiospores from those prototrophs which fruited successfully were diluted and plated, and their phenotypes were established. It can be seen from Table 4-1 that in almost every instance the fruiting prototrophs yielded only prototrophs in the next generation, indicating that they were wild-type recombinants rather than heterokaryons. This was evident even with His3 x Arg3 in which the corresponding double auxotrophic recombinant was not recovered. Of the 15 prototrophs from Nic1 x Rib1, 7 yielded either nicotinamide (Nic^-) or riboflavin (Rib^-) auxotrophs in addition to prototrophs, but in no case did a single prototroph yield both auxotrophs or the double auxotrophic recombinant. However, when rigorous precautions were not taken to avoid conidial contamination and clumping of basidiospores, some of the recovered prototrophs proved to be heterokaryons which, upon fruiting, yielded all four phenotypes.

It has been reported previously that the number of sterigmata per basidium in some strains of P. chrysosporium varied between 2 and 8 (27,41). This result was confirmed in the present study for several P. chrysosporium strains by using light and electron microscopy. Strains ME-446, FP-104297, BKM-F-1767, ML-21, and OGC101 all had between two and seven sterigmata per basidium at the time examined. However, in each strain the majority of basidia appeared to have four sterigmata.

Mating System. All strains of P. chrysosporium examined by fluorescent microscopy had multinucleate septate mycelia with as many as 15 nuclei randomly dispersed throughout each cell. None of the strains

examined had clamp connections, confirming an earlier report for ME-446 (46).

We previously reported that 100% of colonies obtained from single, predominantly uninucleate conidia of strain OGC101 produced fruit bodies and basidiospores on fruiting medium (156). These results have now been extended to sonicated conidia from other strains of P. chrysosporium (Table 4-2), although in several of these strains, conidia from MGPT slants were predominantly binucleate rather than uninucleate.

The results of fruiting colonies arising from single basidiospores of various strains of P. chrysosporium are also shown in Table 4-2. All such colonies from ME-416, FP-104297, and P-2843f strains fruited normally, suggesting that these strains are homothallic. Basidiospores from ME-446, P-1271, OGC101, and ML-21 gave rise to a significant number of colonies which grew normally on fruiting medium but which did not produce basidiospores, or did so only very sparingly. Some of these isolates produced basidia which did not mature to the point of sporulation. All these isolates have been defined as nonfruiters, in contrast to the fruiting isolates, which produced viable basidiospores almost as efficiently as the wild type. In addition, many of the basidiospores from strain OGC101 yielded isolates which did not grow on cellulose and could not be induced to fruit under other conditions. These have been defined as Cel^- isolates. Finally, in most cases basidiospores obtained from fruiting auxotrophs gave rise to colonies which all fruited.

To determine whether the fruiting isolates recovered from OGC101 were the result of complementation between nonfruiting phenotypes, due

TABLE 4-2. Fruiting of single conidia and basidiospores from various strains of Phanerochaete chrysosporium [a]

Strain	Conidia			Basidiospores		
	Colonies fruiting	Total	% Uninucleated conidiospores [b]	Fruiting isolates [c]	Nonfruiting isolates [d]	Cel^- [e]
OGC101	87	87	62	18	6	23
ME-446	38	38	75	6	8	0
ML-21	10	10	25	7	9	0
ME-416	9	9	25	19	0	0
FP-104297	27	27	60	16	0	0
P-2843F	23	25	50	10	0	0
BKM-F-1767	27	27	<10	4	1	0
P-1271	27	27	90	5	11	0

[a] Basidiospores and sonicated conidia were plated out; resulting colonies were transferred to fruiting medium as described in the text.

[b] Estimated by fluorescence microscopy after staining with mithramycin A as described in the text or after Giemsa staining as described previously (11).

[c] Fruiting isolates produce fruit bodies and basidiospores similar to those of the parent wild-type strain.

[d] Nonfruiting isolates fruit very little or not at all.

[e] Cel^- isolates do not grow on cellulose and have not been induced to fruit under other conditions.

either to heterokaryotic basidiospores or to colonies arising from multiple spores, progeny basidiospores from fruiters were plated out, and the resultant colonies were transferred to fruiting medium. Fruiting isolates arising from the original wild-type cross produced basidiospores which all fruited (Fig. 4-2). This usually happened in the first generation, although it was sometimes necessary to continue to select fruiting isolates for several generations before the nonfruiting and Cel$^-$ phenotypes were eliminated, indicating that in these cases the original fruiting phenotype included other genotypes as well. This was probably a result of colonies arising from clumped spores.

Occasionally, pairs of nonfruiting and Cel$^-$ isolates produced basidiospores when allowed to grow together on fruiting medium. The basidiospores from such crosses gave rise to colonies of all three phenotypes. Pairs of nonfruiting isolates originating from the same strain did not produce basidiospores. However, in some cases, pairs consisting of a nonfruiter from OGC101 and a nonfruiter from another strain of P. chrysosporium fruited very well when inoculated together. Pairs of Cel$^-$ isolates did not complement on fruiting medium.

Heterokaryosis in Strain OGC101. OGC101 nonfruiting isolates differed from each other, as well as from the fruiting and Cel$^-$ isolates and the parental wild-type strain, in several characteristics (Table 4-3). Growth of the fruiting isolate, as measured by dry weight, was 72% of that of the parental wild type. In contrast, the dry weights of two nonfruiting isolates were 50 and 55% of the wild type. The Cel$^-$ isolate was only 27% of the wild-type dry weight. The fruiting, nonfruiting, and Cel$^-$ isolates had linear growth rates that were 88, 78, and 48% that

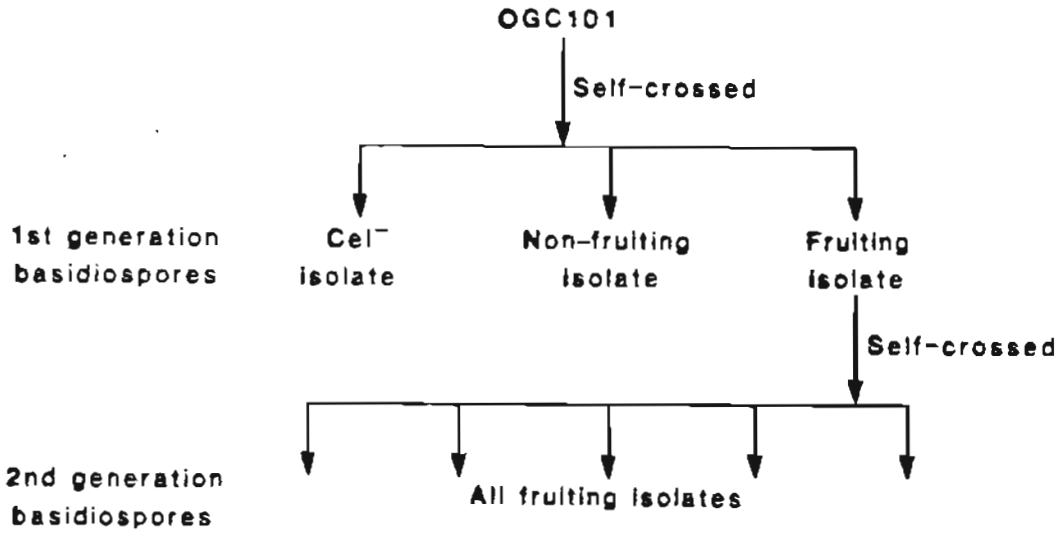

FIGURE 4-2. Fruiting isolates recovered from basidiospores of wild-type strain OGC101 were self-crossed and the progeny phenotypes determined as described in the text.

TABLE 4-3. Comparison of wild-type, fruiting, nonfruiting, and Cel^- strains of *P. chrysosporium* OGC101 [a]

Strain	Dry wt (g) [b]	Linear growth (mm/h) [c]	Asexual sporulation (spores/ml)	Fruiting of colonies from single asexual spores	Basidiospore formation (spores/plate)
Wild type [d] (OGC 101)	0.121	1.04	2.1×10^7	87/87 fruited	1.7×10^8
Fruiter [d]	0.087	0.91	4.2×10^6	15/15 fruited	1.1×10^8
Nonfruiter 1 [e]	0.066	ND [f]	1.0×10^5	0/20 fruited	0
Nonfruiter 2 [e]	0.061	0.81	1.5×10^7	ND	5.0×10^6
Nonfruiter 3 [e]	ND	ND	1.9×10^6	ND	0
Cel^- [e]	0.033	0.50	3.0×10^4	16/16 Cel^-	0

[a] Isolates are as defined in Table 4-2. Comparisons were made as described in the text.

[b] Dry weights were determined as described in the text; average of two flasks.

[c] Linear growth was measured for 12 days at 28°C as described in the text.

[d] Asexual spores are primarily conidia.

[e] Asexual spores are primarily arthrospores.

[f] ND, Not determined.

of the wild type, respectively. The fruiting isolate produced only 20% as many conidia as the wild type, whereas nonfruiting isolates varied in asexual sporulation from 0.5 to 71% of the wild type. Most Cel^- isolates produced essentially no asexual spores. Conidia from the fruiting isolate were morphologically identical to those of the wild type. In contrast, the nonfruiting and Cel^- isolates produced an excess of cylindrical and irregular bodies which appeared to be arthrospores (41,46) and were similar to those produced by many auxotrophic mutants of P. chrysosporium. Spore suspensions from nonfruiting isolates also contained large numbers of chlamydospores (46) as compared with the wild type. In all cases, colonies arising from spores, of whatever type, were identical to the parental strain with regard to their ability to fruit (Table 4-3). The fruiting isolates released approximately 65% as many basidiospores as the wild type. A few of the nonfruiting isolates occasionally released a small number of basidiospores, never exceeding about 3% of the number produced by the wild type. However, the nonfruiting isolates grew very well on fruiting medium. The Cel^- isolates exhibited little or no growth with cellulose as the sole carbon source. The isolates also differed from the wild type and from each other in their ability to evolve $^{14}CO_2$ from ^{14}C-side chain-labeled lignin (Fig. 4-3). Whereas the fruiting isolate was very similar to the wild type in this respect, different nonfruiting isolates varied considerably. In no instance was $^{14}CO_2$ evolution detected from Cel^- cultures.

Discussion

Basidiospore Formation. In most instances, prototrophs recovered from crosses between two auxotrophic strains of P. chrysosporium, when fruited, yielded basidiospores which gave rise to only prototrophic colonies (Table 4-1). This was true even in the case of His3 x Arg3 in which neither Arg^- nor the double auxotrophic recombinant were recovered. This strongly suggests that the prototrophs are true wild-type recombinants rather than complementary heterokaryons and that the binucleate basidiospores are therefore homokaryotic, arising via a postpackaging mitotic event. Of 15 fruiting prototrophs from Nic1 x Rib1 7 yielded either Nic^- or Rib^-. However, both auxotrophs were not recovered from a single prototroph, nor was the double auxotroph ever recovered. Therefore, in these instances it is likely that, despite the precautions taken, a wild-type and an auxotrophic spore were clumped and underwent plasmogamy upon germination.

That the basidiospores of P. chrysosporium are homokaryotic is supported by other evidence. As previously reported, the results of recombination experiments between auxotrophic mutants strongly suggest that the basidiospores are homokaryotic (156). Were the binucleate basidiospores to arise by the random migration of two nuclei into each spore, a large preponderance of prototrophs would be expected from such crosses. However, in most cases, the ratio of phenotypes approached 1:1:1:1, indicating that the nuclei in each spore are identical.

The possibility of an occasional heterokaryotic spore cannot be completely ruled out. The existence of variable postmeiotic mitosis

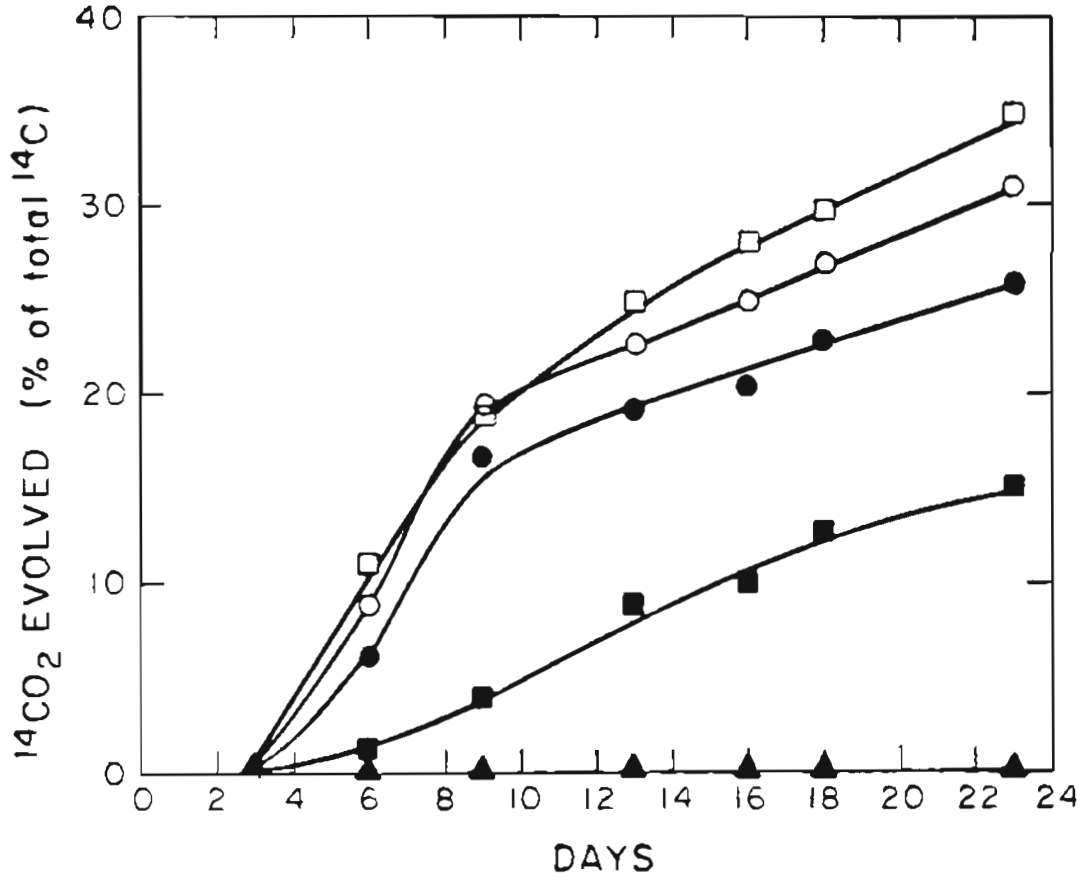

FIGURE 4-3. $^{14}CO_2$ evolution from ^{14}C-sidechain-labeled lignin by wild-type (○), fruiting (●), non-fruiting 1 (■), non-fruiting 2 (□) and Cel^- (▲) strains of P. chrysosporium OGC101. Stationary cultures containing 20 ml of medium in 250 ml flasks were purged periodically with 100% O_2 and $^{14}CO_2$ evolution was measured as described in the text.

occurring in either the basidium or the spore, resulting in basidiospores that can be either homokaryotic or heterokaryotic, has been reported for other basidiomycetes (167,168). The occurrence in P. chrysosporium of an irregular number of sterigmata per basidium is most unusual among basidiomycetes (41) and could conceivably be related to the packaging of nuclei into the spores. However in many of the basidia examined, the fifth sterigma appeared to have poorly developed—perhaps vestigial—spores, and it has been reported that in cases in which the basidia of P. chrysosporium bear six sterigmata, the spores mature in two distinct groups of three each (41). At any rate, our evidence indicates that homokaryotic basidiospores are the norm in this organism.

Mating System. Our results also indicate that P. chrysosporium exhibits a homothallic or self-fertile mating system. Typically, heterothallic hymenomycetes form a true dikaryon with one pair of compatible nuclei per cell, with or without clamp connections (7). In contrast, we observed as many as 15 nuclei per hyphal cell in various strains of P. chrysosporium. With the exception of strain P-2843f, predominantly uninucleate monosporous colonies always fruit normally, and 92% of the P-2843f colonies fruited (Table 4-2). In addition, many auxotrophic strains of P. chrysosporium fruit, and presumably each of these strains arose via a mutation in a single haploid nucleus. Fruiting heterokaryons have been obtained with every attempted combination of complementary auxotrophs, again making heterothallism unlikely. The fact that basidiospores from P. chrysosporium ME-416, FP-104297, and P-2843f give rise to colonies which all fruit is also strong evidence for homothallism (Table 4-2). In strains which segregate out

basidiospores of different phenotypes, the fruiting isolates continue to yield all fruiters in succeeding generations (Fig. 4-2). Since fruiting isolates recovered in this way do not segregate out nonfruiting phenotypes in subsequent generations, it is unlikely that fruiting is the result of complementation between different nuclei. Again, this indicates that the basidiospores are homokaryotic and that the organism is homothallic. Secondary homothallism can be ruled out by the existence of homokaryotic basidiospores which fruit.

The fact that pairwise combinations of nonfruiting isolates from the same strain never fruit indicates that these isolates do not represent mating types, nor are they able to complement one another. Nonfruiting isolates from OGC101 do occasionally fruit normally when inoculated together with nonfruiting isolates from other strains or with Cel^- isolates, suggesting that in these instances complementation has occurred. Crosses between nonfruiting and Cel^- isolates can produce basidiospores of the fruiting phenotype which yield only fruiting isolates in subsequent generations. This suggests that recombination has occurred between alleles that enable normal fruiting to take place.

While homothallism is not uncommon among the higher basidiomycetes, it has not been well studied (7). In some species, different strains can exhibit different mating systems (59), and our results do not preclude the existence of heterothallism in other strains of P. chrysosporium.

Heterokaryosis in Strain OGC101. This strain of P. chrysosporium appears to be a heterokaryon composed of fruiting, nonfruiting, and Cel^- nuclei. Such heterokaryosis has been reported for other homothallic

basidiomycetes (155). Fruiting, nonfruiting, and Cel^- basidiospore isolates segregate out in approximately equal numbers. Again, this indicates that the basidiospores are homokaryotic since, if they were heterokaryotic, complementation should result in a preponderance of fruiting isolates.

Nonfruiting isolates differ markedly among themselves in a variety of traits, including fruit body formation and asexual sporulation (Table 4-3) and $^{14}CO_2$ evolution from radiolabeled lignin (Fig. 4-3). This suggests that each of these isolates is the result of unique recombination events between genetically distinct nuclei. The presence of fruit bodies and the occasional release of basidiospores in some nonfruiting isolates may also imply that these isolates are recombinants for traits that may be only indirectly related to fruiting.

Nonfruiting and Cel^- phenotypes are not observed with conidial isolates from the wild-type strain OGC101. However, these phenotypes do segregate out when conidial isolates are fruited, indicating that they were present in the conidiospores, many of which are multinucleate. It is therefore likely that conidia containing these nuclei do not germinate unless they are heterokaryotic. In contrast to the wild type which conidiates profusely, the nonfruiting and Cel^- isolates, if they sporulate at all, appear to make many more arthrospores and chlamydospores than conidia (46). Since arthrospores are formed by the fragmentation of a hypha and chlamydospores are formed from hyphal cells, they apparently do not require complementary nuclei to germinate.

The growth and patterns of asexual sporulation seen with the nonfruiting and Cel^- isolates of OGC101 are reminiscent of those seen

with many of the auxotrophic mutants of P. chrysosporium that were obtained from either OGC101 or ME-446. This may reflect the genotypes of the nuclei from which the auxotrophs arose. It may also explain why some auxotrophs cannot be induced to fruit.

Further research is needed to characterize these isolates and determine their significance for fruiting studies and lignin degradation in P. chrysosporium. For example, it is unclear whether Cel^- isolates are truly defective in fruiting as well as in other metabolic functions. Several strains, including ME-466, appear to segregate out fruiting and nonfruiting isolates, but not the Cel^- phenotype. It would be of interest to determine whether the latter phenotype is the result of mutation in strain OGC101 alone or whether it is present in other strains but is perhaps not viable as a homokaryon. A report of high-frequency cellulase mutations in P. chrysosporium (27) may, in fact, reflect the presence of these nuclei in other strains.

Since P. chrysosporium mycelia are multinucleate, and even under optimum conditions, the conidia are only 62% uninucleate (11), obtaining large numbers of mutants with this organism has proved difficult. However, the homokaryotic basidiospores should provide a useful means for isolating mutants or transformants that might otherwise be masked by a wild-type or complementary nucleus. Fruiting the organism after mutagenesis or transformation could allow for the segregation of homokaryotic isolates of the desired genotype.

Similarly, homothallism in P. chrysosporium has advantages for genetic studies, provided appropriate genetic markers are available. Homothallism suggests that any two strains of this organism should be

capable of undergoing plasmogamy and recombination. Additionally, as with Aspergillus nidulans, homothallism in P. chrysosporium will allow for the self-crossing of transformants to test directly for meiotic instability after transformation (169).

CHAPTER 5: TRANSFORMATION BY COMPLEMENTATION OF AN ADENINE AUXOTROPH OF THE LIGNIN-DEGRADING BASIDIOMYCETE PHANEROCHAETE CHRYSOSPORIUM

The white rot basidiomycete Phanerochaete chrysosporium has been the focus of numerous studies on lignin degradation (4,5) and cellulose metabolism (146). The purification and characterization of two P. chrysosporium extracellular enzymes involved in lignin degradation, lignin peroxidase (87,88,149) and manganese peroxidase (89,91,170), and elucidation of their catalytic mechanisms (171,172) have contributed enormously to our understanding of the biochemistry of this process. Sequences for cDNA clones (95,96) and a genomic clone (100) of LiP have recently been published and a MnP cDNA has also been cloned and sequenced in this laboratory (106). 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 utilization of this organism for industrial bioprocessing.

Previous genetic studies have included the isolation of auxotrophic marker strains of P. chrysosporium (11) and their use in genetic recombination experiments (156) 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 (70,173). We also reported the preparation, fusion and regeneration of mycelial protoplasts of P. chrysosporium (16). 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 (174) 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 (11). The leu2 auxotroph deficient in β-isopropylmalate dehydrogenase (163) was crossed with a ura5 mutant deficient in orotidylic acid pyrophosphorylase (unpublished results) as previously described (173), with subsequent screening for a leucine auxotroph which produced abundant basidiospores. Ade2leu2 double mutants were then obtained by crossing the single auxotrophs. Auxotrophic mutants were maintained on slants as previously described (156) and were induced to fruit on one-sixth strength modified Vogel medium (151) containing 4.5% Walseth cellulose with the appropriate nutritional supplement(s) as previously described (49,173).

Transforming DNA. Plasmids containing two different adenine biosynthetic genes from S. commune, pADE2 and pADE5 (123), were generously provided by R. Ullrich, Univ. of Vermont. These genes were isolated by complementation of S. commune adenine auxotrophs using an S. commune genomic library cloned into the BamH1 site of plasmid pRK9 (175)

and subsequently recovered in E. coli (123). Plasmid DNA was prepared by the alkaline lysis method (176) and purified via ethidium bromide-CsCl equilibrium gradient centrifugation.

Chemicals. CsCl was obtained from Var Lac Oid Chemical Co., Bergenfield, N.J. Novozym 234 was obtained from Novo Industries, Denmark, Cellulase CP from Sturge Enzymes, U.K., and Cellulase TV from Miles Laboratories. Polyethylene glycol was obtained from Sigma Chemical Co. Restriction endonucleases were obtained from Bethesda Research Laboratories. [^{32}P]dCTP was obtained from New England Nuclear.

Preparation of Protoplasts. Protoplasts were prepared from basidiospores by a modification of the procedure developed for S. commune (121,123). The spores were washed from the lids of fruiting plates in 1-2 ml modified Vogel medium containing 3% malt extract, 0.15% yeast extract (MYV), pH 4.8, at concentrations of ~1 x 10^7 spores/ml. Spores were swollen at 36°C for 4-5 hr on a rotary shaker (150 rpm). The suspension was then centrifuged at 400 x g for 15 min at room temperature, and the pellet resuspended in 1 ml 0.5 M $MgSO_4$, 0.05 M maleic acid, pH 5.9 (MgOsm). Novozym 234 batch #1035 and Cellulase CP, 10 mg each, were added and the sample incubated at 38°C for 4 hr on a rotary shaker (100 rpm). Substitution of Cellulase TV for Cellulase CP had no apparent affect on protoplast formation, regeneration or transformation frequency. The protoplast suspension was centrifuged for 5 min at 270 x g at RT to pellet spores and cell wall debris. The supernatant containing the protoplasts was then added to 2 ml 1 M sorbitol, 20 mM MOPS, pH 6.3 (SorbOsm). The pellet was washed in 1 ml MgOsm, recentrifuged and the second supernatant added to the above. The

protoplasts in SorbOsm were pelleted by centrifuging 15 min at 270 x g at RT. The pellet was washed with 3 ml SorbOsm, recentrifuged as above and resuspended in SorbOsm + 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 10mM Tris, 1 mM EDTA [TE] + 0.04 M $CaCl_2$, pH 8) was added to ~2 x 10^6 protoplasts in 100 μl SorbOsm + $CaCl_2$, mixed gently and incubated on ice for 10 min. Control protoplasts were treated with 60 μl TE + $CaCl_2$. Samples were underlain with 160 μl 44% polyethylene glycol 3350 in 10 mM MES, pH 6.75, incubated on ice for 10 min, mixed gently and incubated an additional 10 min. Protoplasts were diluted to a concentration of 2.5 to 5.0 x 10^5/ml in asparagine-glucose-salts minimal medium (MM) (139) + 0.5 M $MgSO_4$, pH 4.8, and 0.1 ml added to each top agar tube containing 4 ml MM, 0.4 M $MgSO_4$, 1% agar, at 48°C. The top agar was poured over plates containing MM, 0.5 M $MgSO_4$, 1.5% agar. Serial dilutions of protoplasts were plated as above in medium containing 0.001% adenine to determine protoplast regeneration. Plates were incubated at 37°C. Colonies were visible on supplemented plates in 2-3 days, whereas transformant colonies first appeared about day 5 and additional colonies arose over the following two weeks.

Analysis of DNA from transformants. Spore suspensions of putative transformants, as well as the wild-type strain OGC101 (173) and the Ade2 auxotrophic strain, were used to inoculate 100 ml modified Vogel medium containing 2% glucose, 0.04% yeast extract plus 0.001% hypoxanthine for adenine auxotrophs. Cultures were incubated for 24 hr at 37°C with shaking (150 rpm). Cells were harvested and the DNA extracted according

to a published procedure (177), except that the RNase A treatment (35 μl of 20 mg/ml) was for 30 min at 37°C. Restriction endonucleases were used according to the manufacturer's recommendations; gel electrophoresis and Southern transfers to Gelman Bio Trace RP nylon filters were by standard methods (178). Random priming of plasmid DNA with [^{32}P]dCTP (179), Southern and slot blot hybridizations and autoradiography were by standard procedures (176).

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 (11). 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 (173). To test for the presence of the Ade$^-$ allele in transformants, Ade2leu2 double auxotrophs and Ade2leu2 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 (173).

Results

Protoplasting and Regeneration of Swollen Basidiospores. Optimal swelling (approximately 70%) of P. chrysosporium basidiospores was obtained in 4 - 5 hr at 36°C with concentrations of 1 x 10^7 spores/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 hr 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%. This was comparable to the regeneration frequencies obtained with P. chrysosporium mycelial protoplasts (16). 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 (11) 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 strain 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, nor from Ade2 protoplasts treated with pRR9 DNA alone or with the 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 x 10^6 protoplasts per μg of DNA. No increase in the frequency of transformation per μg of DNA was observed with increased amounts of either DNA or protoplasts. However, pADE2 linearized with EcoR1 resulted in a higher frequency of transformation than did concatemers of circular plasmid. Use of Novozym 234 alone appeared to decrease the frequency of transformation while not significantly affecting either the degree of 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 carrier plasmid DNA (pRK9) nor increasing the transformation incubation time from 10 min 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. 5-1) or pRK9 DNA (Fig. 5-2). 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 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. 5-1) or with [^{32}P]-labeled pRK9 (data not shown), even under low stringency conditions.

In the Southern blot in Fig. 5-1, 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 x 10^7 base pairs (64). In general, the bands obtained with 500 ng

FIGURE 5.1. 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 HindIII and fractionated by electrophoresis in 0.7% agarose. The blot was hybridized with radiolabeled pADE2 DNA. Lane 1, 214 pg of the plasmid pADE2 restricted with HindIII + 500 ng of DNA from the Ade2 auxotroph. Lanes 2-6, DNA (500 ng) from the Ade2 auxotroph, WT, and Transformants 2, 6, and 8 respectively. Lane 7, 500 ng of undigested DNA from Transformant 8. Bars to the left and right indicate the positions of molecular weight standards, from top to bottom: 23, 9.4, 6.6 4.4, 2.3 and 2.0 kilobases.

FIGURE 5.2. Southern analysis of DNA from a P. chrysosporium transformant. Transformant 2 (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. 500 ng of transformant DNA or 10 ng of pADE2 was added to each lane. Lane 1, undigested T2; lanes 2 and 3, pADE2 and T2 digested with PstI, respectively; lanes 4 and 5, pADE2 and T2 digested with SalI, respectively; lanes 6 and 7, pADE2 and T2 digested with EcoRI, respectively. Bars to the left and right indicate the positions of molecular weight standards, from top to bottom: 23, 9.4, 6.6 4.4, 2.3 and 2.0 kilobases.

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).

HindIII was used to linearize pADE2 for the Southern blot shown in Fig. 5-1. HindIII digests of DNA extracted from various transformants gave one strong band at about 14 kb and very faint, slightly smaller bands when probed with [^{32}P]-labeled pADE2 (Fig. 5-1) or pRK9 (data not shown). This 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. 5-2).

Mitotic and Meiotic Stability of Transformants. P. chrysosporium basidiospores are binucleate and this organism readily forms heterokaryons (173). In addition, the DNA-treated protoplasts were plated out in high concentrations on a medium that was not colony-inducing (8), 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

fruiting and selecting prototrophic colonies that arose from single homokaryotic basidiospores (173). Conidia and basidiospores arising from such colonies were all prototrophic, indicating the mitotic and meiotic stability of the transforming character.

Many of the Ade2leu2 double auxotrophs transformed to adenine prototrophy did not require purification, probably due to 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 5-1). 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. As can be seen in Table 5-1, all such crosses yielded some adenine auxotrophs, suggesting 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 the parent cross (Table 5-1). Crosses between Ade^{+}leu2 transformants and arginine and histidine auxotrophs likewise produced some Ade^{-} progeny (data not shown).

TABLE 5-1. Genetic recombination of transformed and nontransformed auxotrophs of *P. chrysosporium*.[a]

Crosses	Progeny							
	Proto-trophs	Ade^-	Leu^-	Rib^- or Nic^-	Ade^-Leu^-	Ade^-Rib^- or Ade^-Nic^-	Leu^-Rib^- or Leu^-Nic^-	$Ade^-Leu^-Rib^-$ or $Ade^-Leu^-Nic^-$
Ade2leu2 x Rib1	16	16	7	9	5	1	2	2
T7 x Rib1	23	7	15	9	5	5	6	0
T8 x Rib1	9	6	15	1	2	0	2	1
Ade2leu2 x Nic1	4	6	2	7	12	3	2	5
T8 x Nic1	11	5	11	20	5	4	3	0
T11 x 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 two years there have been reports on the transformation of the basidiomycetes S. commune (121,123) and Coprinus cinereus (122,124). We have adapted the system developed for the transformation of protoplasted basidiospores from S. commune (121,123) 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 to the frequencies now obtainable in other fungal systems (123,180), it is comparable to those obtained in initial transformation experiments with Aspergillus nidulans (169), Neurospora crassa (181) and S. commune (121). Since pADE2 is a large plasmid (14.0 kb), the transformation frequency per μg 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 has utilized a heterologous gene and promoter, it is possible that subsequent experiments utilizing 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 x 10^6 protoplasts is comparable to results with C. cinereus, in which 3 μg of DNA per 10^7 - 10^8 protoplasts was found to be saturating, although no inhibition of transformation was

observed with higher DNA concentrations (122). In that system, about 100 abortive transformants were obtained for every stable transformant, whereas we observe very few unstable transformants, and no background of abortive transformants on selection plates, such as are sometimes found in other systems (136).

Analysis of DNA from Transformants. As seen in Figs. 5-1 and 5-2, the transforming DNA appears to be chromosomally integrated rather than replicated autonomously. This is consistent with results obtained in other filamentous fungi (121,122,182,183). In addition, multiple copies of the plasmid appear to be integrated (Fig. 5-1). 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 (124).

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 5-1). 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 (121). Heterologous integration of tandem multiple copies of transforming DNA has also been reported in A. nidulans (184). 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 (122), although in transformations with the isocitrate lyase gene (acu7), no homologous integration of the whole plasmid was observed among the 40 transformants analyzed (124).

Analysis of genetic recombination data with P. chrysosporium is complicated by the fact that the organism is homothallic (173). In addition, clumping of basidiospores and low germination frequencies can influence recombination data (156). 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 due to multiple copies of the transforming DNA (124).

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 (123), 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 (174,185). 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 (174) as well as in bacteria (185), 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 the sixth and seventh steps 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 (186). 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. 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.

CHAPTER 6: TRANSFORMATION OF *PHANEROCHAETE CHRYSOSPORIUM* AND *NEUROSPORA CRASSA* WITH ADENINE BIOSYNTHETIC GENES FROM *SCHIZOPHYLLUM COMMUNE*

The white rot basidiomycete *Phanerochaete chrysosporium* has been the focus of numerous studies on lignin degradation (3-5) and cellulose metabolism (146). The purification and characterization of two *P. chrysosporium* extracellular enzymes involved in lignin degradation, lignin peroxidase and manganese peroxidase, and elucidation of their catalytic mechanisms (3,4) have contributed significantly to our understanding of the biochemistry of this process. The sequences of cDNA clones (95-97) and genomic clones (100-103) for lignin peroxidase and for manganese peroxidase (106,108) (Godfrey et al.; manuscript in preparation) have shown these enzymes to be encoded by genes belonging to two related gene families. The recent development of a transformation system for *P. chrysosporium* (187) will facilitate studies on the regulation and expression of these genes and genetic approaches to structure-function studies of the enzymes.

Previous genetic studies in our laboratory have included the isolation of auxotrophic marker strains of *P. chrysosporium* (11) and their use in genetic recombination experiments (156), as well as studies indicating that some strains of this organism have a primary homothallic mating system (i.e., are self-fertile) and produce binucleate homokaryotic basidiospores (173). In addition, at least some wild-type

strains of P. chrysosporium are heterokaryotic, containing more than one distinct genome (70,173). Our previously reported transformation system (187) utilized an adenine biosynthetic gene (Ade2) from the basidiomycete Schizophyllum commune to complement a P. chrysosporium adenine auxotrophic strain (Ade2). In this report, we describe the transformation of a second P. chrysosporium adenine auxotroph (Ade1) by complementation with another S. commune adenine biosynthetic gene (Ade5) and identification of the enzyme encoded by this gene, via transformation of a biochemically-characterized adenine auxotroph of the ascomycete Neurospora crassa (ade2). In addition, we have subcloned and mapped the S. commune adenine genes, thereby facilitating the construction of vectors for further transformation experiments with P. chrysosporium.

Materials and Methods

Strains. P. chrysosporium wild-type strain OGC101 (173) and the auxotrophic strains OGC107-1 (Ade1), OGC128-2 (Ade2) and OGC923-5 (Ade4) (11) were induced to fruit as previously described (17,49). N. crassa strains #2281 (ade1), #673 and #674 (ade2), #15, #3458 and #3459 (ade5), #663 and #664 (ade6), #665 (ade7), and #952 (ade9) were obtained from the Fungal Genetics Stock Center (Univ. of Kansas Medical Center).

Chemicals. Novozym 234 was obtained from Novo Industries, Denmark, and Cellulase CP from Sturge Enzymes, U.K. ^{32}P-dCTP was obtained from New England Nuclear. Benomyl (99.8% Tech) was generously provided by E. I. DuPont de Nemours & Co.

Transforming DNA. Plasmids containing two different adenine biosynthetic genes from S. commune, pADE2 and pADE5 (123), were

generously provided by R. Ullrich, Univ. of Vermont. Restriction fragments of these plasmids were electroeluted from agarose gels and fragments that were capable of transforming P. chrysosporium adenine auxotrophs to prototrophy were ligated into the multiple cloning site of pUC18. Subclones were isolated by transformation of E. coli strain DH5αF'. Plasmid DNA was prepared by the alkaline lysis method (176) and mapped by gel electrophoresis of restriction digests. Plasmids were linearized with restriction endonucleases for transformation of P. chrysosporium. The N. crassa benomyl resistance plasmid pBT6 (188) was generously provided by C. Yanofsky, Stanford Univ.

Transformation of P. chrysosporium. Protoplasts of swollen basidiospores were transformed as previously described (187) except that the transformation mixtures were underlaid with 320 μL of 44% polyethylene glycol (PEG) and chilled on ice for 10 min, after which the protoplasts were spun into the PEG at 270g for 5 min (189), mixed gently and incubated on ice for an additional 10 min before diluting and plating.

Analysis of DNA from P. chrysosporium Transformants. DNA from the wild type and putative transformant strains was extracted as previously described (187), digested, gel electrophoresed and transfered to Southern blots by standard methods (176). Random priming of plasmid DNA with ^{32}P-dCTP (179), Southern hybridizations and autoradiography (176) were performed by standard procedures.

Genetic Analysis of P. chrysosporium 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 (11). 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 (173).

Transformation of N. crassa. Protoplasts of germinated conidia were prepared using 2.5 mg/ml Novozym 234 as described elsewhere (180,190) and stored at -70°C in 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 6.3) containing 1 M sorbitol, 50 mM $CaCl_2$, 8% PEG 3350, 0.025% heparin, and 1% (v/v) dimethylsulfoxide at a concentration of 2 x 10^8 protoplasts per ml. In order to eliminate background regeneration of protoplasts on minimal medium, cotransformations using 5 μg of the benomyl resistance plasmid pBT6 and 5 μg of pADE2 or pADE5 in 10 mM Tris, 1 mM EDTA (TE) (pH 8) were performed according to Schweizer et al. (190). The transformation mixture, consisting of DNA, 2 x 10^7 protoplasts, and 1 ml of 40% PEG solution, was added to 150 ml of regeneration agar (1X Vogels, 1 M sorbitol, 2% sorbose, 0.05% fructose, 0.05% glucose, 0.02% inositol, 1.5% agar) and overlayered on 10 plates of regeneration agar without sorbitol but containing 1.5 μg/ml benomyl. A 1:1000 dilution of the transformation mixture was plated on medium lacking benomyl but containing 0.01% adenine, to determine the protoplast regeneration frequency. Plates were incubated at 30°C until colonies had conidiated. Putative transformants were picked to minimal slants.

Results

Transformation of P. chrysosporium Ade1 with pADE5. Optimization of our protoplasting and transformation protocols led us to repeat our earlier attempts to transform other adenine-requiring auxotrophs of P. chrysosporium with pADE5 (187). Putative transformants were obtained with pADE5 and the Ade1 strain and these colonies were transferred to minimal slants to verify prototrophy. Colonies did not arise on minimal medium with protoplasts that did not receive DNA or with Ade1 protoplasts treated with pRK9 vector DNA alone or with the plasmid pADE2. Protoplasts of P. chrysosporium strains Ade2 and Ade4 did not give rise to colonies on minimal medium when treated with linearized pADE5. Using 2×10^6 protoplasts, transformation frequencies of approximately 100 transformants per μg of DNA were obtained with uncut pADE5 and at least 300 transformants per μg were obtained with the BamHI-linearized plasmid. Protoplasts plated on medium supplemented with adenine regenerated at a frequency of ~10%; thus, on the average, between 0.05% and 0.15% of viable protoplasts were transformed.

Subcloning pADE5. The S. commune plasmid pADE5 (123), as rescued from E. coli, consisted of an approximately 5.1-kb insert in the pBR322-derived vector pRK9 (191). This plasmid was digested into two fragments with KpnI and BamHI, which each cut once within the insert, and the fragments were electroeluted from a gel. The smaller 3.8-kb fragment, which transformed P. chrysosporium Ade1 with the same frequency as linearized pADE5, was ligated into the multiple cloning site of pUC18. This plasmid, designated pADE5-2g (Fig. 6-1), was linearized with KpnI

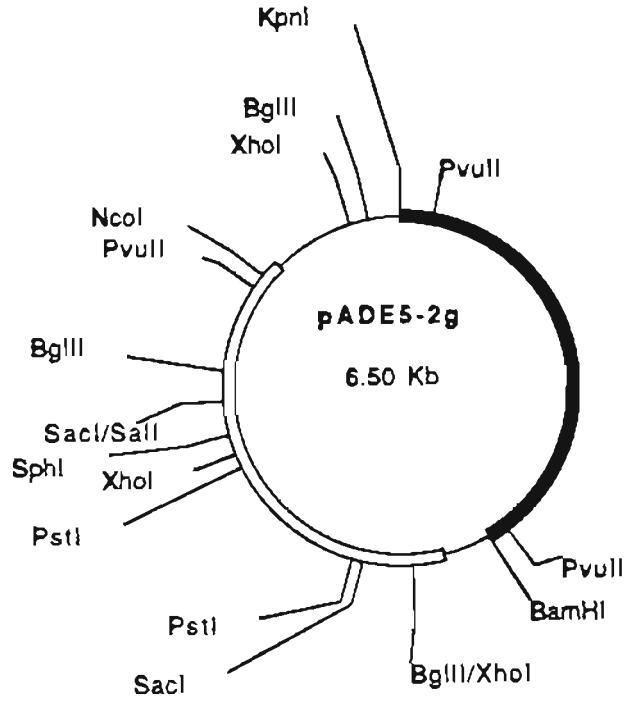

FIGURE 6-1. Restriction map of pADE5-2g. Bold line represents pUC18; double line indicates approximate location of the Ade5 gene.

and used in subsequent transformations of the P. chrysosporium Ade1 strain.

The maximum transformation frequency obtained with pADE5-2g was approximately 300 transformants per μg of DNA and averaged about 100 transformants per μg. The majority of transformants appeared between day 4 and day 7 after plating. Conidia arising from transformants were all prototrophic, demonstrating that the transforming DNA was mitotically stable. Colonies arising from single basidiospores of three transformants were all prototrophic, indicating both that the transforming DNA was meiotically stable and that the transformant colonies did not contain untransformed nuclei. A fourth transformant had to be purified of untransformed nuclei. This was accomplished by selecting a prototrophic colony that arose from a single basidiospore (173) and fruiting it. These second-generation basidiospores were all prototrophic.

Fig. 6-2 shows Southern blots of DNA extracted from four Ade1 transformants and hybridized with the [^{32}P]-labeled pADE5-2g insert. The absence of a rapidly migrating band in samples of uncut transformant DNA indicated that the transforming plasmid was integrated into the chromosome rather than replicated as an autonomous plasmid. DNA extracted from P. chrysosporium wild-type strain OGC101 (Fig. 6-2A, lane 2) or the Ade1 strain (data not shown) did not hybridize with the probe in Southern analyses, even under low-stringency conditions.

The Southern blots in Fig. 6-2A (lanes 1 and 7) and B (lane 1) included pADE5-2g at a concentration of approximately one copy per genome in 500 ng of DNA. The genome size of P. chrysosporium has been

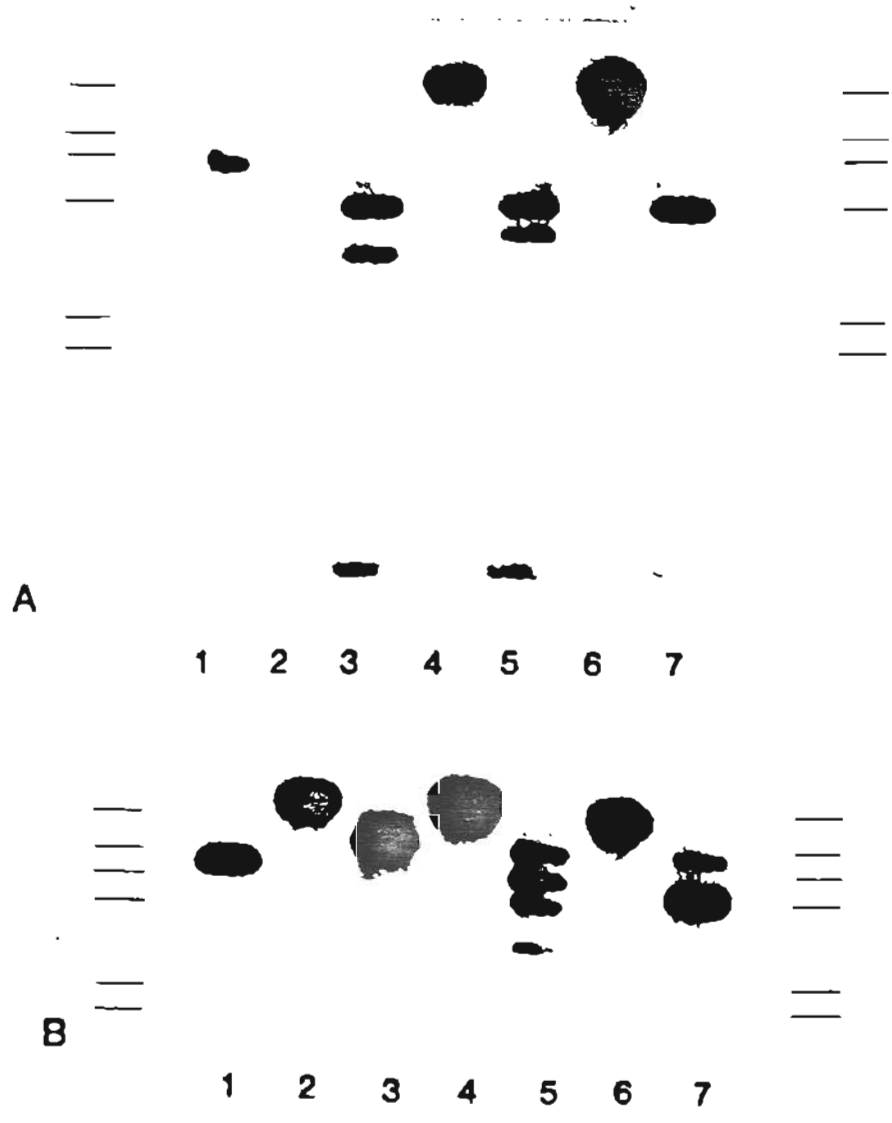

FIGURE 6-2. Southern analysis of DNA from P. chrysosporium Adel transformants. Samples of total DNA were digested with restriction enzymes and fractionated by electrophoresis in 0.7% agarose. The blot was hybridized with ^{32}P-labeled DNA from the pADE5-2g insert. A: pADE5-2g digested with KpnI and PstI (lanes 1 and 7 respectively); undigested wild type, T4 and T3 DNA (lanes 2, 4 and 6 respectively); T4 and T3 digested with PstI (lanes 3 and 5 respectively). B: pADE5-2g linearized with EcoRI (lane 1); T1, T2 and T3 undigested DNA (lanes 2, 4 and 6 respectively); T1, T2 and T3 digested with EcoRI (lanes 3, 5 and 7 respectively). Plasmid lanes contained 70 pg of DNA; all other lanes contained 500 ng each. Bars indicate positions of molecular weight standards; from top to bottom: 23, 9.4, 6.6, 4.4, 2.3, 2.0 kilobases.

determined to be ~4.4 x 10^7 base pairs (64). The bands obtained with 500 ng of transformant DNA appear stronger than those in the pADE5-2g lanes, suggesting the presence of multiple plasmid copies in an equivalent amount of transformant DNA. The patterns obtained with transformants 3 (T3) and 4 (T4) digested with PstI (Fig. 6-2A, lanes 5 and 3), with strong bands corresponding to the plasmid bands and additional lighter bands, suggest that in these transformants, the multiple copies were present as tandem duplications or insertions rather than as multiple insertions at different sites. The strong bands presumably represent identical multiple copies of the plasmid bands, with the fainter bands corresponding to the two ends of the array. The fact that the fainter bands are different in the two transformants suggests that the plasmid integrated at different sites in the two genomes.

However, EcoRI digests of transformants exhibited a more complicated pattern. Digests of transformant 1 (T1) and T3 (Fig. 6-2B, lanes 3 and 7) exhibited bands corresponding to the uppermost band in the EcoRI digest of transformant 2 (T2) (Fig. 6-2B, lane 5). This band is of higher molecular weight than the linearized plasmid. Identical hybridization patterns were obtained with different genomic DNA preps of each transformant. The EcoRI digest of T4 (data not shown) exhibited the same single strongly-hybridizing band as T1. EcoRI digests of T2 and T3 (Fig. 6-2B, lanes 5 and 7) also exhibited bands of lower molecular weight than the linearized plasmid. There are several possible explanations for these hybridization patterns, including: multiple plasmid integrations in a "head-to-head" arrangement; deletion of EcoRI sites upon integration; or various plasmid rearrangements. In

addition, the multiple bands obtained with the EcoRI digest of T2 (Fig. 6-2B, lane 5) could result from plasmid integration at multiple sites, either in a single genome or in different nuclei.

Fig. 6-1 shows a restriction map of pADE5-2g, as determined by single and double restriction digests. Transformations with various plasmid digests were used to try to determine the approximate location of the S. commune gene. NcoI, which linearizes the plasmid, resulted in a transformation frequency that was only 43% of the control transformation with pADE5-2g linearized with KpnI. SphI, which cuts once within the insert and once in the multiple cloning site, resulted in a transformation frequency that was 80% of the control. SalI, which also yields two fragments, resulted in a transformation frequency that was 18% of the control. Digests using XhoI, SacI, BglII or PstI, each of which cuts pADE5-2g into three fragments, resulted in transformation frequencies that were 22%, 17%, 9% and 7% of the control, respectively. Thus, at least one site for each restriction enzyme used in these experiments, appears to be located within the gene or within a region affecting gene expression. In general, transformants arising from plasmid digests appeared later than transformants from the linearized control plasmid.

Subcloning pADE2. The S. commune plasmid pADE2, as rescued from E. coli, consisted of a 9.7 kb insert in the vector pRK9 (123). pADE2 digested with KpnI, which cuts twice within the insert, transformed P. chrysosporium Ade2 at frequencies equal to or greater than the linearized pADE2. A 4.7-kb transforming fragment from this digest was electroeluted and ligated into the multiple cloning site of pUC18. This

plasmid, designated pADE2-3b (Fig. 6-3), was linearized with EcoRI and used in subsequent transformations of the P. chrysosporium Ade2 strain.

The maximum transformation frequency obtained with pADE2-3b was approximately 350 transformants per μg of DNA and averaged about 150 transformants per μg. Transformant colonies were evident four days after plating and continued to arise over the next few days. Conidia arising from transformants were all prototrophic, demonstrating that the transforming DNA was mitotically stable. Colonies arising from single basidiospores of several transformants were all prototrophic, indicating both that the transforming DNA was meiotically stable and that the transformant colonies did not contain untransformed nuclei.

Fig. 6-4 shows Southern blots of DNA extracted from 2 transformants and hybridized with the entire pADE2-3b subclone labeled with ^{32}P-dCTP. The absence of a rapidly migrating band in samples of uncut transformant DNA again indicated that the transforming plasmid was integrated into the chromosome rather than replicated as an autonomous plasmid. DNA extracted from P. chrysosporium wild-type strain OGC101 or the Ade2 strain did not hybridize with [^{32}P]-labeled pADE2-3b in Southern analyses, even under low-stringency conditions (data not shown).

The Southern blots in Fig. 6-4A and B (lanes 1) included pADE2-3b at a concentration of approximately one copy per genome in 500 ng of DNA. The bands obtained with 500 ng of transformant DNA again appear stronger than those obtained with 90 pg of pADE2-3b, suggesting the presence of multiple plasmid copies in an equivalent amount of transformant DNA. The PstI and the EcoRI digests of transformant 1 (T1)

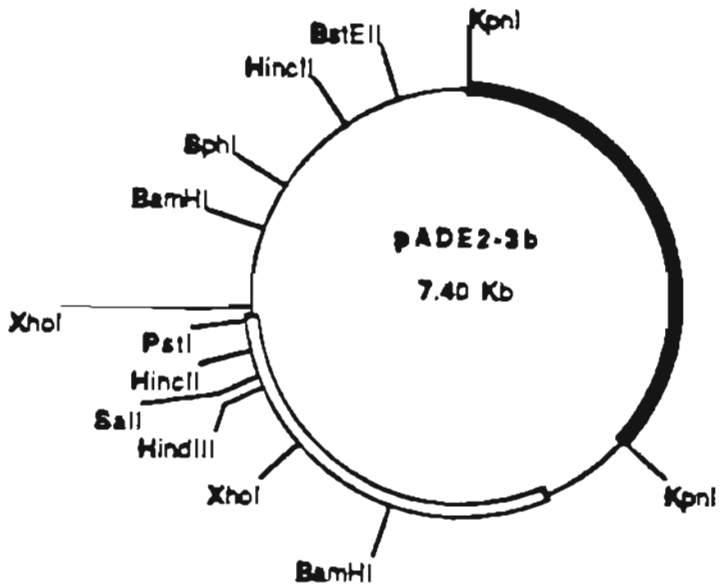

FIGURE 6-3. Restriction map of pADE2-3b. Bold line represents pUC18; double line indicates approximate location of the Ade2 gene.

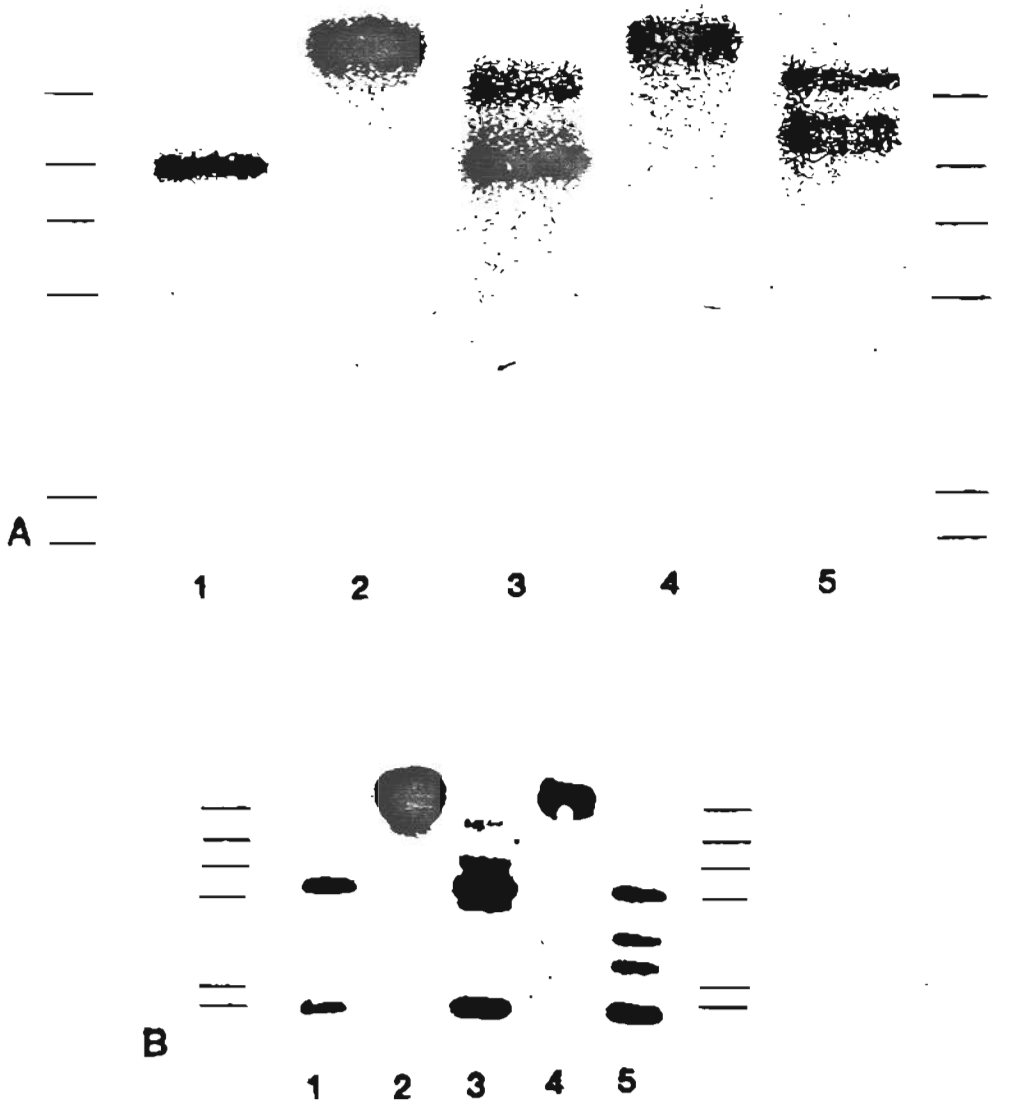

FIGURE 6-4. Southern analysis of DNA from P. chrysosporium Ade2 transformants. Samples of total DNA were digested with restriction enzymes and fractionated by electrophoresis in 0.7% agarose. The blot was hybridized with ^{32}P-labeled pADE2-3b. A: pADE2-3b linearized with EcoRI (lane 1); T1 and T2 undigested (lanes 2 and 4 respectively) and digested with EcoRI (lanes 3 and 5 respectively). B: pADE2-3b digested with PstI (lane 1); T1 and T2 undigested (lanes 2 and 4 respectively) and digested with PstI (lanes 3 and 5 respectively). Plasmid lanes contained 90 pg of DNA; all other lanes contained 500 ng each. Size markers as in Fig. 6-2.

and the PstI digest of transformant 2 (T2) show strong bands corresponding in size to the plasmid bands. T1 exhibits several less intense bands, suggesting that the multiple plasmid copies may have integrated at different sites, either within a single genome or within different nuclei of the transformant. The PstI digest of T2 exhibits two less intense bands, presumably corresponding to the ends of a single tandem array. The fact that all of the bands obtained with the EcoRI digest of T2 are of higher molecular weight than the linearized plasmid, could be the result of "head-to-head" plasmid integrations, deletion of the plasmid EcoRI site, or plasmid rearrangement. The different patterns obtained with these transformants suggest that the plasmid can integrate at different sites in the P. chrysosporium genome.

Fig. 6-3 shows a restriction map of pADE2-3b, as determined by single and double digests using various restriction enzymes. The approximate location of the S. commune gene was determined from the transformation efficiency obtained with various plasmid digests. Since pADE2-3b digested with BamHI did not transform the P. chrysosporium Ade2 strain, one BamHI site was assumed to be located within the gene. PstI, SalI, HindIII, XhoI and HincII digests of pADE2-3b all resulted in transformation frequencies that were reduced by 50-90% compared with the EcoRI-linearized plasmid. The PstI and SalI fragments were electroeluted from agarose gels and used in transformations. In both cases, the fragments transformed P. chrysosporium Ade2 in combination but not as single fragments. Thus, these sites, as well as one of the HincII sites and one of the XhoI sites in the insert, are thought to be located within the gene or in a region affecting gene expression. SphI

and BstEII digests of pADE2-3b resulted in normal transformation of the P. chrysosporium auxotroph.

Identification of the S. commune Ade5 Gene via Transformation of N. crassa. In order to identify the enzymes encoded by the transforming DNA, we attempted to complement known adenine biosynthetic mutations in N. crassa with the S. commune adenine plasmids. Transformations were attempted with each N. crassa auxotrophic strain in the pathway from phosphoribosyl pyrophosphate to inosinic acid (IMP), except ade3A and ade3b ("purple ades") and ade4, using pADE5, pADE2 and the two subclones. In order to eliminate the background regeneration observed with some auxotrophic strains, cotransformations were performed utilizing the plasmid pBT6 containing the N. crassa benomyl resistance gene. Protoplast regeneration on medium supplemented with 0.01% adenine varied between 5% and 20%. Transformation to benomyl resistance with pBT6 alone varied between 50 and 400 transformants per μg of DNA. In these experiments, pADE5 transformed N. crassa ade2 strains #673 and #674 to prototrophy with or without cotransformation for benomyl resistance. Although as many as 500 transformants per μg of DNA were obtained in cotransformation experiments, many of these transformants failed to conidiate, or failed to grow when transferred to minimal slants. Mitotically stable prototrophic transformants arose at frequencies ranging from 50 to 150 transformants per μg of DNA. Frequencies were increased slightly when pBT6 was omitted and no benomyl was used in the medium. The subclone pADE5-2g transformed at about the same freqency as pADE5. Linearizing either plasmid did not seem to significantly affect transformation.

Neither pADE2 nor pADE2-3b complemented N. crassa auxotrophs in these experiments.

Discussion

Recently we reported the development of a transformation system for the lignin-degrading basidiomycete Phanerochaete chrysosporium, utilizing an adenine biosynthetic gene (Ade2) from the basidiomycete Schizophyllum commune to complement an adenine auxotroph (Ade2) of P. chrysosporium (187). We have now successfully transformed the P. chrysosporium adenine auxotroph Ade1 with the S. commune plasmid pADE5. Frequencies of approximately 100 transformants/μg of DNA were obtained with pADE5 and the frequency increased to at least 300 transformants per μg when the plasmid was linearized. These results are similar to those obtained with the plasmid pADE2 and the P. chrysosporium auxotroph Ade2 (187).

Both our protoplast regeneration and our transformation frequencies have improved since our previous report. Although a number of variations in the protoplasting and transformation procedures were tested (189), the only factors that significantly improved transformation frequencies were increasing the volume of PEG used and sedimentation of the transformation mixture into the PEG rather than adding the PEG to the mixture. It is common in fungal transformations to use up to 10 volumes of 40% PEG 4000 (119); however, we have found two volumes of 44% PEG 3350 to be optimal.

Since both pADE2 and pADE5 are large plasmids (14 and 9.4 kb, respectively), it was desirable to subclone fragments containing the S.

commune adenine biosynthetic genes into the smaller vector pUC18. This would facilitate construction of transformation vectors for various P. chrysosporium genes, such as the peroxidase genes, for which there exist no simple selection protocols. The resulting subclones, pADE5-2g and pADE2-3b, are shown in Figs. 6-1 and 6-3, respectively. Between 0.05% and 0.15% of viable P. chrysosporium protoplasts were transformed with these subclones, which is similar to the percentage of viable protoplasts transformed with S. commune (189).

The transforming DNA is mitotically and meiotically stable, as seen by analysis of conidia and basidiospores derived from self-crosses. Few if any abortive transformants are observed in these experiments, in contrast to transformations of the basidiomycete Coprinus cinereus (122). However, P. chrysosporium Ade1 and Ade2 basidiospores and protoplasts regenerate on minimal medium, without growing up, so background regeneration could easily mask the appearance of abortive transformants.

Southern blots of DNA extracted from transformants obtained with pADE5-2g and pADE2-3b (Fig. 6-2 and 6-4 respectively) indicate that the transforming DNA is chromosomally integrated as is the case with most other filamentous fungi (119). This is in contrast to a recent report claiming low frequency transformation of P. chrysosporium with an autonomously replicating plasmid (133).

Southern analysis of transformants obtained with both pADE5-2g and pADE2-3b (Fig. 6-2 and 6-4) suggest that the transforming DNA is present in more than one copy per genome, as was the case with our initial transformations with pADE2 (187). It is possible that since these

transformations are employing a heterologous gene and promoter, single copy tranformants may not grow well enough to be detected against the background of untransformed regenerating protoplasts. Nevertheless, multiple copy integrations are common in transformations of filamentous fungi (119). The PstI digests of T4 and T3 obtained with pADE5-2g (Fig. 6-2A, lanes 3 and 5) and T2 obtained with pADE2-3b (Fig. 6-4B, lane 5) suggest that, as with our initial transformations (187), the multiple copies are often the result of tandem duplications or insertions, rather than multiple insertions at different sites. However, the different patterns obtained with different transformants suggest that the transforming plasmid can insert at various sites in the recipient genomes. In addition, some transformants appear to contain either "head-to-head" insertions or multiple insertions at different sites. This can be seen with the EcoRI digest of Ade1 T2 (Fig. 6-2B, lane 5) and with the PstI digest of Ade2 T1 (Fig. 6-4B, lane 3). It is possible that this is a result of transformation with linearized DNA, which may facilitate integrations at multiple genomic sites. Our earlier transformation experiments (187) utilized concatameric DNA, which may favor multiple tandem insertions at a single site in the recipient genome. Razanamparany and Begueret (192) have suggested that tandem integrated copies may be the result of concatamer formation prior to integration. However, the transformants chosen for Southern analysis, although free of untransformed nuclei, were not purified via basidiospore formation. Therefore, these transformants could be heterokaryons arising in one of two ways: Since the protoplasted basidiospores are binucleate (173), and the regenerating protoplasts may

be multinucleate by the time of plasmid integration, the integration sites may actually be located in separate nuclei. Alternatively, these transformants may have arisen via the fusion of two transformed protoplasts. The presence of PEG in the transformation mixture could promote protoplast fusion as has been shown previously for mycelial protoplasts of P. chrysosporium (16). In fact, it has been suggested that it is PEG-induced protoplast fusion that results in internalization of the DNA during fungal transformation (193). In any case, apparently random, multiple, tandem or nontandem DNA integrations are common in transformations of filamentous fungi (119,122,124,194-196).

The patterns obtained with EcoRI digests of the transformant DNA (Figs. 6-2B and 6-4A) could result from the plasmids integrating in opposite orientations, from loss of the plasmid EcoRI site, or from plasmid rearrangement. Sequence rearrangements have been frequently reported in fungal transformations (124,193,195) and deletions of plasmid sequences at the sites of nonhomologous integration have been reported in transformations of Podospora anserina with the cloned ura5 gene (197).

In our previous report (187), genetic methods were used to show that the transforming DNA was integrating at site(s) other than the resident adenine biosynthetic gene. In the present study, Southern analysis of transformants obtained with both pADE5-2g and pADE2-3b indicates that the DNA is integrating at various sites in the P. chrysosporium genome. These results, as well as our observations that the S. commune adenine genes and the corresponding P. chrysosporium genes lack the necessary sequence similarity for cross hybridization on

Southern blots (Fig. 6-2A, lane 2) (187), make it unlikely that the transforming DNA is integrating at the sites of the resident biosynthetic genes. In transformation of the basidiomycete Coprinus cinereus, predominately ecotopic (heterologous) integration is observed even though the transforming DNA is homologous, having been cloned from the same fungal strain (122,124).

Figs. 6-1 and 6-3 show restriction maps of the subclones pADE5-2g and pADE2-3b, respectively. Attempts were made to localize the adenine biosynthetic genes by transforming P. chrysosporium auxotrophs with various restriction digests of the appropriate plasmids. In every case, gel electrophoresis indicated that the digests were complete. However, none of the pADE5-2g digests completely abolished transformation. NcoI, which linearizes the plasmid by cutting within the insert, reduced the transformation frequency to 43% of the control plasmid, KpnI-linearized pADE5-2g. The transformation frequency with the SalI digest, which cuts the plasmid into two fragments, was 80% of the control. The restriction digests which cut pADE5-2g into several fragments, all reduced the transformation frequency to less than one quarter of the control. This suggests that the digests are religating or are incorporated together into the P. chrysosporium genome. A SalI digest of the pADE5-2g insert destroyed transforming activity in S. commune (123), so it is possible that the presence of the pUC18 vector increases the likelihood of religation or cointegration. Alternatively, P. chrysosporium may have more endogenous DNA ligase activity than S. commune. The fact that transformants from plasmids digested into multiple pieces, in general,

arose later than those from the linearized plasmid control may be relevant in this regard.

As with pADE5-2g, most digests of the larger plasmid, pADE2-3b, reduced, but did not eliminate transformation. However, a BamHI digest destroyed all transforming activity. SphI and BstEII digests resulted in normal transformation frequencies and these sites are therefore assumed to be outside of both the coding and noncoding regions of the Ade2 biosynthetic gene. The fact that SalI and PstI fragments each transformed the Ade2 auxotroph when used in combination but not when used alone, again suggests that the fragments are religating, either spontaneously or via endogenous P. chrysosporium ligase activity, or are cointegrating.

The biosynthetic pathway leading from phosphoribosyl pyrophosphate to AMP has twelve steps and many of the enzymes involved have not been well characterized in fungi (198). 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. Steps 8 and 12 (ade13 in Saccharomyces cereviseae, purB in E. coli and ade4 in N. crassa) are thought to be catalyzed by a single bifunctional enzyme, adenylosuccinate lyase (174,185,198). Since step 12 is in the pathway of hypoxanthine conversion to AMP it is unlikely that either of the S. commune genes, Ade2 or Ade5, encodes this enzyme. Furthermore, neither the S. commune Ade2 nor the S. commune Ade5 gene complements either of the "purple ade" Schizophyllum auxotrophs (R. Ullrich; personal communication), which are deficient in the sixth and seventh steps of the pathway. Such mutants are visibly identifiable due to a build-up of

aminoimidazole ribotide which polymerizes to a dark red pigment on exposure to air (186). To our knowledge, no such P. chrysosporium mutants have been isolated.

We therefore attempted to complement known adenine auxotrophs of N. crassa using the S. commune plasmids, in order to identify the biosynthetic genes involved in the transformations. Because of the high background regeneration of some of the N. crassa auxotrophs, these experiments were performed as cotransformations with the benomyl resistance plasmid pBT6. Regeneration frequencies of the N. crassa protoplasts were similar to those reported elsewhere (199). pADE5 and pADE5-2g transformed N. crassa ade2 strains #673 and #674 to prototrophy, with or without cotransformation to benomyl resistance. Although abortive transformants were observed in these experiments, as is typical with N. crassa transformations (183), mitotically stable prototrophic transformants were obtained at frequencies of 50 to 150 transformants per μg of DNA. This indicates that the S. commune Ade5 biosynthetic gene which transforms P. chrysosporium strain Ade1, encodes the enzyme phosphoribosylaminoimidazole synthetase which converts phosphoribosylformylglycinamidine (FGAM) to phosphoribosylaminoimidazole (AIR) (200). This corresponds to the S. cereviseae ade7 gene (174).

This is, to our knowledge, the first report of complementation of an N. crassa mutation using a basidiomycete gene. Hynes (127) has recently reported the successful transformation of another ascomycete, Aspergillus nidulans, with the acu7 gene encoding isocitrate lyase from the basidiomycete C. cinereus. In initial experiments, pADE2 and pADE2-3b have failed to transform any of the N. crassa adenine auxotrophs.

However, cross-expression of ascomycete and basidiomycete genes may be a variable phenomenon. For example, although it has been reported that various A. nidulans genes, including the isocitrate lyase gene **acuD**, and **trpC**, do not appear to be expressed in C. cinereus (125), we have successfully transformed a **ura5** mutant of P. chrysosporium with the **ura5** gene from the ascomycete P. anserina (unpublished results). Attempts to identify the Ade2 gene are continuing in our laboratory.

Currently, we are utilizing our transformation systems for the construction of expression vectors, as well as developing transformation of P. chrysosporium with homologous genes and promoters. Ultimately, use of these transformation systems should assist in elucidation of regulatory mechanisms in this organism.

CHAPTER 7: CONCLUDING REMARKS

Summary of Research

The goal of this work has been to contribute to the classical and molecular genetics of the lignin-degrading basidiomycete Phanerochaete chrysosporium. Toward this end, we have demonstrated that genetic recombination readily occurs between auxotrophic marker strains; that this organism is coenocytic, producing multinucleate, heterokaryotic conidia and binucleate homokaryotic basidiospores; that wild-type strains are heterokaryotic; and that at least some strains of P. chrysosporium possess a primary homothallic mating system. In addition, we have characterized the six leucine auxotrophs of P. chrysosporium genetically and biochemically. Finally, we have developed a transformation system for P. chrysosporium, based on complementation with adenine biosynthetic genes from Schizophyllum commune, and identified one of these genes as encoding phosphoribosylaminoimidazole synthetase, by transformation of a Neurospora crassa ade2 strain.

Future Directions

Auxotrophic Mutants. The commonly-used P. chrysosporium strains, OGC101, ME-446 and BKM-F-1767, are all heterokaryotic. As a result of this, mutant strains have varying genetic backgrounds and cannot be backcrossed to the parental strain. In addition, in some cases

homokaryotic isolates may have been mistakenly-identified as mutants. It would be very useful if, in the future, mutants were obtained from a single homokaryotic strain. This would facilitate the selection of mutants against a known genetic background.

It has been shown that homokaryotic isolates of the wild-type strains OGC101 and ME-446 vary greatly in the expression of their lignin-degrading systems (Chapter 4; ref. 32). Thus it is possible that strains containing specific lignin-degrading phenotypes could be constructed; for example, strains in which only certain LiP and MnP isozymes are expressed. Since P. chrysosporium is homothallic, it is difficult to conduct genetic crosses without nutritionally-forcing heterokaryons or fusing protoplasts. However, auxotrophic marker strains with differing lignin-degrading phenotypes could be crossed to construct such strains.

Mating System. It is apparent that there are unanswered questions concerning the P. chrysosporium mating system and it is possible that this organism could be utilized to study the mechanisms of homothallism. Additional experimental crosses between homokaryotic isolates from various wild-type strains could yield further insights into the mating type mechanisms. Of particular interest is the P. chrysosporium type culture HHB-6251, in which neither single basidiospores nor conidia give rise to strains which fruit. It would also be of interest to attempt to isolate genes responsible for sexual development in P. chrysosporium, using heterologous probes derived from the mating type genes of other basidiomycetes. For example, the flanking regions of the Aα4 gene from S. commune (38) and the Aα-β cosmid from Coprinus cinereus are known to

cross-hybridize (C. Specht; personal communication). Five homothallic strains of Neurospora contain fragments that hybridize only to the A-specific mating type probe from N. crassa; whereas a sixth homothallic strain contains segments that hybridize to both A- and a-specific probes (201).

Genome Analysis. Some of the discrepancies concerning the heterokaryotic nature of P. chrysosporium could be resolved by further analysis of restriction fragment length polymorphisms. In particular, it should be possible to obtain homokaryotic conidial isolates, using medium which promotes production of mononucleated conidia (11) and sonication to unclump the spores. RFLP analysis of these strains should reveal the number and genotypes of parental types present in the wild-type strains ME-446 and OGC101. In addition, RFLP analysis could be used to determine the homokaryotic or heterokaryotic nature of other P. chrysosporium strains, and to further map the P. chrysosporium genome using additional probes from isolated genes.

Pulsed field gel electrophoresis (202) would also be useful for mapping the P. chrysosporium genome. It could be used to determine the number of chromosomes as well as to visualize linkages between LiP and MnP genes and other identified markers. It could also be used to reveal any chromosome anomalies between strains. Contour-clamped homogenous electric field (CHEF) gels (203) were recently used to obtain an electrophoretic karyotype of N. crassa (204).

Transformation. The adel gene, encoding phosphoribosylamino-imidazole synthetase, has recently been isolated from P. chrysosporium (Mayfield, Nipper, Alic and Gold; unpublished), by probing a λ EMBL3

genomic library with a probe made from the S. commune **ade5** gene. We plan to compare tranformation frequencies obtained with this gene, to those obtained with pADE5-2g. We also plan to look for homologous integration of the **ade1** gene into the P. chrysosporium genome. In addition, the P. chrysosporium Ade2 and **ura5** genes are currently being cloned, using heterologous probes from S. commune and Podospora anserina respectively.

Expression vectors containing the LiP and MnP genes have been contructed using the subclone pADE5-2g (Chapter 6) (Godfrey, Ritch and Gold; unpublished) and Ade^+ tranformants obtained with these vectors are being analyzed for expression of the peroxidase genes, as well as for integration sites within the P. chrysosporium genome. It is possible that expression and/or regulation of these genes may be site-specific. Evidence has recently been obtained for site-specific expression of the isocitrate lyase gene of C. cinereus (196) and for site-specific regulation of a conidiation-specific gene from Aspergillus nidulans (205).

It may be possible to increase production of lignin-degrading peroxidases by multiple-copy transformation. It may also be possible to increase enzyme production by placing the genes under the regulation of strong promoters such as those of the triosephosphate isomerase (206) or glyceraldehyde-3-phosphate dehydrogenase (GPD) (207) genes, both of which are currently being cloned from P. chrysosporium in our laboratory. In yeast, GPD can account for at least 5% of the total cellular protein (208). Promoters from the **ade1** or β-tubulin genes, which have already been isolated in our laboratory, may also be used in

constructs with the LiP and MnP genes in attempts to obtain peroxidase expression during primary metabolism.

Vectors utilizing the reporter gene β-glucuronidase (209,210) are being developed in our laboratory for analysis of MnP gene promoter sequences and their regulation by Mn and nitrogen. Such vectors could also be utilized for analysis of general secondary metabolic regulation.

Transformation vectors based on complementation of adenine or other auxotrophs could also be utilized with modified LiP or MnP genes to study structure-function relationships. For example, a LiP gene from which the propeptide has been removed could be used in such a construct. X-ray crystallographic studies of LiP and MnP are currently underway (Poulos and Gold; unpublished). These studies should provide information for the design of experiments involving site-directed mutagenesis (211,212) of LiP and MnP genes. Probing of the Mn-binding site of MnP or the ability of LiP to oxidize methoxybenzenes with very high redox potentials (3,213) are two of the many possible experimental questions that could be approached using site-directed mutagenesis.

Since no simple selection exists for LiP or MnP expression, the success of experiments involving the ligninase genes or their promoters will depend on the availability of a suitable transformation system with a selectable marker. Complementation of auxotrophic mutants with either single-vector constructs or cotransformations with independent plasmids can be used. However such experiments will depend on the construction by genetic recombination, of recipient auxotrophic strains suitable for expression of the ligninase genes being studied. Transformation systems for wild-type strains, utilizing positive selection systems such as

resistance to hygromycin B (129), phleomycin (131,132) or G418 (133) are also possible.

Depending on the frequency of homologous recombination in transformations of P. chrysosporium, experiments involving gene disruptions and replacements (194,214,215) could prove very useful, both for creating auxotrophic mutants and for creating specific mutations in the lignin-degrading system. For example a MnP gene interrupted with an S. commune Ade2 or ade5 gene, that shares very little homology with the P. chrysosporium ade gene, could be transformed into the P. chrysosporium adenine auxotroph. If some of the Ade^+ transformants integrated homologously at the MnP gene site, the endogenous MnP gene would be disrupted. Similar experiments could be carried out with LiP genes. Such experiments could facilitate examination of the role of MnP and LiP isozymes in the degradation of lignin, or provide null backgrounds for the introduction of modified MnP or LiP genes. Ura3 and ura5 mutants are particularly useful for experiments involving gene disruptions and replacements, because 5-fluoro-orotate resistance (13) serves as a positive selection for mutations in these genes (215,216).

Several different roles in lignin degradation have been suggested for veratryl alcohol (4,217-219) (Wariishi and Gold; J. Biol. Chem., in press). Since veratryl alcohol is synthesized from L-phenylalanine in P. chrysosporium (220), mutants deficient in phenylalanine lyase (PAL), the enzyme catalyzing the first step of this pathway, would be very useful for analyzing the role of this secondary metabolite. The pal gene is available from the basidiomycete yeast Rhodosporidium torloides (221) and could be used to attempt to clone the P. chrysosporium gene by

heterologous hybridization. Gene disruptions could then be used to create veratryl alcohol-negative mutants for further study.

It is possible that LiP and MnP could be expressed in heterologous systems which lack endogenous ligninases, such as the yeast expression system Pichia pastoris (222,223) or Aspergillus, and that structure-function studies could be carried out in these organisms. Since our expression vectors contain an S. commune biosynthetic gene, we intend to transform S. commune **ade5** strains with these LiP and MnP vectors. However correct insertion of the heme into LiP and MnP may be difficult. This is particularly true of the LiP apoenzyme, which has not been reconstituted in vitro (Akileswaran, Wariishi and Gold; unpublished). We therefore intend to try expressing these enzymes, either via cotransformation or with new vector contructions, in the basidiomycete C. cinereus, which is known to secrete an extracellular heme-containing peroxidase (224) and therefore may be able to correctly insert the heme into LiP.

Regardless of the results with heterologous expression, it is likely that the study of LiP and MnP regulation, as well as analysis of the roles of individual components of the lignin degrading system, will require the use of P. chrysosporium. The classical genetic results and the transformation system presented in this thesis should facilitate such studies, as well as the eventual utilization of P. chrysosporium for biotechnological applications.

REFERENCES

1. Sarkanen, K. V., and Hergert, H. L. (1971) in Lignins: Occurrence, Formation, Structure, and Reactions (Sarkanen, K. V., and Ludwig, C. H., eds) pp. 43-94, Wiley-Interscience, New York
2. Crawford, R. L. (1981) Lignin Biodegradation and Transformation, John Wiley & Sons, New York
3. Gold, M. H., Wariishi, H., and Valli, K. (1989) in Biocatalysis in Agricultural Biotechnology (Whitaker, J. R., and Sonnet, P. E., eds), pp. 127-140, American Chemical Society, Washington DC
4. Kirk, T. K., and Farrell, R. L. (1987) Annu. Rev. Microbiol. **41**, 465-505
5. Buswell, J. A., and Odier, E. (1987) CRC Crit. Rev. Biotech. **6**, 1-60
6. Hammel, K. E. (1989) Enzyme Microb. Technol. **11**, 776-777
7. Fincham, J. R. S., Day, P. R., and Radford, A. (1979) Fungal Genetics. 4th ed., University of California Press, Berkeley
8. Gold, M. H., and Cheng, T. M. (1978) Appl. Environ. Microbiol. **35**, 1223-1225
9. Lederberg, J., and Lederberg, E. M. (1952) J. Bacteriol. **63**, 399-406
10. Beadle, G. W., and Tatum, E. L. (1945) Am. J. Bot. **32**, 678-686
11. Gold, M. H., Cheng, T. M., and Mayfield, M. B. (1982) Appl. Environ. Microbiol. **44**, 996-1000

12. Liwicki, R., Paterson, A., MacDonald, M. J., and Broda, P. (1985) J. Bacteriol. 162, 641-644

13. Boeke, J. D., LaCroute, F., and Fink, G.R. (1984) Mol. Gen. Genet. 197, 345-345

14. Skrzynia, C., Binninger, D. M., Alspaugh II, J. A., and Pukkila, P. J. (1989) Gene 81, 73-82

15. Peberdy, J. F. (1979) Annu. Rev. Microbiol. 33, 21-39

16. Gold, M. H., Cheng, T. M., and Alic, M. (1983) Appl. Environ. Microbiol. 46, 260-263

17. Krejci, R. (1987) Folia Microbiol. 32, 177-180

18. Ander, P., and Eriksson, K.-E. (1976) Arch. Microbiol. 109, 1-8

19. Gold, M. H., Mayfield, M. B., Cheng, T. M., Krisnangkura, K., Shimada, M., Enoki, A., and Glenn, J. K. (1982) Arch. Microbiol. 132, 115-122

20. Eriksson, K.-E., Johnsrud, S. C., and Vallander, L. (1983) Arch. Microbiol. 135, 161-168

21. Fenn, P., and Kirk. T. K. (1981) Arch. Microbiol. 130, 59-65

22. Glenn, J. K., and Gold, M. H. (1983) Appl. Environ. Microbiol. 45, 1741-1747

23. Gold, M. H., Glenn, J. K., and Alic, M. (1988) Methods Enzymol. 161, 74-78

24. Kuwahara, M., and Asada, Y. (1987) in Lignin Enzymic and Microbial Degradation (Odier, E., ed.) pp. 171-176, Institut National de la Recherche Agronomique, Paris

25. Ander, P., and Eriksson, K.-E. (1975) Sven. Papperstid. **18**, 643-652

26. Ander, P., Hatakka, A., and Eriksson, K.-E. (1980) Arch. Microbiol. **125**, 189-202

27. Johnsrud, S. C., and Eriksson, K.-E. (1985) Appl. Microbial. Biotechnol. **21**, 320-327

28. Ramasamy, K., Kelley, R. L., and Reddy, C. A. (1985) Biochem. Biophys. Res. Commun. **131**, 436-441

29. Kelley, R. L., Ramasamy, K., and Reddy, C. A. (1986) Arch. Microbiol. **144**, 254-257

30. Kirk, T. K., Croan, S., Tien, M., Murtagh, K. E., and Farrell, R. L. (1986) Enzyme Microb. Technol. **8**, 7-32

31. Kirk, T. K., Tien, M., Johnsrud, S. C., and Eriksson, K.-E. (1986) Enzyme Microb. Technol. **8**, 75-80

32. Raeder, U., Thompson, W., and Broda, P. (1989) Mol. Microbiol. 3:, 919-924

33. Oberwinkler, F. (1982) in Basidium and Basidiocarp: Evolution, Cytology, Function, and Development (Wells, K., and Wells, E. K., eds) pp. 9-35, Springer-Verlag, New York

34. Ross, I. K. (1979) Biology of the Fungi, McGraw-Hill, New York

35. Whitehouse, H. L. K. (1949) New Phytol. **48**, 212-244

36. Raper, J. R. (1966) Genetics of Sexuality in Higher Fungi, Ronald Press, New York

37. Raper, J. R. (1953) Quart. Rev. Biol. **28**, 233-259

38. Giasson, L., Specht, C. A., Milgrim, C., Novotny, C. P., and Ullrich, R. C. (1989) Mol. Gen. Genet. 218, 72-77

39. Gilbertson, R. L. (1971) in Evolution in the Higher Basidiomycetes (Petersen, R. H., ed) pp. 275-307, Univ. of Tenn. Press, Knoxville

40. Boidin, J. (1971) in Evolution in the Higher Basidiomycetes (Petersen, R. H., ed) pp. 129-148, Univ. of Tenn. Press, Knoxville

41. Stalpers, J. A. (1984) Stud. Mycol. 24, 1-105

42. Lentz, P. L. (1971) in Evolution in the Higher Basidiomycetes (Petersen, R. H., ed) pp. 99-127, Univ. of Tenn. Press, Knoxville

43. Burdsall, H. H. Jr. (1985) A Contribution to the Taxonomy of the Genus Phanerochaete, J. Cramer, Braunschweig

44. Ainsworth, A. M. (1987) in Evolutionary Biology of the Fungi (Rayner, A. D. M., Brasier, C. M., and Moore, D., eds) pp. 285-299, Cambridge Univ. Press, Cambridge

45. Eriksson, J., Hjortstam, K., and Ryvarden, L. (1978) The Corticiaceae of North Europe 5, 989

46. Burdsall, H. H., Jr., and Eslyn, W. E. (1974) Mycotaxon. 1, 123-133

47. Burdsall, H. H. Jr. (1981) Mycologia 73, 675-680

48. Thompson, W., and Broda, P. (1987) Trans. Br. Mycol. Soc. 89, 285-294

49. Gold, M. H., and Cheng, T. M. (1979) Arch. Microbiol. 121, 37-41

50. Esser, K. (1966) in The Fungi: An Advanced Treatise Vol.II (Ainsworth, G. C., and Sussman, A. S., eds) pp.661-676, Academic Press, New York

51. Raper J. R., and Miles, P. G. (1958) Genetics 43, 530-546

52. Chase, T. E., Ullrich, R. C., and Korhonen, K. (1985) Mycologia 77, 975-977

53. Carlile, M. J. (1987) in Evolutionary Biology of the Fungi (Rayner, A. D. M., Brasier, C. M., and Moore, D., eds) pp. 203-214, Cambridge Univ. Press, Cambridge.

54. Coates, D., Rayner, A. D. M., and Todd, N. K. (1981) Trans. Br. Mycol. Soc. 76, 41-51

55. Rayner, A. D. M., Coates, D., Ainsworth, A. M., Adams, T. J. H., Williams, E. N. D., and Todd, N. K. (1984) in The Ecology and Physiology of the Fungal Mycelium (Jennings, D. H., and Rayner, A. D. M., eds) pp. 509-540, Cambridge Univ. Press, Cambridge

56. Coates, D., and Rayner, A. D. M. (1985) J. Gen. Microbiol. 131, 207-219

57. Talbot, P. H. B. (1973) in The Fungi: An Advanced Treatise Vol.IVB (Ainsworth, G. C., Sparrow, F. K., and Sussman, A. S., eds) pp. 327-349, Academic Press, New York

58. Lemke, P. A. (1969) Mycologia 61, 57-76

59. Ullrich, R. C. (1973) Mycologia 65, 1234-1249

60. Korhonen, K. (1987) in Evolutionary Biology of the Fungi (Rayner, A. D. M., Brasier, C. M., and Moore, D., eds) pp. 301-310, Cambridge Univ. Press, Cambridge

61. Ullrich, R. C., and Raper, J.R. (1975) Genetics 80, 311-321

62. Lemke, P. A. (1966) Ph.D. Dissertation, Harvard Univ.

63. Kurtzman, C. P. (1985) in Gene Manipulations in Fungi (Bennett, J. W., and Lasure, L. L., eds) pp. 35-63, Academic Press, Orlando

64. Raeder U., and Broda, P. (1984) Curr. Genet 8, 499-506

65. Muller, B., and Gautier, F. (1975) Eur. J. Biochem. 54, 385-394

66. Broda, P., MacDonald, M., Raeder, U., Liwicki, R., Haylock, R., McCarthy, A., Paterson, A., Birch, O., Peace, E., Ramsey, L., and Cookson, I. (1984) Appl. Biochem. Biotech. 9, 321-322

67. Raeder, U., and Broda, P. (1988) Methods Enzymol. 161, 211-220

68. Lewin, B. (1985) Genes 2nd. ed., John Wiley & Sons, New York

69. Perkins, D. D. (1988) Fungal Genet. Newsl. 35, 44-46

69a. Ullrich, R. C., and Anderson, J. B. (1978) Exp. Mycol. 2. 119-129

70. Raeder U., and Broda, P. (1986) EMBO J. 51, 1125-1127

71. Davis, R. H. (1966) in The Fungi: An Advanced Treatise Vol.II (Ainsworth, G. C., and Sussman, A. S., eds) pp.567-588, Academic Press, New York

72. Raeder, U., Thompson, W., and Broda, P. (1989) Mol. Microbiol. 3, 911-918

73. Botstein, D., White, R. L., Skolnick, M., and Davis, R. W. (1980) Am. J. Hum. Genet. 32, 314-331

74. Metzenberg, R. L., Stevens, J. N., Selker, E. U., and Morzycka-Wroblewska, E. (1984) Neurospora Newsl. 31, 35-39

75. Metzenberg, R. L., Stevens, J. N., Selker, E. U., and Morzycka-Wroblewska, E. (1985) Proc. Natl. Acad. Sci. USA 82, 2067-2071

76. Berlin, V., and Yanofsky, C. (1985) Mol. Cell. Biol. 5, 849-855

77. Haylock, R., Liwicki, R., and Broda, P. (1985) J. Microbiol. Meth. 4, 155-162

78. Haylock, R., and Broda, P. (1988) Methods Enzymol. 161, 221-227

79. Raeder, U., Thompson, W., and Broda, P. (1987) Phil. Trans. R. Soc. Lond. A321, 475-483

80. McClintock, B. (1945) Am. J. Bot. 32, 671-678

81. Carmi, P., Holm, P. B., Koltin, Y., Rasmussen, S. W., Sage, J., and Zickler, D. (1978) Carlsberg Res. Commun. 43, 117-132

82. Perkins, D. D. (1985) in Molecular Genetics of Filamentous Fungi (Timberlake, W. E., ed) pp.277-294, Alan R. Liss, New York

83. Caten, C. E. (1987) in Evolutionary Biology of the Fungi (Rayner, A. D. M., Brasier, C. M., and Moore, D., eds) pp. 215-229, Cambridge Univ. Press, Cambridge

84. Struhl, K., Cameron, J. R., and Davis, R. W. (1976) Proc. Natl. Acad. Sci. USA 73, 1471-1475

85. Vapnek, D., Hautala, J. A., Jacobson, J. W., Giles, N. H., and Kushner, S. R. (1977) Proc. Natl. Acad. Sci. USA 74, 3508-3512

86. Ballance, D. J. (1986) Yeast 2, 229-236

87. Tien, M., and T. K. Kirk (1984) Proc. Natl. Acad. Sci USA 81, 2280-2284

88. Gold, M. H., Kuwahara, M., Chiu, A. A., and Glenn, J. K. (1984) Arch. Biochem. Biophys. 234, 353-362

89. Glenn, J. K., and Gold, M. H. (1985) Arch. Biochem. Biophys. 242, 329-341

90. Renganathan, V., Miki, K., and Gold, M. H. (1985) Arch. Biochem. Biophys. 241, 304-314

91. Paszczynski, A., Huynh, V.-B., and Crawford, R. (1986) Arch. Biochem. Biophys. 244, 750-765

92. Leisola, M. S. A., Kozulic, B., Meussdoerffer, F., and Fiechter, A. (1987) J. Biol. Chem. 262, 419-424

93. Zhang, Y. Z., Zylstra, G. J., Olsen, R. H., and Reddy, C. A. (1986) Biochem. Biophys. Res. Commun. 137, 649-656

94. Zhang, Y. Z., and Reddy, C. A. (1988) Methods Enzymol. 161, 228-237

95. Tien, M., and Tu, C.-P. D. (1987) Nature 326, 520-523

96. de Boer, H. A., Zhang, Y.Z., Collins, C., and Reddy, C.A. (1987) Gene 60, 93-102

97. Andrawis, A., Pease, E.A., Kuan, I., Holzbaur, E., and Tien, M. (1989) Biochem. Biophys. Res. Commun. 162, 673-680

98. de Boer, H. A., Zhang, Y. Z., Collins, C., and Reddy, C. A. (1988) Gene 69, 369

99. Fuller, R. S., Sterne, R. E., and Thorner, J. (1988) Ann. Rev. Physiol. 50, 345-362

100. Smith, T. L., Schalch, H., Gaskell, J., Covert, S., and Cullen, D. (1988) Nucleic Acids Res. 16, 1219

101. Walther, I., Kalin, M., Reiser, J., Suter, F., Fritsche, B., Saloheimo, M., Leisola, M., Teeri, T., Knowles, J. K. C., and Fiechter, A. (1988) Gene 70, 127-137

102. Schalch, H., Gaskell, J., Smith, T. L., and Cullen, D. (1989) Mol. Cell. Biol. 9, 2743-2747

103. Asada, Y., Kimura, Y., Kuwahara, M., Tsukamoto, A., Koide, K., Oka, A., and Takanami, M. (1988) Appl. Microbiol. Biotechnol. 29, 469-473

104. Brown, A., Sims, P. F. G., Raeder, U., and Broda, P. (1988) Gene 73, 77-85

105. Holzbaur, E. L. F., and Tien, M. (1988) Biochem. Biophys. Res. Commun. 55, 626-633

106. Pribnow, D., Mayfield, M. B., Nipper, V. J., Brown, J. A., and Gold, M. H. (1989) J. Biol. Chem. 264, 5036-5040

107. von Heijne, G. (1985) J. Mol. Biol. 184, 99-105

108. Pease, E. A., Andrawis, A., and Tien, M. (1989) J. Biol. Chem. 264, 13531-13535

109. Dynan, W. S., and Tjian, R. (1985) Nature 316, 774-778

110. Wingender, E. (1988) Nucl. Acids. Res. 16, 1879-1902

111. Lindquist, S. (1986) Annu. Rev. Biochem. 55, 1151-1191

112. MacDonald, M. J., Paterson, A., and Broda, P. (1984) J. Bacteriol. 160, 470-472

113. MacDonald, M. J., Ambler, R., and Broda, P. (1985) Arch. Microbiol. 142, 152-156

114. Sims, P., James, C., and Broda, P. (1988) Gene 74, 411-422

115. Sims, P., Brown, A., James, C., Raeder, U., Schrank, A., and Broda, P. (1988) in Biochemistry and Genetics of Cellulose Degradation (Aubert, J.-P., Beguin, P., and Millet, J., eds) pp. 365-373, Academic Press, London

116. Mishra, N. C., and Tatum, E. L. (1973) Proc. Natl. Acad. Sci. USA 70, 3875-3879

117. Hinnen, A., Hicks, J. B., and Fink, G. R. (1978) Proc. Natl. Acad. Sci. USA 75, 1929-1933

118. Case, M. E., Schweizer, M., Kushner, S. R., and Giles, N. H. (1979) Proc. Natl. Acad. Sci. USA 76, 5259-5263

119. Fincham, J. R. S. (1989) Microbiol Rev 53, 148-170

120. Ullrich, R. C., Novotny, C. P., Specht, C. A., Froeliger, E. H., and Muñoz-Rivas, A. M. (1985) in Molecular Genetics of Filamentous Fungi (Timberlake, W. E., ed) pp.39-57, Alan R. Liss, New York

121. Muñoz-Rivas, A., Specht. C. A., Drummond, B. J., Froeliger, E., Novotny, C. P., and Ullrich, R. C. (1986) Mol. Gen. Genet. 205, 103-106

122. Binninger, D. M., Skrzynia, C., Pukkila, P. J., and Casselton, L. A. (1987) EMBO J. 6, 835-840

123. Froeliger, E. H., Muñoz-Rivas, A. M., Specht, C. A., Ullrich, R. C., and Novotny, C. P. (1987) Curr. Genet. 12, 547-554

124. Mellon, F. M., Little, P. F. R., and Casselton, L. A. (1987) Mol. Gen. Genet. 210, 352-357

125. Casselton, L. A., and de la Fuente Herce, A. (1989) Curr. Genet. 16, 35-40

126. Bégueret, J., Razanamparany, V., Perrot, M., and Barreau, C. (1984) Gene 32, 487-492

127. Hynes, M. J. (1989) Exp. Mycol. 13, 196-198

128. Punt, P. J., Oliver, R. P., Dingemanse, M. A., Pouwels, P. H., and van den Hondel, C. A. M. J. J. (1987) Gene 56, 117-124

129. Wang, J., Holden, D. W., and Leong, S. A. (1988) Proc. Natl. Acad. Sci. USA 85, 865-869

130. Genilloud, O., Garrido, M. C., and Moreno, F. (1984) Gene 32, 225-233

131. Mattern, I. E., and Punt, P. J. (1988) Fungal Genet. Newsl. 35, 25

132. Kolar, M., Punt, P. J., van den Hondel, C. A. M. J. J., and Schwab, H. (1988) 62, 127-134

133. Randall, T., Rao, T. R., and Reddy, C. A. (1989) Biochem. Biophys. Res. Commun. 161, 720-725

134. Grindley, N. D. F., and Joyce, C. M. (1980) Proc. Natl. Acad. Sci. USA 77, 7176-7180

135. Rao, T. R., and Reddy, C. A. (1984) Biochem. Biophys. Res. Commun. 118, 821-827

136. Turner, G., and Ballance, D. J. (1985) in Gene Manipulations in Fungi (Bennett, J. W., and Lasure, L. L., eds) pp. 259-278, Academic Press, Orlando

137. Katayose, Y., Shishido, K., and Ohmasa, M. (1986) Biochem. Biophys. Res. Commun. 138, 1110-1115

138. Pontecorvo, G., Roper, J. A., Hemmon, L. M., MacDonald, K. D., and Bufton, A. W. J. (1953) Adv. Genet. 5, 141-239

139. Raper, J. R., and Hoffman, R. M. (1974) in Handbook of Genetics Vol.1 (King, R. C., ed) pp. 597-626, Plenum Press, New York

140. Guerdoux, J. L. (1974) in Handbook of Genetics Vol.1 (King, R. C., ed) pp. 627-636, Plenum Press, New York

141. Gross, S. R. (1965) Proc. Natl. Acad. Sci. USA 54, 1538-1546

142. Calvo, J. M., and Gross, S. R. (1970) Methods Enzymol. 17A, 791-793

143. Ulm, E. H., Böhme, R., and Kohlhaw, G. (1972) J. Bacteriol. 110, 1118-1126

144. Taylor, A. L., and Trotter, C. D. (1974), in Handbook of Genetics Vol.1 (King, R. C., ed) pp. 135-156, Plenum Press, New York

145. Kohlhaw, G. B. (1980) in Current Developments in Yeast Research. (Stewart, G. G. and Russell, I., eds) pp. 199-204, Pergamon Press, New York

146. Eriksson, K.-E. (1981) in Trends in the Biology of Fermentation for Fuels and Chemicals (Hollaender, A., and Rabson, R., eds) pp. 19-32, Plenum Press, New York

147. Kirk, T. K., and Chang, H.-M. (1981) Enzyme Microb. Technol. 3, 189-196

148. Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983) Biochem. Biophys. Res. Commun. 114, 1077-1083

149. Kuwahara, M., Glenn, J. K., Morgan, M. A., and Gold, M. H. (1984) FEBS Lett. 169, 247-249

150. Tien, M., and Kirk, T. K. (1983) Science 221, 661-663

151. Smith, M. H., and Gold, M. H. (1979) Appl. Environ. Microbiol. 37, 938-942

152. Tatum, E. L., Barratt, R. W., Fries, N., and Bonner, D. (1950) Am. J. Bot. 37, 38-46

153. Snider, P. J., and Raper, J. R. (1958) Am. J. Bot. 45, 538-546

154. Raper, J. R., and Krongelb, G. S. (1958) Mycologia 50, 707-740

155. Burnett, J. H. (1975) Mycogenetics, John Wiley & Sons, London

156. Alic, M., and Gold, M. H. (1985) Appl. Environ. Microbiol. 50, 27-30

157. Ryan, E. D., Tracy, J. W., and Kohlhaw, G. B. (1973) J. Bacteriol. 116, 222-225

158. Vogel, H. J. (1964) Am. Nat. 96, 435-446

159. Parsons, S. J., and Burns, R. O. (1970) Methods Enzymol. 17A, 793-799

160. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275

161. Satyanarayana, T., Umbarger, H. E., and Lindegren, G. (1968) J. Bacteriol. 96, 2012-2017

162. Ryan, E. D., and Kohlhaw, G. B. (1974) J. Bacteriol. 120, 631-637

163. Molskness, T. A., Alic, M., and Gold, M. H. (1986) Appl. Environ. Microbiol. 51, 1170-1173

164. Slater, M. L. (1976) J. Bacteriol. 126, 1339-1341

165. Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978) Arch. Microbiol. 117, 227-285

166. Ryan, F. J., Beadle, G. W., and Tatum, E. L. (1943) Am. J. Bot. 30, 784-799

167. Burnett, J. H., and Boulder, M. E. (1963) New Phytol. 62, 217-236

168. Sequeira, L. (1954) Mycologia 46, 470-483

169. Tilburn, J., Scazzacchio, C., Taylor, G. G. Zabicky-Zissman, J. H., Lockington, R. A., and Davies, R. W. (1983) Gene 26, 205-221

170. Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986) Arch. Biochem. Biophys. 251, 688-696

171. Kersten, P. J., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985) J. Biol. Chem. 260, 2609-2612

172. Renganathan, V., and Gold, M. H. (1986) Biochemistry 25, 1626-1631

173. Alic, M., Letzring, C., and Gold, M. H. (1987) Appl. Environ. Microbiol. 53, 1464-1469

174. Jones, E. W. and Fink, G. R. (1982) in The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp. 181-299, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

175. Muñoz-Rivas, A. M., Specht, C. A., Ullrich, R. C., and Novotny, C. P. (1986) Curr. Genet. 10, 909-913

176. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

177. Raeder, U., and Broda, P. (1985) Lett. Appl. Microbiol. 1, 17-20

178. Southern, E. M. (1975) J. Mol. Biol. 98, 503-517

179. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13

180. Akins, R. A., and Lambowitz, A. M. (1985) Mol. Cell. Biol. 5, 2272-2278

181. Dhawale, S. S., Paietta, J. V., and Marzluf, G. A. (1984) Curr. Genet. 8, 77-79

182. Ballance, D. J., and Turner, G. (1985) Gene 36, 321-331

183. Kinsey, J. A., and Rambosek, J. A. (1984) Mol. Cell. Biol. 4, 117-122

184. Durrens, P., Green, P. M., Arst, H. N. Jr., and Scazzocchio, C. (1986) Mol. Gen. Genet. 203, 544-549

185. Ebbole, D. J., and Zalkin, H. (1987) J. Biol. Chem. 262, 8274-8287

186. Silver, J. M., and Eaton, N. R. (1969) Biochem. Biophys. Res. Commun. 34, 301-305

187. Alic, M., Kornegay, J. R., Pribnow, D., and Gold, M. H, (1989) Appl. Environ. Microbiol. 55, 406-411

188. Orbach, M. J., Porro, E. B., and Yanofsky, C. (1986) Mol. Cell. Biol. 6, 2452-2461

189. Specht, C. A., Muñoz-Rivas, A., Novotny, C. P., and Ullrich, R. C. (1988) Exp. Mycol. 12, 357-366

190. Schweizer, M., Case, M. E., Dykstra, C. C., Giles, N. H., and Kushner, S. R. (1981) Proc. Natl. Acad. Sci. USA 78, 5086-5090

191. Schechtman, M. G., and Yanofsky, C. (1983) J. Mol. Appl. Gen. 2, 83-99

192. Razanamparany, V., and Bégueret, J. (1986) Curr. Genet. 10, 811-817

193. Timberlake, W. E., and Marshall, M. A. (1989) Science 244, 1313-1317

194. Paietta, J. V., and Marzluf, G. A. (1985) Mol. Cell. Biol. 5, 1554-1559

195. Wernars, K., Goosen, T., Wennekes, L. M. J., Visser, J., Bos, C. J., van den Broek, H. W. J., van Gorcom, R. F. M., van den Hondel, C. A. M. J. J., and Pouwels, P. H. (1985) Curr. Genet. 9, 361-368

196. Mellon, F. M., and Casselton, L. A. (1988) Curr. Genet. 14, 451-456

197. Razanamparany, V., and Bégueret, J. (1988) Gene 74, 399-409

198. Perkins, D. D., Radford, A., Newmeyer, D., and Björkman, M. (1982) Microbiol. Rev. 46, 426-570

199. Vollmer, S. J., and Yanofsky, C. (1986) Proc. Natl. Acad. Sci. USA 83, 4869-4873

200. Buchanan, J. M. (1960) Harvey Lectures 54, 104-130

201. Glass, N. L., Vollmer, S. J., Staben, C., Grotelueschen, J., Metzenberg, R. L., and Yanofsky, C. (1988) Science 241, 570-573

202. Lai, E., Birren, B. W., Clark, S. M., Simon, M. I., and Hood, L. (1989) Biotechniques 7, 34-42

203. Chu, G., Vollrath, D., and Davis, R. W. (1986) Science 234, 1582-1585

204. Orbach, M. J., Vollrath, D., Davis, R. W., and Yanofsky, C. (1988) Mol. Cell. Biol. 8, 1469-1473

205. Miller, B. L., Miller, K. Y., Roberti, K. A., and Timberlake, W. E. (1987) Mol. Cell. Biol. 7, 427-434

206. McKnight, G. L., O'Hara, P. J., and Parker, M. L. (1986) Cell 46, 143-147

207. Punt, P. J., Dingemanse, M. A., Jacobs-Meijsing, B. J. M., Pouwels, P. H., and van den Hondel, C. A. M. J. J. (1988) Gene 69, 49-57

208. Krebs, E. G., Rafter, G. W., and Junge, J. M. (1953) J. Biol. Chem. 200, 479-492

209. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987) EMBO J. 6, 3901-3907

210. Roberts, I. N., Oliver, R. P., Punt, P. J., and van den Hondel, C. A. M. J. J. (1989) Curr. Genet. 15, 177-180

211. Smith, M. (1985) Annu. Rev. Genet. 19, 423-462

212. Gerlt, J. A. (1987) Chem. Rev. 87, 1079-1105

213. Millis, C. D., Cai, D., Stankovich, M. T., and Tien, M. (1989) Biochemistry 28, 8484-8489

214. Miller, B. L., Miller, K. Y., and Timberlake, W. E. (1985) Mol. Cell. Biol. 5, 1714-1721

215. Kronstad, J. W., Wang, J., Covert, S. F., Holden, D. W., McKnight, G. L., and Leong, S. A. (1989) Gene 79, 97-106

216. Kelly, R., Miller, S. M., Kurtz, M. B., and Kirsch, D. R. (1987) Mol. Cell. Biol. 7, 199-207

217. Harvey, P. J., Schoemaker, H. E., and Palmer, J. M. (1986) FEBS Lett. 195, 242-246

218. Faison, B. D., Kirk, T. K., and Farrell, R. L. (1986) Appl. Environ. Microbiol. 52, 251-254

219. Tonon, F., and Odier, E. (1988) Appl. Environ. Microbiol. 54, 466-472

220. Shimada, M., Nakatsubo, F., Kirk, T. K., and Higuchi, T. (1981) Arch. Microbiol. 129, 321-324

221. Gilbert, H. J., Clarke, I. N., Gibson, R. K., Stephenson, J. R., and Tull, M. (1985) J. Bacteriol. 161, 314-320

222. Cregg, J. M., Barringer, K. J., Hessler, A. Y., and Madden, K. R. (1985) Mol. Cell. Biol. 5, 3376-3385

223. Cregg, J. M., Digan, M. E., Tschopp, J. F., Brierley, R. A., Craig, W. S., Velicelebi, G., Siegel, R. S., and Thill, G. P. (1989) in Genetics and Molecular Biology of Industrial Microorganisms (Hershberger, C. L., Queener, S. W., and Hegeman, G., eds) pp. 343-352, American Society for Microbiology, Washington, DC

224. Morita, Y., Yanashita, H., Mikami, B., Iwamoto, H., Aibara, S., Terada, M., and Minami, J. (1988) J. Biochem. 103, 693-699

BIOGRAPHICAL NOTE

The author was born December 11, 1949 in Oak Park, Illinois. She attended Sarah Lawrence College and the University of Michigan, before graduating from Reed College in 1972, with a B. A. in biology.

Prior to coming to OGI as a research technician in 1982, the author held several research assistant positions in chemistry and immunology and taught a course at Portland State University on the history of women in science. During this period, the author wrote a book on the history of women in science (see below). For the past 15 years, she has worked as a radio music programmer.

In addition to the work presented in this dissertation, the author's publication list includes the following:

Gold, M. H., Glenn, J. K., and Alic, M. (1988) Methods Enzymol. 161, 74-78

Gold, M. H., Cheng, T. M., and Alic, M. (1983) Appl. Environ. Microbiol. 46, 260-263

Alic, M. (1986) Hypatia's Heritage: A history of women in science from antiquity to the late nineteenth century. The Women's Press, London; Beacon Press, Boston; German, Italian, and Swedish translations

Alic, M. (1982) Women's Studies International Forum 5:75-81

Alic, M. (1981) Women's Studies International Quarterly 4:305-312

The author began her graduate studies at OGI in September, 1985.