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EXTRACHROMOSOMALLY MAINTAINED TRANSFORMATION VECTORS OF THE LIGNIN DEGRADING FILAMENTOUS FUNGUS PHANEROCHAETE CHRYSOSPORIUM

presented by

THOMAS ALLAN RANDALL

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Microbiology and Public Health

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EXTRACHROMOSOMALLY MAINTAINED TRANSFORMATION VECTORS OF THE LIGNIN DEGRADING FILAMENTOUS FUNGUS PHANEROCHAETE CHRYSOSPORIUM

BY

THOMAS ALLAN RANDALL

A DISSERTATION

Submitted to
Michigan State University
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Department of Microbiology and Public Health

1990

ABSTRACT

EXTRACHROMOSOMALLY MAINTAINED TRANSFORMATION VECTORS OF THE LIGNIN DEGRADING FILAMENTOUS PHANEROCHAETE CHRYSOSPORIUM

BY

THOMAS ALLAN RANDALL

Phanerochaete chrysosporium is a lignin degrading white rot basidiomycete fungus. There is considerable interest in this organism due to its applications in biopulping, xenobiotic degradation and in bioconversion of lignocellulosic materials to feeds, fuels, and chemicals. Hence, the physiology, genetics, and molecular biology of this organism is being studied extensively. To further the molecular genetic analysis of this fungus, we constructed vector pRR12 that transforms P. chrysosporium to G418 resistance. Vector pG12-1, a derivative pRR12, was isolated from a pRR12 transformant and was also shown to transform P. chrysosporium. This vector appeared to result from a rearrangement between pRR12 and an endogenous

plasmid(pME). Vector pG12-1 was shown to be maintained in P. chrysosporium in a low copy, circular, extrachromosomal state and was recovered intact from fungal transformants by E. coli transformation. The vector was stably transmitted to basidiospore progeny of the transformants. A similar mode of transformation was found to occur with two other vectors, YIp5-kan^r and pAN7-1, which transform P. chrysosporium to G418 and hygromycin resistance, respectively. Plasmid p12-6, a common derivative of pG12-1 and YIp5-kan^r, was also shown to transform P. chrysosporium. Southern blot analysis of p12-6 transformants shows directly that the vector is maintained extrachromosomally, although not autonomously. Two classes of plasmids(p12-6 and p511), were found to be recovered by E. coli transformation using total DNA of p12-6 transformants. The lacZ gene inserted into p12-6, was shown to be passed intact through P. chrysosporium suggesting that p12-6 has a potential as a shuttle vector. When the kan^r determinant was inserted into the coding region of a genomic clone containing the lignin peroxidase H2, the resulting plasmid was shown to transform P. chrysosporium to G418 resistance, was maintained in a high copy number autonomous form in the transformants, and was recoverable by E. coli transformation.

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INTRODUCTION

Lignin is a highly complex, heterogeneous, amorphus, three dimensional aromatic polymer that is a major structural component of all woody plants(1). Lignin is second only to cellulose in being the most abundant structural component of terrestrial plants. Its role in these plants is to provide structural rigidity and because of its recalcitrance to biodegradation, to provide the first line of defense against plant pathogens(2). Lignin, because of its relative resistance to microbial degradation, is an important limiting factor in the earth's carbon cycling and in the efficient conversion of plant biomass to feeds, fuels, and chemicals(1). Lignin degradation in nature is predominantly carried out by the white rot fungi. There has been much interest in the lignin degrading system (LDS) of the basidiomycete fungus Phanerochaete chrysosporium because it is able to rapidly and completely degrade lignin(3); grows well at a relatively high temperature (39-40^{o}C); and conidiates profusely(4-6). For those reasons and others, P. chrysosporium has become a model organism for the study of lignin degradation(2,3). The interest in the lignin degrading capacity of this fungus is primarily due to the potential industrial applications of lignin

degradation(2,3,7). P. chrysosporium has also attracted world wide attention recently because the LDS of this fungus has also been implicated in the detoxification of a broad range of recalcitrant man-made environmental pollutants(8,9).

The LDS of P. chrysosporium is expressed strictly during secondary metabolism in response to nutritional starvation(4). Recent investigations have focused on the biochemistry, genetics, and the molecular biology of the lignin degrading system of P. chrysosporium in order to better understand the process of lignin degradation in nature(8,9). Two major families of extracellular enzymes, designated lignin peroxidases(LIP) and manganese dependent peroxidases(MNP), which are involved in lignin degradation have been identified(8,9). Genes encoding several LIP and MNP isozymes have been cloned and sequenced. Both LIP and MNP gene families consist of multiple genes with very high sequence homology. Many other enzymes, such as glucose oxidase(gox), glyoxal oxidase, β-etherases, laccases, ring cleavage enzymes, and demethylases, that have been implicated in the lignin degradation pathway, have been described(8,9). Various secondary metabolic mutants altering the pattern of expression of LIPs, MNPs, glucose oxidase have been isolated in order to better define the regulatory pathway(s) controlling the expression of these genes(10-14).

The transformation systems for the well studied yeasts

and filamentous fungi have led to advances in two broad areas. First, the varied types of vectors used in both the yeast and fungal transformation systems has been utilized in concert with the classical genetic approaches to yield considerable advances in the understanding of the genetics and physiology of these lower eukaryotes. Secondly, these transformation systems have been the models for the development of similar systems for many of the less well characterized fungi, many of which are important in agriculture and medicine and for which gene transfer systems are either not available or are primitive. To facilitate more effective genetic manipulation of these fungi, of which P. chrysosporium is a prime example, recombinant DNA technology can in many cases be used to develop these fungi to their full potential. The first step in exploiting the biotechnological potential of these organisms is to develop a DNA transformation system for these organisms and to clone and characterize specific genes of interest.

Comparisons between the transformation systems developed for single celled yeasts and the filamentous fungi have also illustrated marked differences in the mechanism by which DNA transformation is achieved in these organisms. This has resulted in detailed knowledge concerning the genetic processes operating in these organisms. Transformation has also been used to understand the regulation of transcription of genes of interest and to

understand the nature of important elements of the chromosome. Attempts to extend this knowledge to the filamentous fungi has lead to a better understanding of the transformation process in filamentous fungi and towards the development of more sophisticated and useful vectors. These transformation systems are now being utilized in the genetic analysis of various filamentous fungi. This literature review will be concerned with two areas of interest in relation to this thesis: 1) the recent advances in lignin degradation by P. chrysosporium; and 2) the phenomenon of DNA transformation in fungi.

LITERATURE REVIEW

I. Lignin Degradation by P. chrysosporium

1. The Structure of Lignin

Lignin exists in nature as a major cell wall component of all vascular plants, accounting for 25% of the dry weight of plants(1). The other major components of plants, cellulose and hemicellulose, account for approximately 50 and 25%, respectively. Lignin in plants has two major roles: 1) it provides much of the structural support needed for trees to stand; 2) it provides a barrier to the attack of plants by bacterial and fungal pathogens.

The lignin polymer is a heterogenous aromatic polymer formed through the peroxidase mediated dehydrogenative polymerization of three cinnamyl alcohol derivatives: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol(2; Fig. 1). These three compounds are present in variable amounts in different plant species and within the tissue of a single plant(3,4). The lignin monomers are linked primarily by C-C and C-O-C linkages which are relatively resistant to microbial degradations. The amounts of the individual lignin monomers as well as the relative proportions of intermonomer linkages are also highly variable between different species of plants and between different tissues of the same plant. Furthermore, the

subunits which comprise lignin are organized in a very irregular fashion with no repeating linkages(Fig. 1). This is in contrast to the other major components of wood, cellulose and hemicellulose, which are have an ordered arrangement of defined linkages. Thus, the heterogeneous, amorphous, and three dimensional structure of lignin ideally serves a protective role in nature against plant pathogens

2. Microbiology of Lignin

Relatively few groups of bacteria and fungi have been reported to degrade lignin, and none have been convincingly shown to use lignin as a sole energy source for growth in pure culture(4,5). White rot fungi are known to degrade all the three major components of wood: cellulose, lignin, and hemicellulose(3,4,7). Among the white rot fungi, P. chrysosporium has been shown to completely mineralize lignin to CO_2 more rapidly than most others(6). P. chrysosporium has become the model system for the study of lignin degradation for many reasons. It is one of the fastest growing of the white rot fungi; has a rapid rate of lignin degradation; the physiological and cultural parameters for maximizing growth and lignin degradation have been defined; the enzymes associated with lignin degradation and the genes encoding several of them have been extensively documented(8,9). In addition, genetic studies on P. chrysosporium, while primitive compared to

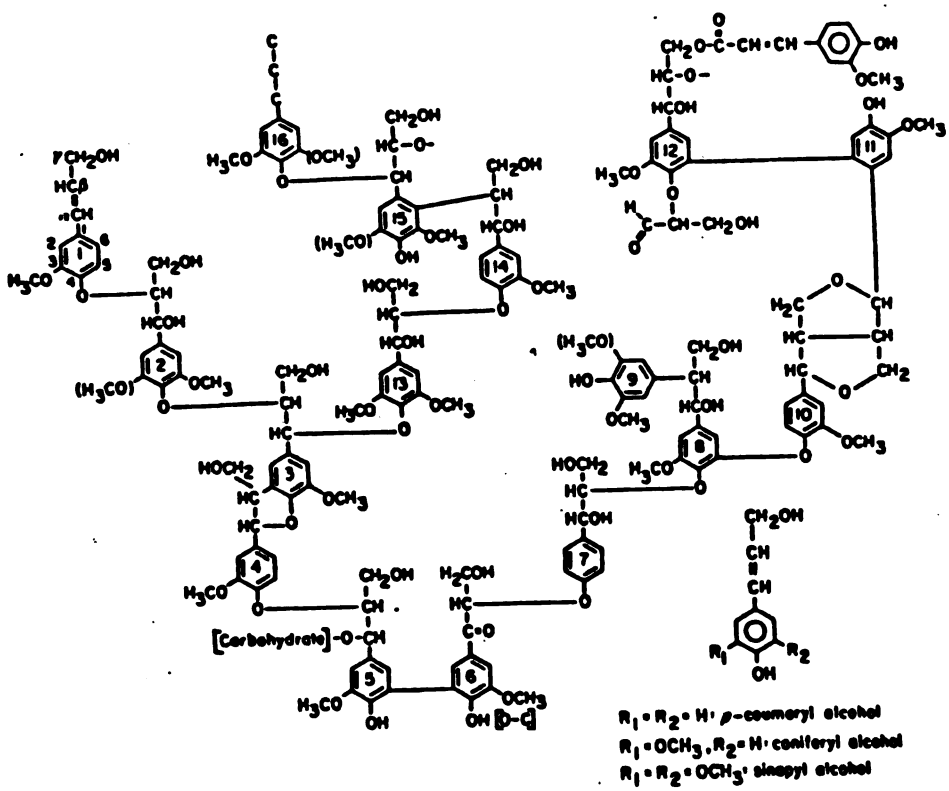


Fig. 1. The structure of lignin. The structure shown illustrates the major linkages of a representative angiosperm lignin. At bottom left are the structures of the three precursor alcohols which predominate in the lignin structure. Figure is taken from ref. 9.

other fungi, have been extensive, as compared to other white rot fungi, and many auxotrophic(15-18) and secondary metabolic(10-14) mutants have been characterized.

Soft rot and brown rot fungi also attack wood but have not been studied in detail. The soft rot fungi cause softening of the wood tissue, primarily by attacking the polysaccharide components, although low levels of lignin degradation have been observed(19). The brown rot fungi mainly degrade the polysaccharides but also cause a limited degradation of lignin. This limited degradation is thought to be due to the inability of the brown rot fungi to efficiently attack the aromatic rings of the cinnamyl alcohol derivatives(20).

Several genera of eubacteria, including Streptomyces, Nocardia, Pseudomonads, and Flavobacteria have been reported to degrade different forms of natural or modified lignins(2,21); however the degradation is relatively slow and there is no convincing evidence for the complete mineralization of lignin to CO_2 by bacteria in pure culture.

3. The Physiology of Lignin Degradation in P. chrysosporium

Lignin degradation in P. chrysosporium is a tightly regulated event and occurs only in a limited range of physiological conditions. Lignin degradation is strictly a secondary metabolic event(22-24). The appearance of the lignin degrading system(LDS; measured as the degradation of

^{14}C-labelled synthetic lignin[DHP] to CO_2) is triggered in response to nitrogen, carbon, or sulfur limitation(22,25,26). Lignin degradation in nitrogen limited cultures is strongly repressed by the addition of exogenous nitrogen sources such as NH_4^+ or L-glutamate(26, 27) and the latter has been shown to play an important role in the regulation of secondary metabolism in P. chrysosporium(27). The onset of secondary metabolism in P. chrysosporium is preceded by a ten fold increase in the intracellular levels of cyclic AMP(cAMP) suggesting that cAMP plays a role in the onset of lignin degradation(28,29), but the exact role of cAMP is not known.

Since lignin degradation is an oxidative process, oxygen is an important requirement. A comparison of the rates of lignin degradation under different oxygen partial pressures showed that the amount of lignin degraded at 5% O_2 is only 10% of that degraded at 21% O_2, and that cultures grown under 100% O_2 showed the highest level of lignin degradation (26). Agitation of cultures has been found to completely inhibit lignin degradation in wild type strains in certain culture media(30). This inhibition can be relieved by the addition of low levels of detergents such as Tween-20 or Tween-80 to shaking cultures, although the rate of agitation still remains critical for optimal ligninolytic activity in these cultures(31,32).

Hydrogen peroxide plays an important role in lignin degradation by wood-rotting fungi(33-36). Several wood

rotting fungi have been shown to produce substantial levels of H_2O_2 from various substrates(37,38). An increase in the specific activity for H_2O_2 production coincides with the onset of ligninolytic activity in low nitrogen cultures(39). The critical involvement of H_2O_2 was further established with the isolation of lignin peroxidases(see below) which are strictly dependent on H_2O_2 for activity(40,41). Evidence has been published which indicates that glucose oxidase, and perhaps glyoxal oxidase and other enzymes, play an important role in lignin degradation by P. chrysosporium(42,43).

The appearance of the enzymes of the LDS, as measured by ^{14}C lignin to $^{14}CO_2$, is not dependent on the presence of lignin in the medium, although higher levels of these enzymes are produced when lignin is present(44). As lignin itself cannot cross the cell membrane, it was thought that this effect may be due to a by-product of lignin degradation. Lignin monomers, dimers, and several secondary metabolites produced during lignin degradation were then tested and it was found that lignin dimers as well as veratryl alcohol, a typical secondary metabolite produced by P. chrysosporium, increased the level of lignin degradation in nitrogen limited cultures(45,46).

4. Enzymes involved in Lignin Degradation

A. Lignin Peroxidases(LIP)

Lignin peroxidases are a family of H_2O_2 dependent, heme containing, glycosylated extracellular enzymes(M_r of 39-43 kD), which catalyze the C_a-C_b cleavage of lignin model compounds and other phenolic and aromatic compounds(40,41,47-49). Lignin peroxidases are relatively non-specific and contain one protoheme IX(prosthetic group) per molecule. Various spectral analyses(50,51) showed that the native enzyme contains a high-spin iron and the reduced enzyme contains a high-spin, pentacoordinate ferrous iron.

The number of lignin peroxidase isozymes produced by different strains of P. chrysosporium varies from 3 to 15 (30,49,52). All of these isozymes catalyze oxidation of veratryl alcohol to veratrylaldehyde in the prescence of H_2O_2(30), the typical assay for lignin peroxidase activity. The levels of different LIP isozymes vary under different culture conditions(30,53) indicating a very complex regulation of the production of these enzymes. It has been found that the highest lignin peroxidase enzymatic activity is seen when the cultures are grown under air during primary metabolism and then shifted to 100% O_2 during secondary metabolism(45). Addition of veratryl alcohol to secondary metabolic cultures has been shown to result in up to a four fold increase in the level of lignin peroxidases produced(54). It was subsequently found that veratryl

alcohol increased the levels of some of the lignin peroxidase isozymes while having no effect on others suggesting that it could induce the production of a higher level of a specific subset of the lignin peroxidases(30). The addition to cultures of certain trace minerals was also shown to enhance lignin degradation(30) and levels of lignin peroxidase activity.

The lignin peroxidases of P. chrysosporium strain BKM-F have been more completely characterized than those of other strains. The FPLC profile of the extracellular fluid from BKM-F cultures grown for 5 days in low nitrogen medium showed multiple heme protein peaks. Six of which(designated H1,H2,H6,H7,H8, and H10) have veratryl alcohol oxidizing activity(26) while the four others(H3, H4, H5, and H9) were shown to have manganese peroxidase activity(see below). The lignin peroxidases H2 and H8 are the major proteins produced in dimethylsuccinate buffered cultures, and H2 and H6 show the highest specific activity. Polyclonal antibody raised against H8 has been found to crossreact to all lignin peroxidases(30) suggesting their structural relatedness. V8 protease digestion analyses indicate that H1 and H2 produce almost identical peptide patterns; H8 was quite similar to these except for two missing peptides and H6 and H8 produced patterns different from H1, H2, and H10(30). These results suggested that multiple genes encode lignin peroxidases, although in some cases the multiplicity of these enzymes due to post-transcriptional differences could not be ruled out. These

enzymes have several potential biotechnological applications. LIP enzymes, either purified or in culture with other enzymes of the LDS could be utilized in the production of a range of small molecular weight chemical feedstocks from the lignin polymer. In commercial pulping procedures, which produce high levels of toxic halogenated aromatic byproducts, a biopulping alternative using LIPs has been shown to dehalogenate these chemicals, thus LIP enzymes have potential in processes to detoxify these toxins.

B. Mn^{++} Dependent peroxidases(MNP)

A second group of extracellular glycosylated heme proteins which require both H_2O_2 and Mn^{++} to oxidize a variety of lignin model compounds and model dyes such as poly R-481 and poly B-411(55,56) are designated manganese dependent lignin peroxidases. These are different from lignin peroxidases in that they do not exhibit veratryl alcohol oxidation activity(57). The Mn of the MNPs is oxidized from Mn^{II} to Mn^{III}. The Mn^{III} then transfers a single electron to the lignin model compound resulting in an unstable radical which, as in the case of lignin peroxidases, is then resolved to several different breakdown products. The exact contributions of MNPs to the lignin degradation process is still unclear, although studies with a Lip$^-$ mutant containing only MNP activity can still degrade ^{14}C lignin to $^{14}CO_2$, although at a lower

level(13). Ligninolytic cultures reportedly produce as many as six Mn^{++} peroxidases, which, based on their similarity in peptide digestion patterns and amino acid sequences, are probably encoded by distinct but highly related genes(52). The availability of these two classes of peroxidases should allow for a more selective production of chemical feedstocks and treatment of waste byproducts once more is known concerning the specific substrate differences between the LIPs and MNPs, and the possible differences in activities between the isozymes of each family. These aspects are currently under investigation.

C. H_2O_2 Producing Enzymes

There is a temporal correlation between the appearance of H_2O_2 production and lignin degradation activity. The fact that the key lignin degrading enzymes(LIPs and MNPs) require H_2O_2 for their catalysis clearly showed that H_2O_2 plays an integral role in lignin degradation but the physiological source of H_2O_2 in lignin degrading cultures was not known. Kelley et.al.(33,42) found that the primary enzyme involved in the production of H_2O_2 during secondary metabolism in P. chrysosporium was glucose oxidase. This catalyzes the oxidation of D-glucose to H_2O_2 and D-glucolactone in the prescence of molecular oxygen. Glucose oxidase activity, like LIP and MNP activity, was found to be a secondary metabolic event triggered by either nitrogen or carbohydrate starvation and was repressed by adding

exogenous nitrogen(NH_4^+ or glutamate) to lignin-degrading cultures.

Since the identification of glucose oxidase as the primary source of H_2O_2 during lignin degradation, several other H_2O_2 producing enzymes have been identified. The Mn^{++} dependent peroxidase described above has also been shown to produce H_2O_2 by the oxidation of NADH or NADPH(58), although this enzyme is present in the extracellular fluid in very low levels and it appears unlikely that the cell would have an abundant supply of NADPH as a source of H_2O_2. Two others enzymes produced by P. chrysosporium have been shown to produce H_2O_2; these are fatty acyl CoA oxidase(59) and glyoxal oxidase(43), both of which are present at low levels and their actual significance in producing H_2O_2 in lignin degrading cultures is not clear at present.

II. Genetics and Molecular Biology of P. chrysosporium

1. Introduction

The enzyme system involved in degrading lignin to CO_2 is necessarily very complex and is believed to consist of a large number of enzymes. The genes encoding the lignin degrading enzyme complex have not been well characterized to date. Several lignin peroxidase cDNAs have been cloned

and sequenced(60-62) and six different structural genes for lignin peroxidases alone, each encoding a different isozyme, have recently been described by Zhang and Reddy(63). Several other groups have reported the cloning, sequencing, and structural analysis of the lignin peroxidase gene encoding H8 protein(64-70). Two manganese peroxidase cDNAs have recently been cloned and sequenced(71,72). A detailed molecular genetic analysis of the lignin peroxidase and manganese peroxidase genes, including the regulation of expression of these genes and an understanding of the genetic regulation of secondary metabolism in P. chrysosporium and the ability to manipulate it, would not only lead to the development of strains with increased lignin degrading capacity, but would provide information concerning how secondary metabolism is controlled in fungi.

2. Genetic analysis of P. chrysosporium

A. Life Cycle.

P. chrysosporium is a filamentous basidiomycete fungus(6). As in other filamentous fungi, the structure of the mycelium leads to an undefined intracellular environment with respect to the traditional concept of cellular growth. It grows vegetatively as a mycelium, and lacks clamp connections. Cell walls are septate, but not regularly spaced, and the fungus is coenocytic. Vegetative

growth occurs by apical extension at the leading edge of the mycelium. P. chrysosporium can different into several major spore types. Conidia, which are products of mitotis, are produced under most culture conditions starting at approximately the third day of growth and a majority contain one to two nuclei per conidium(15). Basidiospores, which are haploid meiotic products containing two genetically identical(homokaryotic) nuclei, are produced in a specialized fruiting structure termed the basidium. The diploid stage of growth of P. chrysosporium is transient(restricted to karyogamy in the basidium). The production of basidiospores can be induced only in certain environmental conditions, for example by growing the fungus on cellulose, at 30^{o}C and under continuous light(73).

B. Genetics.

Much of our knowledge concerning the genetic system of P. chrysosporium and the methods for its' genetic analysis have been developed by Gold, Broda, and coworkers. Whether P. chrysosporium is homothallic or heterothallic has not been determined unequivocally(74,75). A genetic system has been developed, based on either mycelial fusion(anastomosis), or forced fusion of protoplasts to investigate genetic phenomena such as complementation(15-17,76). Media to induce colonial growth have been developed. These generally contain sodium deoxycholate(0.01%) and sorbose(1-10%), which have been

shown to induce colonial growth in many fungi(77). A replica plating technique has also been developed(78). P. chrysosporium is haploid, and cells are multinucleate, with up to 15 nuclei per hyphal cell(15). The nuclear content of conidia varies; approximately 60 % of conidia are mononucleate with the rest being bi-or multinucleate(15). The multinucleate conidia are often heterokaryotic, containing nuclei of differing genotypes, and result in the development of stable heterokaryotic progeny from a single conidium. Some of the strains such as ME446(ATCC 34571), which has been extensively studied by a number of laboratories have been shown to be naturally heterokaryotic(79). Since the basidiospores have been shown to be binucleate and homokaryotic(74), isolation of basidiospores from heterokaryotic strains can be used to produce homokaryotic isolates of a desired genotype. Isolation of auxotrophs has been done by standard mutagenic techniques(15-17). Complementation studies between auxotrophs have resulted in classification of a collection of leucine auxotrophs into three complementation groups(18). Complementation between auxotrophs can be maintained simply by having populations of two or more mutant nuclei within a mycelium, due to the cross feeding of nuclear products within a common cytoplasm. The parental genotypes can be re-isolated from such a heterokaryon by inducing basidiospore formation. No recombination can be achieved between two haploid nuclei of different genotypes unless meiosis is induced and

basidiospores are produced. No linkage analysis between auxotrophs has been performed and the number of chromosomes is still undetermined. To bypass the traditional accumulation of linkage data based on the isolation of mutants, Broda and co-workers have begun work on the determination of a linkage map of the P. chrysosporium chromosomes using restriction fragment length polymorphisms(RFLPs; 79,80). These studies showed that many of the LIP genes are closely linked in P. chrysosporium ME446(81); evidence to date suggests the presence of at least seven linkage groups(80).

C. Ligninolytic and cellulolytic mutants

In recent years, a number of investigators have focused their attention on isolating a variety of metabolic mutants which would serve as important tools in understanding the biochemistry and molecular biology of lignin degradation by this fungus. Two classes of pleiotrophic mutants designated phenol oxidase(pox; 10,11) and glucose oxidase(gox; 12,35) have been isolated. The gox mutants lack LIPs, MNPs, glucose oxidase and other secondary metabolic activities. Revertants of these mutants have resulted in the restoration of all the lost activities, suggesting that the gox mutants are lacking a global regulatory element involved in expression of secondary

metabolic activities, including the LDS. Another class of regulatory mutants which specifically lack the ability to produce LIP enzymes but retain MNP enzyme activity has been isolated(13). The decoupling of LIP and MNP activity in these mutants suggests a further level of regulation controlling the exression of these seperate classes of enzymes. Lignin degradation is limited to secondary metabolism and is induced by nitrogen depletion while high levels of nitrogen are strongly inhibitory to the lignin degrading enzyme system. A mutant has recently been isolated which is able to express the lignin degrading complex in high nitrogen cultures and is designated a nitrogen deregulated mutant(der; 14). This mutation appears to result from the constitutive expression of a regulatory protein controlling the LDS enzymes and genetic studies to confirm this are in progress. This der mutant may be the first step toward the development of a strain which can constitutively degrade lignin. A different approach toward strain improvement has also been applied. Growth of P. chrysosporium in a medium containing a lignin model compound covalently bound to glycine found that the cleavage of glycine from this compound was dependent on the appearance of the LDS. This may allow for a selection of a strain able to use this glycine adduct as a sole nitrogen source, in which presumably the LDS would be deregulated(82).

Major potential industrial uses of P. chrysosporium include applications in the biopulping of paper and

degradation of recalcitrant xenobiotics such as dioxins(83). To produce paper, lignin has to be selectively stripped from wood, leaving the cellulose and hemicellulose. Present chemical treatments required to accomplish this produce many toxic aromatic by-products, which P. chrysosporium has been shown to detoxify(84-86). Several cellulase deficient mutants of P. chrysosporium have been described which retain high levels of lignin degrading activity, yet do not degrade or have marginal ability to degrade cellulose(87,88). Such a seperation of cellulase degradation from lignin degradation would be absolutely necessary in an industrially useful strain since the wild type would degrade the cellulose and hemicellulose along with the lignin. While wild type P. chrysosporium strains are among the fastest and most efficient lignin degraders in nature, this degradation is relatively slow. The development of a genetically altered strain combining high levels of LDS with a minimum of industrially counterproductive enzyme activities(cellulases and hemicellulases) may allow the development of a process for biopulping of wood which would, from an environmental standpoint, be a major improvement over present industrial processes and save the industry billions of dollars in energy and chemical costs.

3. Molecular Biology of P. chrysosporium

To complement the above described attempts at studying the genetics of lignin degradation in P. chrysosporium, procedures for the isolation and study of the genes encoding various enzymes involved in lignin degradation are needed. In addition, a means of reintroducing these genes back into P. chrysosporium to analyze the expression and regulation of these genes and of secondary metabolism in general is required.

The genome size of P. chrysosporium is approximately 4 $x10^7$ bp, twice that of S. cerevisiae(89). Recently protocols have been developed for the isolation of DNA and RNA from P. chrysosporium(90-92). The initial isolation of cDNAs of lignin peroxidase genes used oligonucleotide probes. These were synthesized based on the amino acid sequence of tryptic digests of the major lignin peroxidase, H8. The isolation of cDNAs encoding two structurally different lignin peroxidases has been accomplished from a library of clones enriched for a population of mRNAs expressed specifically during secondary metabolic growth in P. chrysosporium(60,91). Two classes of cDNAs, CLG4 and CLG5, were isolated which code for two distinct lignin peroxidase genes, H2 and H10, respectively, based on comparison of the N terminal amino acid sequence of these proteins to the respective cDNA sequence(53). These cDNAs have 71.5% DNA homology, and 68.5% protein homology to each other(61). Another cDNA(ML-1) with significant homology to

the CLG5 cDNA, and which appears to encode lignin peroxidase H8, has also been isolated(62).

Recently, Zhang, et.al. have cloned six genomic clones corresponding to lignin peroxidases from P. chrysosporium BKM-F(63, Zhang, Y.Z., C.A. Reddy, A. Rasooly, submitted). This was done by screening a genomic library of strain BKM-F in YRp12 with both CLG5 and CLG4 cDNA clones. The genes isolated were found to form two gene familes based on DNA hybridization. Five of the genes are highly homologous to one another and the CLG5 cDNA, while the other showed hybridization only to the CLG4 cDNA clone. All genes have structural differences, based on major differences in their restriction maps. Two of these genes, GLG1(corresponding to CLG4 and encoding H2) and GLG2(corresponding to CLG5 and encoding H10), have been sequenced. GLG2 was shown to have eight introns, ranging in size from 50-62 bp(63, Zhang, Y.Z., C.A. Reddy, A. Rasooly, submitted), quite similar in size and structure to introns in other filamentous fungal genes(93). The GLG1 gene has nine introns ranging is size from 50-63 bp(Naidu, P., Y.Z. Zhang, and C.A. Reddy, manuscript in prep.). Several laboratories have cloned and sequenced the lignin peroxidase gene encoding the H8 protein or its close relatives(64-70; Table 1). A summary of these results indicates that these genes are regulated at the RNA level(59,63) with 1.5 kb mRNA transcripts homologous to the above genes being present only in nitrogen limited cultures in secondary metabolism but not in nitrogen limited cultures prior to the onset of secondary metabolism or in

Table 1.

Nucleotide and amino acid homology among *P. chrysosporium* lignin peroxidase genes

		AMINO ACIDS[b]										
		LIP2	LIP5	LIP6	0282	ML4	ML5	GLG3	GLG6	LIG1	PLG1	Reference
NUCLEOTIDES[a]	LIP2		72	66	70	71	71	72	72	70	72	Padmavathy and Reddy, unpublished
	LIP5	73		82	95	99	91	98	88	90	99	62, 66, 68
	LIP6	72	80		81	81	79	81	82	79	82	Zhang et al., submitted
	0282[c]	73	87	77		95	89	95	86	89	95	69
	ML4[c]	73	98	80	87		91	99	88	90	98	64
	ML5	73	85	79	83	85		91	86	93	91	64
	GLG3[c]	73	99	80	87	99	86		88	90	99	Padmavathy and Reddy, unpublished
	GLG[6]	72	82	79	80	81	80	82		88	88	Padmavathy and Reddy, unpublished
	LIG[d]	71	82	78	81	83	89	83	81		90	65
	PLG1[d]	73	99	80	87	98	86	99	82	83		67

[a]Percentage of matched nucleotides within exons as determined by GENEPRO program, a modification of the method of Needleman and Wunsch (J. Mol. Biol. 148:443-453, 1970).

[b]Percentage of matched amino acids within two aligned amino acid sequences of LIPs. LIP2, LIP5 and LIP6 genes respectively encode lignin peroxidases H2, H8 and H10. The specific LIP protein encoded by ML5 has not been identified.

c and d are variants of the LIP5 gene from *P. chrysosporium* BKMF and *P. chrysosporium* ME446 strains, respectively.

nitrogen sufficient cultures. S1 analysis has been used to map the 5′ ends of these transcripts and indicated that single(64) or multiple(68) transcription initiation sites can be present. These genes contained typical eukaryotic sequence motifs thought to be essential for transcription(TATA boxes 78-89 bp upstream of the first AUG codon; and CAAT boxes 105-168 bp upstream of the first AUG codon), and, where data are available, a potential polyadenylation signal in the 3′ untranslated portion of these genes. Lignin peroxidase genes appear to constitute a multigene family. In P. chrysosporium BKM-F, 5 of the 6 LIP genes which have been cloned are highly homologous to each other based on Southern analysis and sequence data, and in general have 80-90 % homology at the sequence level(Naidu and Reddy, unpublished data), while the sixth gene has approximately 70 % sequence homology to the above family. Similar levels of homology are seen at the protein level. Features similar to other cloned peroxidase genes are seen in these LIP genes, in particular a potential active site containing two histidine residues. The multiplicity of LIP structural genes should, for the most part, account for the abundance of LIP isozymes present in P. chrysosporium; attempts to make a one to one correlation of gene to protein are being made, although it is likely that not all of the genes encoding LIP isozymes have yet been cloned.

Recent evidence has indicated that other white rot

basidiomycetes also have genes that have significant homology to the LIP genes of P. chrysosporium. A lignin peroxidase cDNA from Phlebia radiata with 60% homology to the P. chrysosporium lignin peroxidases has been described(94). Based on cross hybridization with the genes from P. chrysosporium, several genes from Trametes versicolor have recently been cloned. This was done using the CLG5 cDNA as an heterologous probe to screen a lambda EMBL3 genomic library and indicated that this fungus appeared to contain a LIP gene family. More interesting, where some genetic linkage of LIP genes in P. chrysosporium ME446 based on RFLP data has been demonstrated(81), six of the T. versicolor genes appear to be very tightly linked in three subgroups. Three different subclones of the EMBL3 library each have two genes with LIP homology seperated by 1 to 2 kb. Sequence data from one of these genes shows that it is most related to the CLG4 cDNA of P. chrysosporium and appears in all aspects to be similar to the LIP genes discussed above(Black and Reddy, manuscript in preparation).

Two cDNAs encoding seperate manganese peroxidase genes have recently been sequenced(71,72); Southern blotting data indicate that these enzymes also form a family of related genes(72). A potential peroxidase active site containing two histidine residues has been found in each, and the cDNAs appear to encode proteins with an M_r of 37 kD. Similar to the LIP isozymes, these genes appear only during secondary metabolism and appear to be transcriptionally

regulated(72). The reason for the multiplicity of lignin peroxidases and manganese peroxidases is not clear at this time, but several laboratories are studying this issue both at the biochemical and genetic level.

III. Fungal Transformation Systems

1. Transformation of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, a budding yeast with well defined haploid and diploid stages, has long been a focus of genetic analysis due to its well studied genetic system. This has allowed detailed genetic analyses due to the fact that it can be technically manipulated with relative ease. *S. cerevisiae* is the model for the development of transformation systems for lower eukaryotes and this subject has been reviewed in depth(95,96). An extensive variety of transformation vectors have been developed for *S. cerevisiae*. Transformation protocols and strategies for the development of vectors for this organism have been the basis for later protocols developed for other yeasts and filamentous fungi. For these reasons, a general outline of yeast transformation systems will be given as an introduction to a discussion of the transformation systems of filamentous fungi.

Cell walls of yeasts are almost exclusively composed of mannose(mannoproteins) and glucose polymers (glucans;97). The most commonly used transformation procedure

involves the degradation of the yeast cell wall using cellulolytic and other cell wall digesting enzymes. This creates spheroplasts which are then incubated with polyethylene glycol in the prescence of $CaCl_2$ to make the cell membrane permeable to the passage of free DNA into the cell. The various enzymes used to digest the cell wall in transformation protocols include cellulase, chitinase, β-glucoronidase, glucanase, and pectinase. These enzymes are used either individually(in relatively pure preparations such as glusulase[β-glucoronidase]) or in combinations in various less well defined commercial preparations such as novozym 234(Novo Laboratories, Inc. Danbury, CT) or "Lysing Enzymes"(Sigma Chemical Co, St. Louis MO.).

The practice of rendering bacterial cells competent for DNA transformation by treatment with various cations(98) led to attempts to reproduce this phenomenon with whole cells of yeast. It was found that Li^+, and to a lesser extent other monovalent alkali cations, could induce compentence of yeast cells(99) and transformation protocols similar in concept to the standard E. coli transformation were developed. These protocols bypassed the need for regeneration of spheroplasts which is often low in spheroplast transformations. However, the transformation frequency obtained in lithium acetate transformations is usually lower than that obtained for the corresponding spheroplast transformation(99).

Several other transformation procedures have been

developed for S. cerevisiae. They include fusion of spheroplasts from different genotypes(100). This procedure would be most useful for gene exchange between yeasts which cannot mate. Fusion of yeast spheroplasts with E. coli minicell spheroplasts(101) has also been induced in the presence of polyethylene glycol. It has been reported that the transformation frequency of an autonomous vector(pGY14, a 2μm plasmid based vector) using this method was found to be several fold higher than the standard spheroplast transformation system. More recently methods to direct transformation to mitochrondria or nuclei by shooting microprojectiles have been developed(102,103). In this procedure, small tungsten particles coated with the plasmid of interest are used to bombard whole yeast cells. A plasmid containing the mitochrondrial gene for cytochrome oxidase III(pQAoxi3) was used to transform a respiration deficient strain of S. cerevisiae(oxi3$^-$) to a respiration competent state and this was shown to be due to integration of the transforming sequences into the mitochrondrial genome(102).

A. Integration Vectors

A number of initial transformation protocols employed vectors which transformed by integrating into the yeast chromosome. These are commonly designated YIp vectors. Hinnen et al.(104) used a plasmid designated pYeleu10. This vector contained bacterial sequences from ColE1, and

the yeast LEU2 gene(encoding β-isopropylmalate dehydrogenase), isolated by complementation of a leuB strain of E. coli. This vector was used to transform a leu2 auxotroph of S. cerevisiae to prototrophy. Southern blotting analyses identified three types of integration events(Fig. 2; after Hinnen et al.; 104). The largest class(Type I; **linked insertion**) of transformants had a single crossover event in a region of homology between the vector and host chromosome resulting in two adjacent copies of this gene (one functional and one nonfunctional) and the integration of the intact ColE1. The second class(Type II; **unlinked insertion**) of transformants were due to recombination of the vector into non-homologous regions of the genome. The third class(Type III; **gene replacement**) were transformants in which the wild type transforming yeast sequence precisely replaced the mutant sequence in the chromosome and all bacterial sequences were lost. This was similar to a gene conversion event or a double crossover. One of the most commonly used integration vectors has been YIp5(105). This contains a chromosomal fragment of yeast containing the URA3 gene and the E. coli plasmid pBR322, which contains an ori and the tetracycline and ampicillin resistance markers. Its usefulness in the construction of more sophisticated yeast vectors will be discussed below.

Homology to yeast DNA was found to be important in the integrative transformation event in S. cerevisiae as the type II transformants which integrated by an apparent non-

Figure 2. Types of integrative transformation events observed in fungi. The thin linear lines represent chromosomal DNA. The thick line labeled m^- or m^+ indicates an arbitrary non-functional and functional fungal selection marker, respectively which are highly homologous at the DNA sequence level. The thin line in the circular vector indicates DNA with no homology to the fungal chromosome represented. A) Type I transformation is strictly by non-homologous recombination into the chromosome resulting in two generally unlinked copies of the selection marker. B) Type II transformation is by a single crossover between regions of homology, resulting in two tightly linked copies of the selection marker. C) Type III transformation is by a double crossover, resulting in complete excision of the non-functional chromosomal copy of "m", leaving one copy of the selection marker in the chromosome. The most common variant of type A and B which are observed in fungi occurs when tandem copies of the vector integrate resulting in multiple linked copies of the selection marker.

homologous event were found to be exceedingly rare. Creating a linear vector by a single restriction cut within the yeast sequence was found to increase the transformation frequency of these vectors, up to a thousand fold compared to the circular integration vector(106). Linearization also directed the integration of the vector to the site at which it was linearized. Both events indicated that the recognition of a homologous stretch of DNA was very important in the integration process in yeast.

Integration events can also be defined by traditional genetic analysis. A haploid transformant(prototroph with *LEU2* complementing gene) can be crossed to a Leu^- haploid of the opposite mating type and the resulting diploid then sporulated to form a four spored ascus. The four meiotic progeny of the cross then segregate in a standard Mendelian segregation pattern of 2 $LEU2^+$:2 $leu2^-$. The stability of the phenotype was also seen through mitosis. When the $LEU2^+$ transformant was grown under nonselective conditions for several generations(usually at least ten) and then screened for the precence of the vector, virtually 100% of the progeny exhibited the complemented phenotype(107).

Vectors integrated into the chromosome of *S. cerevisiae* were generally not recoverable by using total genomic DNA from individual transformants to transform *E. coli*. Recovery was, however, achieved by digesting transformant DNA with a suitable restriction enzyme which leaves the antibiotic resistance marker and the plasmid *ori*

functional, ligating the resulting linear chromosomal fragments, and using the ligation mixture to transform E. coli. Using the appropriate restriction enzyme was also shown to result in the recovery of the original unaltered vector when two or more were tandemly integrated.

B. Autonomous Vectors

Two strategies have been used to develop vectors which can be maintained within the yeast nucleus in an intact, extrachromosomal form. Such vectors serve as shuttles for moving genes into and out of the yeast.
The first approach is based on using portions of the endogenous 2μ plasmid of S. cerevisiae(108) to drive autonomous replication of the vector. The 2μ plasmid is maintained in S. cerevisiae isolates at 30-50 copies per cell, yet has no apparent selective advantage to the organism(109). The advantages of an extrachromosomal YEp(yeast episomal plasmid) vectors are several. These vectors can be easily recovered in E. coli. Libraries constructed in these vectors can be routinely used to isolate genes complementing S. cerevisiae mutations. Genes present on such a vector can also be maintained at a much higher copy number than if in the chromosome. Beggs(110) developed the first 2μ based yeast-E. coli shuttle vectors, pJDB248 and pJDB219. These vectors contained, besides 2μ sequences, pMB9, a derivative of ColE1 of E. coli for replication and selection in bacteria, and the LEU2 gene of

S. cerevisiae.

The second major class of yeast autonomous vectors are called yeast replicating vectors(YRp). These vectors are based on putative chromosomal replication origins termed "ars"; for autonomous replication sequence(105,107). Davis and coworkers(105,111) inserted a fragment of yeast chromosomal DNA containing the TRP1 gene(encoding N-(5'-phosphoribosyl anthranilate isomerase) complementing a trp1 auxotroph into pBR332. It was found that the resulting vector, YRp7, was maintained as an autonomous vector and could transform at a significantly higher frequency(500-5000 Trp^+ yeast transformants per μg DNA for YRp7) than an integrating vector and concluded that the chromosomal fragment complementing the trp1 mutation also contained an ars element, ars1, allowing for this high frequency of transformation. These YRp vectors have similar advantages to those described for YEp vectors. Further studies found that similar sequences allowing YIp5(described above) to be maintained as an autonomous plasmid could be isolated from a variety of eukaryotes(107). All ars' identified in yeast which have been sequenced contain an 11 bp core consensus sequence(5'-[A/T]TTTAT[A/G]TTT[A/T]-3') and these elements are postulated to be chromosomal origins of replication(112). Further studies indicated that ars' generally function as replication origins only in the yeast from which they have been isolated and that ars' are not generalized broad host range replicons(113).

These replicative vectors have several features which

distinguish them from integrative vectors. Southern hybridization analysis of total genomic DNA from transformants containing an autonomously replicating plasmid show that these plasmids are maintained as low molecular weight elements, distinct from the chromosomal DNA(110). These vectors are extremely unstable through both mitosis and meiosis. Meiotic analysis showed a high percentage of abnormal segregation patterns($4^+:0^-$,$3^+:1^-$, $1^+:3^-$, $0^+:4^-$) indicating a marker which is behaving in a non Mendelian manner that is characteristic of extrachromosomal elements. Mitotic analysis showed that such plasmids are lost very quickly during nonselective growth. Plating of a transformant which has undergone 10 generations of nonselective growth on selective media shows very low frequency of prototrophs containing the transforming vector(1-5%; 114). However, variations in relative stabilities of different autonomous plasmids has been reported and this is discussed below.

Vectors based on the 2μ plasmid in general are significantly more stable than those based on <u>ars</u> elements due to plasmid maintenance and partitioning functions(115) resident on the 2μ sequences. An <u>ars</u> vector, YpR141, was examined along with a variety of 2μ vectors and found that the 2μ vectors had a higher stability. A further study comparing an <u>ars</u> vector(pSZ93) to a 2μ vector(YEp13) showed that the <u>ars</u> vector had a much higher segregation bias toward the mother cell than the 2μ vector. This resulted in pSZ93 being maintained in a much smaller fraction of the

cells of a population. Furthermore, this difference in segregation bias between the two types of vectors was found to be eliminated in a strain lacking an endogenous 2μ plasmid. This indicated that 2μ specific genes influence the mitotic segregation of 2μ vectors(114).

There are several other means by which the stability of replicative vectors can be increased. pJDB219 was used to transform S. cerevisiae to leucine prototrophy and an analysis of the types of plasmids present in individual transformants was made(116,117). Some transformants were found to have lost both pJDB219 and the endogenous 2μ plasmid; in this case a new plasmid, pYX, was found which contained only 2μ plasmid sequences and the LEU2 gene with no bacterial DNA sequences. The vector lacking bacterial sequences was found to be maintained non-selectively much better than the parental pJDB219. 2μ vectors, have also been shown on rare occasions were shown to integrate into a resident 2μ plasmid, thus offering a means of replicating along with the stable endogenous plasmid. Similar types of ars based plasmids which contain only yeast sequences have been tested for stability. Vector TRP R1(that contains only the yeast chromosomal fragment complementing trp1$^-$ auxotrophs and a closely linked ars element; 118) is a model for these. This vector is small($\sim$1 kb) and quite stable mitotically, but the lack of bacterial sequences restricts their usefulness as shuttle vectors. However, these vectors were found to be useful for the analysis of the maintenance of plasmids in yeast. Studies showed that

TRP R1 was be maintained stably in yeast during nonselective growth for up to 100 generations at an extremely high copy number(100 per cell).

C. Centromere Vectors

The in depth development of the molecular genetic system of S. cerevisiae has also allowed the construction of other more specialized classes of vectors which are rather unique to the yeast system to date. The first class, centromere vectors, contain a chromosomal centromere on a YRp plasmid and combine the advantages of the integrative and autonomous vectors into one stable, autonomous vector(119). The initial identification of a centromere was that of CENIII from chromosome III(120). CENIII was identified by analyzing a series of plasmids containing overlapping chromosomal DNA fragments from the LEU2-CENIII-CDC10 region of chromosome III inserted in the shuttle vector pLC544. One of these plasmids, pYe(CDC10)1 was found to behave mitotically and meiotically similar to a YIp vector, although it was maintained autonomously as a mini-chromosome. The plasmid pYe(CDC10)1 segregated through meiosis in a $2^{+}:2^{-}$ fashion for all markers tested and showed a mitotic stability of 95-97% compared with less than 1% for pLC544. The presence of a centromere also reduces the copy number of a plasmid to approximately one per cell.

Further development of these vectors, primarily by

linearization and the addition of telomeres has created a class of vectors termed yeast artificial chromosomes(121) which can be shown to behave similarly to actual yeast chromosomes. These are particularly useful in constructing gene libraries of higher eukaryotes(principally mammals) where inserts of 100 kb to 1 Mb are possible, thus reducing screening problems inherent in large bacterial libraries and offering a more accurate analysis of higher mammalian systems(122).

2. Transformation of Filamentous Fungi

In the last ten years, extensive progress has ben made on the DNA transformation systems for many filamentous fungi. These transformation systems, including the methodology, vectors, and selection markers used were first modeled after those developed for S. cerevisiae. There are considerable differences in the nature of maintenance of the transforming vectors in filamentous fungi in comparison to that in single cell yeasts. Studies on transformation of the traditional fungal model systems, such as Neurospora and Aspergillus, as well as more recent results obtained with other genera of fungi of industrial or agricultural importance are reviewed here. Several recent reviews have also extensively covered the progress in this rapidly expanding field(123-127).

Transformation in filamentous fungi was first

demonstrated using modifications of yeast spheroplast transformation systems. Cell walls of filamentous fungi have a diverse range of components including cellulose, chitin, and other various polysaccharides usually complexed with proteins(128), and mannose and glucose polymers. The composition of the cell wall can vary depending on the morphogenic state of the fungus. Thus, with spheroplast based transformation systems, the choice of spheroplasting enzyme is usually dependent on the organism being studied and its morphogenic state. The generation of spheroplasts has been achieved using various β-glucoronidase preparations(glusulase, novozym 234, β-glucoronidase alone). The most common osmotic stabilizers used for suspension of the spheroplasts during and after cell wall degradation are sorbitol and $MgSO_4$. The types of cell wall degrading enzyme and the osmotic stabilizer used were shown to have a marked influence on the regeneration frequency of spheroplasts, which is often quite low and can be the limiting factor in fungal spheroplast transformations(129). Additional modifications, including coating the DNA to be transformed with heparin to limit exposure to nucleases, and the addition of dimethyl sulfoxide to help permeablize the shperoplast membrane, have been used in some protocols(130). Sorbose is often employed in the selective media to restrict mycelial growth and induce colony formation(131). The most complete analyses of the effectiveness of various spheroplasting techniques and osmotic stabilizers has been done using N. crassa and

Podospora anserina as models and should be referred to (129,130). Mycelial transformation procedures, rather than conidial transformation systems, have also been developed for several fungi(130,132,133) and these appear to be effective alternatives.

In addition to spheroplast transformation systems, both lithium acetate(134), liposome mediated(135) and biolistic(103) transformation systems have been developed for N. crassa. In the lithium acetate method, conidia were treated with lithium acetate followed by polyethylene glycol incubation and a heat shock. When the vector pSD10, containing bacterial sequences, the N. crassa ga-2^+ gene, and sequences from a mitochondrial plasmid of N. crassa was used for transformation by the lithium acetate method, the transformation frequency was found to be comparable to the spheroplast transformation system(9 transformants per μg vs. 1-5 per μg for early spheroplast systems; 130). In the liposome protocol, N. crassa total chromosomal DNA without a vector was encapsulated in phosphatidylserine and was then used in the standard spheroplast transformation protocol. Transformation frequencies for DNA coated with liposomes were generally found to be 20-40 fold higher than that for naked DNA molecules.

3. Neurospora crassa

The genetics and cytology of the filamentous ascomycete Neurospora crassa has been extensively studied

since the early part of the century and many advances in the understanding of basic genetic concepts have been accomplished with this fungus(136). Attempts at DNA mediated transformation of N. crassa auxotrophs began in 1961 when the complementation of a pyridoxin mutant was attempted by incubating conidia from this strain with total genomic DNA from the wild type(137,138). Improvements were made in this technique over the next 15 years(135,139-41) but no great advances were made until recombinant DNA technology became available. Complementation of auxotrophs has been the predominant selection marker in Neurospora transformations(Table 2), although the dominant selection markers benomyl(142) and G418(143) have also been successfully used. Benomyl(methyl-1-[butylcarbamoyl]-2-benzimidazole carbamate) specifically binds to β-tubulin, and causes a lethal depolymerization of microtubules. A tubulin gene containing a point mutation in the tubulin protein that is resistant to benomyl-mediated depolymerization and can be used as a selection marker. A series of N. crassa vectors based on benomyl resistance have now been constructed(144) and these have found use in a wide variety of other filamentous fungi(Table 5). G418 is an aminoglycoside antibiotic to which many eukaryotic cells are sensitive. Kanamycin resistance determinants of E. coli encode an aminoglycoside phospotransferase which inactivates both kanamycin as well as G418 and has also been used as selection marker in other filamentous fungi(Table 5). However, sensitivity of N. crassa to other

TABLE 2

Selection markers and transformation vectors used for Neurospora crassa.

Selection Marker	Vector	Reference
qa-2$^+$ (catabolic dehydrogenase)	pVK88	145
"	pALS-1	170
"	pALS-1, pALS-2	171
"	pVK57	130
"	pSD3, pJP102	154
"	pSD3, pVK57, pVK88	152
"	pMK2	173
"	pJP12	149
"	pDV1001	150
am (glutamate dehydrogenase)	C10	150
"	C10	146
"	pBC10, pEB10	143
ben^r (benomyl resistance)	T11b, pBT3	142
"	pSV50	161
"	pJD21,71,81,82, pRM111	144
inl (myo-inositol-1-synthase)	BHB3030	173
trp-1 (AS, IGPS, PRAI[a])	pNC2	152
G418 (geneticin)	pBC10, pEB10	143
Bialaphos	pJA4	336

[a]anthranilate synthase (AS),indoleglycerolphosphate synthase (IGPS), phosphoribosylanthranilate isomerase (PRAI).

common antibiotics such as hygromycin B, which has also been used widely in fungal transformation protocols(Table 5), has not been reported to the best of our knowledge.

A. Integrative Transformation

The first recombinant vector used in N. crassa transformation, pVK88, contained the homologous qa-2$^+$ gene(catabolic dehydroquinase) inserted into pBR322(145) to enable complementation of an N. crassa qa-2$^-$ auxotroph. Similar to the yeast integration vectors, these vectors transformed N. crassa by integration into the chromosome. Genetic analyses of individual transformants showed that the complementing genetic marker generally followed a Mendelian segregation pattern and that the transforming phenotype is stable under nonselective mitotic growth, suggesting integration of the vector in a stable manner into the chromosome. In addition to these stable transformants, a number of abortive transformants, which can be seen on the initial selection plates but cannot be maintained by further transfers onto selective media, were also observed in these experiments. It is thought that in these abortive transformants the phenotype is expressed only transiently and that the marker is not able to stably integrate into the chromosome. The frequency of transformation in N. crassa, using primarily the qa-2$^+$ and am$^+$(glutamate dehydrogenase) genes as markers varied from one to several

thousand transformants per μg DNA based on the selection marker and the reporting laboratory(143,145,146). Further improvements in methodology have raised the transformation frequency in N. crassa up to 50,000 transformants per μg DNA(147). These high frequencies have been found to be dependent on the batch of the cell wall digesting enzyme used and the investigator indicating a considerable variability in both the preparations of enzymes and the protocols used.

The three types of integrative transformation events previously observed in S. cerevisiae were also described with N. crassa. Transformants showing unlinked insertions(type II; Fig. 2) were the most common irrespective of the selective markers used(145,146,148-51). The complementing gene, with or without accompanying bacterial sequences, integrated at a site in the chromosome unlinked to the defective chromosomal allele of the selection marker. Gene replacements(type III) and linked insertions(type I) were also be observed, but much less frequently. Attempts to direct integration at the homologous locus by restriction digestion within the complementing fungal DNA portion of the transforming vector has given mixed results. Marzluf and co-workers(149,151,152) have most completely analyzed this question in N. crassa with several different selection markers and vectors. With the *ga-2*$^{+}$ gene as the marker inserted seperately into three different pBR322 based vectors, pSD3, pVK57, and pVK88, that differed only in the

size of the N. crassa DNA insert, the type II unlinked transformants were predominant. When pSD3 was linearized at any one of several restriction sites within the fungal marker gene of the vector, no increase in the frequency of replacement(type III) or linked insertion(type I) was seen as compared to the circular form of pSD3(151). In a seperate study, vector pJP12, in which the am$^+$ gene of the vector was disrupted with the ga-2$^+$ gene, the feasibility of gene disruption in N. crassa was tested(149). Ten of 117 transformants studied showed a precise gene disruption, indicating that this process occurred, although at a low level. In contrast, when the vector pNC2, containing the trp-1$^+$ gene (a trifunctional polypeptide encoding three enzyme activities in the biosynthesis of tryptophan) was used for transformation, the type of integration event was found to be strictly dependent on the strain of N. crassa used(152). N. crassa trp-1, inl gave a high frequency of type I events, whether the vector was circular or linearized within the fungal marker gene of the vector, while linearization in the pBR322 part of the vector reduced the linked events. In contrast, N. crassa trp-1, ga-2, aro-9, inl gave predominantly unlinked transformants irrespective of whether the vector was circular or linear. A different report found that varying the size of the homologous region in the vector(the am gene in N. crassa inserts ranging from 2.7 kb to 9.1 kb) influenced the frequency of homologous integration. This frequency increased with the size of the insert(153).

In summary, it appears that the type of integration events can be influenced by a number of factors, structure of the transforming plasmid, selection marker and strain used for transformation and the size of the insert. While in both yeast and N. crassa all three types of integrative transformation events have been observed, there are extreme differences in the types of events which predominate. In S. cerevisiae, linked insertions and directed gene disruptions are the rule, due to the high level of homologous recombination between incoming vectors and chromosomal sequences(106). In contrast, no broad generalizations can be made concerning the type of integration events that are permissible in N. crassa with respect to sequence requirements. The mechanism of integration appears to be different considering the high frequency of unlinked transformants seen in N. crassa transformation experiments and the types of events which do occur need to be determined empirically.

Attempts to recover vectors integrated into the chromosome of N. crassa transformants, by E. coli transformation, have generally been unsuccessful(150,154) barring a few exceptions(see below). In one case in which the recovery of a vector by E. coli transformation was reported was later found to be due to contamination of the transformant DNA with an E. coli stock of the transforming vector(155,156). To achieve complementation of an appropriate N. crassa auxotroph transformed with a genomic library a more cumbersome procedure termed sib selection

was developed(147). In this procedure N. crassa genomic library in a plasmid vector is divided into smaller pools and each pool of plasmids is then used to transform the appropriate auxotroph to the desired prototrophy. Whichever pool gives transformants is divided into smaller pools and the procedure is repeated until the pools contain individual plasmids; at this point the plasmid containing the gene complementing the mutation can be identified. This procedure avoids the requirement to recover the actual clone from the individual transformant, which can often be accomplished only by constructing a lambda library of a positive transformant and screening for foreign vector DNA inserts, which can be more time consuming than sib selection. Both structural and regulatory genes have been isolated using this sib selection procedure(157-159). To make the process of sib selection easier, a cosmid has been constructed to reduce the rounds of selection and transformation required to identify a specific gene. The cosmid pJB8, containing pBR322 and a cos site(160), was modified to include the benomyl resistant β tubulin gene of N. crassa(see above) to result in vector pSV50(161; Fig. 3). This cosmid has been used to clone many genes by complementation of structural mutants(161) and has also been used to isolate the A and α mating type idiomorphs from N. crassa(162). It has also been found that cotransformation can be very efficient(163). In this technique, an unselected plasmid along with a seperate selected marker on a different plasmid are both transformed

into the fungus, and in many cases(up to 43% of the transformants) both plasmids can be introduced into the same transformant. This should be useful in conjunction with sib selection when the library of interest does not have an appropriate selectable marker for the Neurospora strain being transformed.

The only generally applicable method used for the recovery of vectors from integrated transformants has been digestion of the total DNA of the fungal transformant DNA by an appropriate restriction enzyme, followed by ligation and transformation of E. coli. As vectors often are inserted randomly into the fungal genome, this always results in the recovery of adjacent fungal sequences also and does not constitute a generalized shuttle vector system(164). Even this direct recovery of transforming vectors by the restriction digestion-religation method was difficult with N. crassa until recently, because transforming DNA was shown to be methylated at cytosine positions to a high degree in N. crassa(165). Consequently, when the transformant fungal DNA was reintroduced into commonly used E. coli cloning strains such as HB101 and LE392, which contain the methylcytosine restriction system, it is immediately degraded. This problem has been overcome using E. coli strains which are lacking methylcytosine restriction systems, defined by the mcrA and mcrB genes encoding endonucleases which cleave only when the C residue in the recognition site is methylated(166).

B. Studies on the development of a shuttle vector sytem.

Shuttle vectors developed for DNA transformation of S. cerevisiae were used as the model for developing shuttle vectors for filamentous fungi. It was assumed that modifications of the procedures used to create efficient yeast shuttle vectors could result in similar success with other fungi. The primary focus at the time was on isolation of replicons suitable for the fungus either by isolating ars elements or by the use of endogenous plasmid sequences as the basis for a replicative vector.

Ars elements, as discussed above, are those genomic sequences that allow autonomous replication of yeast integrating vectors, such as YIp5 in S. cerevisiae. Putative ars elements from N. crassa have been isolated in S. cerevisiae using this approach. However, these heterologous ars elements were found to be ineffective in promoting autonomous replication of vectors when transformed into N. crassa(145). This led to attempts to isolate ars elements by transforming N. crassa with integrating vectors containing libraries of N. crassa genomic DNA in an attempt to duplicate the results obtained with yeast. Paietta and Marzluf(152) developed several N. crassa ars vectors containing N. crassa genomic DNA fragments cloned into pSD3(described above). These ars insertions increased the transformation frequency

approximately 10 fold in comparison to vectors without the putative *ars*. However, these vectors were also found to integrate into the chromosomal DNA of the transformants, suggesting that the putative autonomous replicating vectors might be present only in a very small population of the transformants. Hence, the same investigators then used an alternate approach in which they tried to rescue autonomous vectors by using DNA isolated from pooled colonies of the transformants on a plate to transform *E. coli* followed by isolation of the plasmid from ampicillin resistant *E. coli* transformants. Using this approach, *N. crassa* was transformed with pSD3 and grown in liquid culture for 1,2,3, or 4 days, after which DNA was prepared from the total mycelial mass isolated from each of the four cultures. It was found that the transforming vector could be isolated in a time dependent manner from these cultures, with the isolation being most difficult on the 4th day. This transient isolation of transforming vector was thought by the investigators to represent a stage in the process of transformation when the original vector was still circular, prior to the integration of the vector into the chromosome. Alternatively, it was considered that the transient isolation of the transforming vector was due to the possibility that these vectors recovered in *E. coli* were from abortive fungal transformants in the pooled population in which the plasmid vector was being diluted out. It was not determined in these experiments whether or not the vector recovered had actually replicated in the fungus. In

a further attempt to isolate Neurospora ars elements, N. crassa mycelial spheroplasts were transformed with a genomic library of N. crassa sequences in the transforming vector pFB14, containing the qa-2$^+$ gene in pBR322(130). This resulted in the isolation of genomic fragments by sib selection(see above) which could transform N. crassa at a higher frequency, yet were still found to be integrated into the chromosome(130).

Some wild type strains of N. crassa and Neurospora intermedia have distinct types of relatively small(3-5 kb) endogenous circular mitochondrial plasmids(167) that encode monomer length RNA transcripts(168). These plasmids were frequently lost during cultivation in the laboratory with no apparent effect on growth of the fungus, and thus appear to have no essential function, except that some of these plasmids are implicated in the senescence of strains which contain them(167). These plasmids have been shown to have no homology to mitochondrial DNA. Further analyses of one of these plasmids showed that this plasmid closely resembles Group I mitochondrial introns, which are capable of self splicing and often are able to insert and be deleted from mitochondrial genes at precise locations. The mitochondrial plasmids of N. crassa are structurally similar to these introns and may represent either a progenitor to these introns or spliced introns which have aquired the ability to replicate autonomously(168).

One of the small mitochondrial plasmids, the Labelle plasmid of N. intermedia, has been used as the basis for a

N. crassa shuttle vector designated pALS-1(169). This vector, when transformed into N. crassa, was shown to exist as a circular extrachromosomal element and was visible in Southern blots of total undigested DNA from transformants as an extrachromosomally maintained element. The vector was recovered intact from the DNA of some of the transformants, although a deleted version, pALS-2, which lost all fungal plasmid sequences, was also occasionally recovered from some of the transformants. Subsequent analysis of pALS-2 indicated that it contained rearranged pBR325 sequences and only the ga-2$^+$ fungal selection marker(170).

Another N. crassa vector, lambda C-10 was used to transform N. crassa to am$^+$(146). The results showed that the maintenance of the vector sequences in the transformants was meiotically unstable, which was consistent with autonomous maintenance. However, Southern blot analysis indicated that the sequences were maintained in a high molecular weight form, suggesting chromosomal integration. A similar result using a different am vector, pJR2, was found by the same investigators(171). These contradictory results may be explained by the RIP effect(see below).

Transformation of N. crassa with vector pMK2, a 15.2 kb plasmid containing the N. crassa LaBelle mitochondrial plasmid, pBR322, and the ga-2$^+$ gene for selection, resulted in transformants in which plasmids could consistently be recovered by E. coli transformation(172). In an in depth

analysis of one of these transformants, two types of altered plasmids, pMK2-3(8.4 kb) and pMK2-5(10.5 kb) were recovered; neither could retransform *ga-2*$^-$ *N. crassa* to prototrophy. pMK2-3 was shown to have acquired sequences of the *N. crassa* chromosome. Plasmid pMK2-5 was found to be a dimer consisting mainly of pBR322 sequences. No homology to the *ga-2*$^+$ selection marker or to the LaBelle plasmid was found although homology to the mitochondrial genome was demonstrated in pMK2-5. Both these vector were shown to have replicated in *N. crassa*. This was demonstrated by a *dam* methylation assay based on the following rationale: If the vector had replicated in *N. crassa* then it will have lost the characteristic methylation by the endogenous *dam* methylase at the N^6 position of adenine at the sequence GATC found in *dam*$^+$ strains of *E. coli* in which the transforming plasmid was propagated. Therefore, vector DNA that had replicated in *N. crassa* would be susceptible to digestion by *Mbo*1, which is active on DNA only in the absence of adenine methylation. Southern blots of *Mbo*1 digests of pMK2 transformants containing pMK2-3 and pMK2-5 indicated that vector sequences were digested, thus showing that they had been replicated in *N. crassa*.

Southern blot analysis of total transformant DNA from which pMK2-3 and pMK2-5 were recovered indicated only chromosomal integration(172). These results, along with the fact that pMK2-3 contained chromosomal *N. crassa* sequences not originally present on pMK2, indicated that the recovery of these plasmids was most likely due to

alternate excision products from the chromosome, and not to the presence of automonous plasmids. Genetic analysis of pMK2-3 and pMK2-5 transformants also indicated meiotic instability, which can be attributed to RIP activity and not to true autonomous maintenance of vectors.

A similar type of recovery of altered plasmids was observed in a seperate study(173) in which a cosmid library of N. crassa was used to complement a N. crassa inl$^-$ mutant. Two transformants which complemented this deficiency were obtained and plasmids of various sizes were recovered from both these transformants. Interestingly, none of the recovered plasmids contained the desired inl$^+$ gene. Genetic analyses indicated that these transformants showed some meiotic instability, complete mitotic stability, and were linked to the al-3 locus, which is closely linked to the wild type inl allele. The latter two pieces of data indicate integrative transformation, while the meiotic data could be explained by the RIP effect. Southern analyses indicated that the transforming cosmid was maintained as an extrachromosomal element, although these data were not shown. The structure of the recovered plasmids was not analyzed and it was not ruled out that these were due to excision from the chromosome.

Detailed analysis of other fungal transformation systems(see sections below) with either integrative vectors or putative autonomous vectors have often shown either mitotic or meiotic instability of the vectors which, based on the yeast model, would suggest autonomous maintenance of

vectors. Two explanations for this instability have been made. First, head to tail integration of two or more copies of the transformation vector into the chromosome can generally be accompanied by genetic instability and occasional recovery of the intact vector from transformants. Southern analysis in most of these cases showed only chromosomal integration. Thus, these transformation events are best explained as recombination between adjacent integrated vectors resulting in the excision of intact vectors or of altered vectors containing junction fragments of adjacent chromosomal DNA. Secondly, it has been found that there is an endogenous de novo methylation activity in N. crassa termed RIP(rearrangement induced premeiotically, or repeat induced point mutation; 174-177). In N. crassa, there are few duplicated sequences aside from DNA encoding ribosomal RNA. The transformation of N. crassa generally results in the introduction of a second allele of a single copy gene in a haploid chromosome(174). It was found that duplicated sequences in one haploid were not stable through meiosis. This phenomenon was termed RIP and this activity is specifically active prior to karyogamy(175) in the perithecium(meiotic structure). The RIP activity identifies duplicated sequences, which are then subject to heavy cytosine methylation. Subsequent deamination of methylcytosines results in a high level of G-C to A-T mutations in the targeted duplicated sequences(176). This heavy mutation frequency often results in non-functional copies of genes

which have been duplicated or triplicated, and this non-functionality of these genes mimics instability of the marker through meiosis(177). The exact natural function of the RIP phenomenon in the cell is unknown. However, RIP can be used in to create mutations by transformation in N. crassa. In this approach, the gene to be inactivated would be transformed into N. crassa which results in a transformant with duplicate copies of the gene in question. When meitoic spores are isolated from such transformants, some will have both the transformed gene and endogenous gene irreversibly inactivated due to RIP activity, creating a specific mutation(174). It also appears that methylation of duplicated transformed sequences occurs in other fungi, resulting in similar meiotic instability of some integrated sequences(see below). Thus true evidence for autonomous replication of vectors, or rearranged derivatives thereof appears scarce to date, although some cases of true autonomous replication in fungi have been reported very recently(see below). More detailed specifics of such cases are discussed in the following sections.

4. Aspergillus nidulans

Many species of Aspergillus, such as A. niger, A. awamori, and A. oryzae, are of importance due to their ability to produce large quantities of industrially important extracellular enzymes such as amylases and various secondary metabolites such as aflatoxins(178). A.

nidulans is the most widely studied from both the genetic and molecular standpoint and has offered considerable advances in our understanding of the regulation of gene expression(179) and of developmental processes in filamentous fungi(180). A variety of vectors with multiple applications have now been developed for Aspergillus species, as these organisms are well suited, as an alternative to S. cerevisiae, as hosts for the high level of expression of foreign eukaryotic proteins(181-185). Also, transformation systems developed for Aspergillus species, in combination with those described above for N. crassa, give a comprehensive picture of the types of molecular genetic manipulations and analyses that can be expected to be made in other filamentous fungi which are important in agricultural and industrial applications.

A. Integrative Transformation.

As with N. crassa, the initial transformation protocols developed for A. nidulans were either conidial or mycelial spheroplast transformations using vectors that contained A. nidulans genes which complemented appropriate auxotrophic mutations(see Table 3 for a survey of selection markers used). Ballance et al. used the vector pFB6, which contained the pyr4 gene of N. crassa to complement a pyrG mutant of A. nidulans(186). Additional N. crassa regulatory(187) and structural(188) genes have

TABLE 3

Selection markers and transformation vectors used for Aspergillus nidulans[a].

Selection Marker	Vector	Reference
pyr4 (orotidine-5'-phosphate decarboxylase	pFB6	186
" "	pFB6, pDJB1, pDJB2	132
" "	pDJB1, pRKB1, pRKB2	217
" "	pAIpGM1,4	196
" "	pDJB1, pDJB12.1	194
amdS (acetamidase)	p3SR2	188
"	p3SR2, p3SR2rr, p3SR2mo	187
argB (ornithine transcarbamylase)	pSal43	337
" "	pMA2, pMA2-C	218
" "	pMT201	214
" "	pA1, pAA1	215
" "	pM159	183
trpC (tryptophan biosynthesis complex)	pHY201	190
" "	pKBY2	235
oli^r (ATP synthase, subunit 9)	pMW2, pMW11	228
" "	pMW3deltaE	215
qutE (catabolic dehyroquinase)	pEH1	338
hph (hygromycin phosphotransferase[c])	pAN7-1	210
" "	pDH25	227
prn (proline catabolism gene cluster)	pAN222	339
riboB2 (riboflavin$^-$)	pLO1	222
ben^r	pAIpGM4	195
acuD (isocitrate lyase)	pAcu22	223
acu7[d] "	pHIONA-1	324
niaD (nitrate reductase)	pAN301	325
niaD[e]	pSTA14	237
pki (pyruvate kinase)	pGW402,403	340
sC (adenosine triphosphate sulfurylase)	pFB75	341

[a]Selection marker is from homologous organism unless noted.
[b]from *N. crassa*
[c]from *Escherichia coli* fused to *A. nidulans* transcription signals
[d]from *Coprinus cinereus*
[e] *Aspergillus oryzae*

been found to complement corresponding A. nidulans mutants. In one case the acetyl-coenzyme A synthetase gene from each fungus was cloned and found to respond correctly to regulatory signals in the heterologous fungus(188). Tilburn et al.(189) used the plasmid p3SR2(190), which contains the amdS gene encoding acetamidase, which allows a dominant selection for growth using acetamide as the sole nitrogen source. Vector p3SR2 contains a genomic fragment of A. nidulans with the amdS gene cloned into pBR322 and has become important as a dominant selection marker in a variety of fungal transformation systems(Table 6, Fig. 3) for which no auxotrophic markers are available. The initial reports of A. nidulans transformation showed integration into the chromosome being the norm. Generally, transformation frequencies varied from several to several hundred transformants per μg DNA(132,191). The types of integration events seen were linked insertion and unlinked insertion, with no gene replacements being observed. As seen with N. crassa, both stable and abortive transformants can be observed, with up to 90% abortive in some cases(189). Vectors with different selection markers yielded different proportions of the three classes of transformants. For example, selection for the trpC gene resulted in predominantly direct replacement events, leading to the excision of both the non-functional gene and pBR322 sequences(192,193) while the above reports with p3SR2 gave transformants which were unlinked to the amdS gene.

Homology of the transforming sequences to the chromosome does not appear to be a general requirement for a successful integrative transformation in A. nidulans. Vectors with increased homology to the chromosome(for instance those containing ribosomal repeat DNA) did not show increased transformation frequencies(189) and integrated at non homologous sites in the chromosome, though infrequently. Conversely, strains in which the chromosomal locus for the selection marker has been completely deleted did not show an appreciable decrease in transformation frequency(194). None the less, directed integration of transforming sequences has been achieved in many cases in A. nidulans systems and this has lead to a more detailed analysis of several chromosomal loci through precise gene disruptions at specific sites in the chromosome(195-198), similar to the methodology now standard for S. cerevisiae(199). In one case, a functional analysis of the SpoC1 gene cluster was examined(200). The SpoC1 cluster contains several genes which are highly expressed during sporulation in A. nidulans. To determine if any of the genes in the SpoC1 cluster were essential for conidiation, a vector, pDC6, which contained the argB gene of A. nidulans(encoding ornithine carbamoyl transferase) fused between DNA fragments from the extreme ends of the SpoC1 locus was used to transform A. nidulans to arginine prototrophy. Transformants in which a gene replacement had occurred, (i.e. elimination of the wild type SpoC1 locus and

replacing it with the argB gene), were selected. It was found that a resulting SpoC1$^-$ deletion strain did not differ in growth or in the production of mitotic or meiotic reproductive structures compared to the wild type indicating that the SpoC1 deletion had no demonstrable function in sporulation or conidiation in A. nidulans.

Attempts to utilize the A. nidulans transformation system for the analysis of gene regulation, in a manner similar to that being currently done with S. cerevisiae(201), are being made. Several vectors which allow transcriptional fusions between specific genes of interest and the lacZ gene of E. coli have been constructed(Table 6, Fig. 3). By attaching the promoter region of the trpC gene to the coding region of the lacZ gene and using a vector with this fusion to transform A. nidulans, it was found that β-galactosidase activity could be easily assayed using either an x-gal plate assay or a β-galactosidase enzyme assay(202). To broaden the analysis of gene expression to other promoters, a series of vectors (pAN923-41B, pAN923-42B, and pAN923-43B) which allow in phase fusions of any gene to the lacZ gene were constructed(203,204). In addition to the lacZ gene, these vectors contained the argB gene for selection and pBR329(204). The results showed that approximately 40% of the A. nidulans transformants contained single integrations of these vectors at the homologous argB site in the chromosome. Thus by screening for these homologous insertions, one could eliminate the position effect

variable which would be encountered in any promoter analysis if only random unlinked insertion events in the chromosome were found. Two promoter analyses using such lacZ fusions have been made. A deletion analysis of the trpC promoter showed that seperate DNA elements were present in the upstream region controlling the level of expression and the correct initiation of transcription(205). By fusing the amdS promoter to the lacZ gene, and replacing the resident amdS gene by a two step gene replacement with this fusion, it was found that the level of β-galactosidase production by the transformant could be regulated similarly to acetamidase expression by the wild type(206). These initial studies indicated the usefulness of the above approach to a detailed analysis of the A. nidulans promoters.

One interesting and useful observation made with the A. nidulans transformation system is the fact that multiple integration events often occur in the process of the integration of the transforming vectors into the chromosome. This results in many tandemly repeated inserts at one chromosomal locus(191). This phenomenon appears to be strain specific, and no gene specifically responsible for this type of integration event has yet been identified(191). These studies further showed that multiple copies of the inserted gene can directly be translated into increased enzymatic activity of that gene in a dose dependent manner(191,207). This phenomenon has been taken advantage of in the examination of the genetic

regulation of enzymes involved in nitrogen and carbon catabolite repression in A. nidulans(208,209). It has been found that the introduction of multiple copies of a DNA binding element into A. nidulans can cause titration of the affected DNA binding protein, and that this effect can be reversed by the addition of multiple copies of the gene encoding the DNA binding protein. As A. nidulans is becoming increasingly important in the production of heterologous proteins as an alternative to S. cerevisiae, this phenomenon may allow a means to direct a high level of expression of a particular gene in a stably inherited manner by selecting recombinants with multiple copies of a desired gene. Tandem integration of transforming vectors is a type of integration event seen in many filamentous fungal systems(see below).

Another common phenomenon of fungal transformation, cotransformation, was first demonstrated with Aspergillus species. When an A. nidulans auxotroph was transformed by a vector containing the appropriate complementing gene, it was found that a second, unselected, plasmid could also often be transfromed along with the vector that is being selected for. Southern analysis, or a second screening for the complementation of the previously unselected marker on the second plasmid often demonstrated that the unselected plasmid as well as the original vector could be integrated into the chromosome. Cotransformation was detected up to 50% of the transformants examined and was found to occur independent of the selection marker and

strain(132,210,211). This has been found to be a useful method to insert a gene for which there is no corresponding auxotroph, or in the cloning of genes from heterologous organsims for which there is no direct selection(212).

Mitotic and meiotic analyses of A. nidulans integrative transformants by different investigators showed that transforming sequences are integrated into the chromosome and are stably inherited by spore progeny(186,187). However, transformants in which the vectors have integrated in multiple, tandem repeats do exhibit both mitotic and meiotic instability. The reason for this has not been determined. It may be due to a combination of effects, such as an RIP like event(see above) similar to that seen in N. crassa or the possible inherent instability of the repeats. A further observation which has been made in transformants containing vectors which have integrated in either tandem repeats or multiple copies is the recovery of the intact vector by E. coli transformation. The results suggested that this is due to the precise excision from the chromosome of an intact vector and not due to autonomous replication of the vector in A. nidulans(191,194,213,214).

B. Studies on the development of a shuttle vector system.

Development of shuttle vectors for Aspergillus focused on the isolation of ars elements as no endogenous

plasmids have been reported for A. nidulans. Furthermore, no enhancement of transformation frequency was observed using plasmids containing ribosomal repeats or a mitochondrial origin of replication(189). Ballance and Turner(132) used the vector pFB6, containing the pyr4 gene of N. crassa which complements both pyrG$^-$ mutants of A. nidulans and ura3 strains of S. cerevisiae, to attempt this. An A. nidulans genomic library in pFB6 was used to transform S. cerevisiae to uridine prototrophy. Plasmids which transformed S. cerevisiae at a high frequency were isolated. It was found that one of these plasmids, pFB6-An2, could transform A. nidulans at 5000 transformants per μg, compared to 50-150 transformants per μg observed with pFB6 alone, and this was considered as a potential ars containing vector. This A. nidulans sequence with ars-like activity was designated ans1(Aspergillus nidulans sequence 1). ans1 was cloned into a different vector, pDJB1, containing the pyr4 gene in pBR325, to create pDJB2. Analysis of individual transformants showed that pDJB2 had integrated into the chromosome. Southern blot of A. nidulans genomic DNA using the ans1 sequence as a probe showed that many dispersed regions of the chromosome had homology to ans1, possibly accounting for its ability to increase the transformation frequency by homologous recombination. Further analysis of pDJB2 integration, however, found that integration at the homologous ans1 locus in the chromosome was only rarely observed. The authors also suggested that the increased transformation

frequency is due to the similarity of *ans1* to yeast centromeres(*ans1* has 81% A-T, similar to element II of a yeast centromere; 119) and that the *ans1* locus is closely linked to a centromere(215), but no model for the action of *ans1* was postulated.

Further attempts to develop an Aspergillus shuttle vector utilized mitochrondrial DNA from a ragged(*rgd*$^-$) mutant of *Aspergillus amstelodami*. A specific region of the wild type mitochondrial genome of *A. amstelodamei* undergoes an amplification, similar to the phenomenon observed in petite mutants of *S. cerevisiae*, resulting in the accumulation of two types of small circular molecules containing DNA from two different regions of the mitochondrial genome. This results in the ragged phenotype. Sequence analysis of two circular DNAs, designated *rgd1* and *rgd5* DNA, indicated that both had homology to *S. cerevisiae* as well as to human mitochondrial origins of replication(216). The two circular DNAs were cloned into YIp5(see above; 105) creating pRKB1(*rgd1*) and pRKB2(*rgd5*). Both the latter vectors were shown to transform *S. cerevisiae* at a high frequency indicating that they contain *ars*-like elements. These *ars*-like sequences were recloned into pDJB1(an integrative *A. nidulans* vector; described above) and transformed into *A. nidulans*. Southern analysis showed that the vector integrated into the chromosome at different sites and both single and multiple insertions were observed. Both vectors could be rescued in *E. coli*.

The mitotic stability of the these transformants was reduced and this suggested the presence of tandemly integrated repeats of the vector in these transformants was the most likely cause of this recovery in E. coli(217).

The possibility that a centromere sequence of S. cerevisiae would function in A. nidulans, leading to the establishment of mini-chromosomes as in yeast, has also been examined(218). The centromere of yeast chromosome 11(CEN11) was inserted into the vector pMA2, which contained the argB gene of A. nidulans in pBR329 and this along with pMA2 was used to transform A. nidulans. The results showed that a CEN sequence inserted into pMA2 transformed at similar frequencies to pMA2 and both transformed by integration into the chromosome. No chromosomal instability was observed in such transformants, in contrast to the instability in S. cerevisiae(119) which is caused by the presence of two centromeres on one chromosome. These data suggested that the S. cerevisiae centromere did not function in A. nidulans.

One of the important applications of a transformation system is the ability to use it to clone genes of interest. Genes from A. nidulans have been isolated by complementation of both E. coli(219) and S. cerevisiae(220) auxotrophic mutations. However, an important limitation of this approach is the fact that many of the genes one wishes to clone may not function in these

organisms due to the presence of introns or due to other functional limitations. Another major limitation of most transformation systems of filamentous fungi has been the fact that fungal transforming vectors generally integrate into the chromosome of the recipient and there is no easy and efficient means of recovering complementing genes from the chromosome of transformants. Despite these barriers, the A. nidulans transformation system has been used extensively to clone genes from both A. nidulans(see below) and from other filamentous fungi(187,212).

Several methods have been used to recover vectors from A. nidulans transformants. One of these is by marker rescue(192,212,221-223). Oakley and co-workers have used this technique to clone the riboB2 and pyrG genes(222,223). A genomic library of A. nidulans was constructed in pBR329 and used to transform riboflavin or pyrG$^-$ auxotrophs to prototrophy. In this approach, to recover the complementing gene in the vector which has integrated, a restriction enzyme which does not cut within the vector or the complementing sequence is needed. Since the restriction sites in the complementing gene are not known in most instances, several enzymes are usually selected to maximize the possibility of isolating the intact complementing gene. Total DNA from a transformant is partially digested with each of the several restriction enzymes separately, the genomic DNA from this tranformant is then self-ligated and used to transform E. coli. Individual plasmids recovered from each pool of E. coli

transformants are isolated and used to transform the same A. nidulans riboB2$^-$ strain. The plasmid pool that complements the particular auxotroph contains a functionally intact complementing gene. This pool could then be subdivided and an approach similar to the sib selection procedure for N. crassa could then be used to isolate a single clone containing the intact gene(222).

An alternative to the above approach has been to construct a cosmid library of A. nidulans and use this to transform a specific auxotroph to prototrophy. A cosmid designated pKBY2 which contains a bacteriophage lambda cos site, the trpC$^+$ gene of A. nidulans for selection of fungal transformants, and ampicillin and chloramphenicol resistance genes for selection in E. coli and a bacterial ori was constructed by Timberlake and co-workers(224,225). The cosmid library was then used to transform A. nidulans to Trp$^+$. Transformant DNA was then be packaged into lambda and transduced into E. coli. Individual cosmids recovered in E. coli were used to re-transform A. nidulans to confirm that it carries the desired gene. Occasionally, direct recovery of a vector in E. coli can be accomplished directly from total transformant DNA. In one case, a gene library in the vector pILJ16(pUC8 with the argB gene of A. nidulans) was used to transform a conidiation deficient strain(brlA$^-$) of A. nidulans to Arg$^+$. DNA from one transformant which was conidiation proficient was used to transform E. coli directly and one colony was recovered which contained a genomic fragment of A. nidulans which

complemented the brlA deficiency(226).

One major application of the A. nidulans vectors and selection markers is that they have been used to develop transformation systems for other fungi for which either auxotrophs or homologous selection markers have not been isolated(Table 6). As with N. crassa, several markers have found wide use in the development of fungal transformation systems. One of the most widely used A. nidulans vectors for transforming other fungi is p3SR2(Table 6; Fig. 3), which contains the amdS(acetamidase) gene in pBR322. The amdS gene allows growth of the fungus on acetamide as a sole nitrogen source. Transforming a fungus with p3SR2 and selecting for growth on acetamide has, however, proven to be effective since many filamentous fungi are either very poor utilizers of acetamide as a nitrogen source or do not utilize it at all. Antibiotic markers, though more effective as general purpose markers, were not used in fungal transformations systems initially. This is due to the fact that fungi are often resistant to common antibiotics such as tetracycline, ampicillin, and kanamycin. Furthermore, the most well characterized antibiotic resistance markers are of bacterial origin and would probably not function in fungi unless placed downstream of a suitable fungal promoter. Once the characterization of some highly expressed fungal genes was done, it became possible to create fusions between fungal promoters and bacterial genes. This was initially

accomplished by two different groups using the *E. coli* hygromycin B phosphotransferase gene(*hph*). To construct vector pAN7-1, the *hph* gene was placed between the promoter signal from the glyceraldehyde-3-phosphate dehydrogenase gene(*gpd*) and termination signals from the *trpC* gene and inserted into pUC8(Fig. 3; 210). A similar construction, pDH25(Fig. 3; 227), had the *hph* gene fused to both the transcription start and termination signals from the *trpC* gene. Both pAN7-1 and pDH25 have been used to transform both *A. nidulans* as well as a number of other fungi(Table 6) to hygromycin resistance.

In addition, oligomycin resistance(228) has been used as a selection marker. Oligomycin inhibits the activity of mitochondrial ATP synthase. pMW2, which contains the oligomycin resistant form of the mitochondrial ATP synthase subunit 9 gene in pDJB1(see above). Transformation with pMW2 resulted in insertion events similar to those noted with other markers.

C. Transformation of other *Aspergillus* species

In the genus *Aspergillus*, *A. niger* and *A. oryzae* are the most industrially important species as many useful organic acids, industrially important enzymes, and secondary metabolites are produced in abundance by these organisms(178). As with many industrially important fungi to date, however, the genetics of these two fungi is not highly developed, although many auxotrophs are available

and transformation systems have been described(Table 4).

i. *Aspergillus niger*

Once a transformation system for *A. nidulans* was developed it was only logical that this would be extended to *A. niger*. Transformation of *A. niger* was first demonstrated using heterologous genes from *A. nidulans*. Transformation with vector p3SR2(*amdS* gene; see above and Fig. 3), resulted in integrative transformants(211). The *argB* gene of *A. nidulans* was also used(229). Vectors pDG1, constructed from the cosmid pHC79 into which the *argB* gene was cloned, and pDG3, a derivative of pDG1 to which the kan^r gene of Tn5 was added, both complemented the corresponding *A. niger argB* mutant. Transformation was due to integration into the chromosome and unlinked insertion events into a variety of chromosomal locations were observed in all cases as there was little sequence homology between the selection markers of *A. nidulans* and *A. niger*. On the other hand, integration into the homologous site was observed when the homologous *pyrG* gene was used mutants of *A. niger*, integration into the homologous site was observed(230,231). The *pyrG* cloned in the *E. coli* vector pUN121(vector pGW613) was found to transform *A. niger* only at the homologous locus, either by gene replacement or by linked insertion(230). A corresponding 10 fold increase in the transformation frequency over transformation with the heterologous

TABLE 4

Selection markers and transformation vectors used for other Aspergillus species[a].

Selection Marker	Vector	Reference
Aspergillus niger		
argB[b]	pDG3	229
amdS[b]	p3SR2	211
pyrG	pGW613	230
"	pAB4-1	231
oli[r]	pMW30	234
niaD	pSTA10	232
niaD[c]	pSTA14	237
pyrG[c]	pAO4-2	236
sC (adenosine triphosphate sulfurylase)	pFB75	341
ben[r]	pBT6	233
Aspergillus oryzae		
pyrG[d]	pAB4-1	235
pyrG	pAO4-2	236
argB[b]	pILJ16	238
"	?	181
amdS	p3SR2	"
niaD	pSTA14	237
Aspergillus ficuum		
hph[e]	pAN7-1	242
amdS[b]	p3SR2	"
Aspergillus awamori		
pyrG[d]	pANG1	239
argB[b]	pUC4AP-*argB*	240
Aspergillus giganteus		
hph[e]	pAN7-1	243

[a]Selection marker is from homologous organism unless noted.
[b]from *Aspergillus nidulans*
[c]from *Aspergillus oryzae*
[d]from *Aspergillus niger*
[e]from *Escherichia coli* fused to *A. nidulans* transcription signals
[g]from *Coprinus cinereus*

markers(amdS and argB) was seen, indicating that homology to the chromosome was beneficial in A. niger. Similar results were obtained using the homologous pyrG gene cloned into pUC19(vector pAB4-1) to transform A. niger. Sixty percent of the transformation events were by gene replacement, while the rest were by linked insertion. In contrast, transformation with the vector pSTA10, with the A. niger niaD(nitrate reductase) gene cloned into pUC18 showed that no gene replacement was observed. The data did suggest many linked insertion events(232). A ten fold increase in transformation frequency, (from 100-1000 transformants per μg vector DNA) was obtained when pSTA10, linearized within the niaD gene, was used. Integration was shown to be both by gene replacements and linked insertions in this case(233). In addition, pSTA10 was used to cotransform several unselected genes into A. niger and test for their expression. These include pAN5-41B(containing the lacZ gene[β-galactosidase] fused to the gpd promote of A. nidulans; Fig. 3), pNOM102(the uidA gene[β-glucuronidase] fused to the same gpd promoter; Fig. 3) and plasmid pBT6(with the tubulin-2 gene of N. crassa; Fig. 3), which were all found to be expressed in A. niger. An homologous oligomycin resistance transformation system has been developed(234) for A. niger. Vector pMW30, containing the oligomycin resistant form of mitochondrial synthase subunit 9 in pUCpyr(pUC19 and the N. crassa pyr4 gene) transformed by integrating into the chromosome. The vector DNA was shown to be integrated at the homologous

locus in only 15% of the transformants.

As with N. crassa and A. nidulans, all three of the types of integration events can be observed with integrative vectors. Similar to those fungi, the type of integration observed during transformation with homologous genes in A. niger appears to vary depending on the marker used.

ii. **Aspergillus oryzae**

A. oryzae auxotrophs have been transformed with the pyrG gene of A. niger(235) and A. oryzae(236). With the homologous pyrG gene(vector pOA4-2, with the pyrG cloned into pUC19), 75% of the transformation events were at the homologous locus(type II and III events; 236). About 90% of these integration were by gene replacement. The pyrG gene of A. niger(in pAB4-1, see above) was found to transform A, oryzae. Transformation was shown to be by integration at various sites in the chromosome, and in some transformants multiple integrations were seen(235). Using vector pSTA14, that contained the A. oryzae niaD gene in pUC18 for transformation, a high frequency of gene replacements and linked insertions were found(41% and 23%, respectively). Multiple tandem integration events at the homologous locus were seen occasionally. It was also found that this A. oryzae niaD marker could transform appropriate niaD mutants of A. nidulans, A. niger, and Penicillium chrysogenum to $niaD^{+}$(237). Vectors containing

the argB(181,238) and amdS(181) genes of A. nidulans could transform A. oryzae. The vectors were found to integrate at different sites in the chromosome of individual transformants, and multiple insertions could be observed in some. Cotransformation of A. oryzae was demonstrated in several instances. Cotransformation experiments indicated that the lacZ gene of pAN5-41B and the uidA gene of pNOM102(see above) could be expressed in A. oryzae when cotransformed with pOA4-2(236).

iii. Other Aspergillus species

Successful transformation of A. awamori was shown using pANG1, containing the pyrG gene of A. niger in pUC100 using electroporation. Integration events were observed(239). A. awamori has also been used in the expression of a heterologous protein(2440). Two vectors(pGRG1 and pGRG3) in which the cDNA for bovine prochymosin B was fused behind the transcriptional signals of the glucoamylase gene(glaA) of A. niger gave primarily intracellular expression of chymosin. Making a translational fusion of this cDNA behind the carboxyl terminus of the glaA protein of A. awamori resulted in a high level of secretion of chrymosin, which apparently was released autocatalytically from the galA protein in the extracellular medium to result in an active, native sized enzyme. In addition, the aspartic proteinase aspergillopepsin A gene was cloned from A. awamori and

used to create gene disruptions of this locus(241). Transformation of Aspergillus ficuum using both the amdS gene(p3SR2) and hygromycin resistance marker(pAN7-1) has been shown to be by integration into the chromosome(242). When pAN7-1 was cotransformed with pAN5-41B, subsequent expression of the E. coli lacZ gene was also demonstrated here. Transformation of A. giganteus to hygromycin resistance using pAN7-1(Fig. 3) was shown(243). Although transformation was by integration, pAN7-1 derivatives could be recovered by using undigested DNA from the fungal transformants to transform E. coli. The majority of the plasmids recovered were identical and had a 2.5 kb deletion of pAN7-1, while a small number of the E. coli transformants contained intact pAN7-1.

5. Other filamentous fungi

In the last several years much progress has been made in the development of transformation systems for other filamentous fungi which have also been important in studying the genetic processes of lower eukaryotes. In many cases these transformation systems have been developed using the accumulated genetic data for the particular fungus of interest. However, rapid strides have been made in recent years in developing transformation systems for a variety of filamentous fungi utilizing selection markers that became available through the development of the N. crassa and A. nidulans transformation systems. A finding

of considerable interest was the fact that in many instances, heterologous selection markers from Neurospora or Aspergillus were shown to complement the appropriate auxotrophic mutation in a number of species of filamentouus fungi. It was also found that the transcription signals from A. nidulans were often recognized as such in a number of other fungi examined. This eliminated the need for the isolation and detailed characterization of homologous structural gene to precede the development of a transformation system for some less well characterized fungi. A summary of transformation results and strategies used for filamentous fungi other than Neurospora and Aspergillus which are important genetic models, plant pathogens, or of interest industrially is presented here.

A. Podospora anserina

P. anserina has a well characterized genetic system and has been exploited in recent years because of its usefulness in the study of the senescence phenomenon in fungi(244). The senescence phenomenon is particularly interesting with respect to the development of a transformation system as senescence in P. anserina is associated with the excision of a plasmid from the mitochondrial genome of this organism during aging(245). The senescence plasmid is an intron of the cytochrome c oxidase gene of P. anserina and appears similar to other

fungal plasmids which comprise a class of apparently mobile mitochondrial introns. Initial transformation protocols for this fungus utilized the senescence plasmid alone as the transformation vector(246). One major difficulty associated with such a vector, however, was the fact that the introduction of the senescence plasmids into the vegetative mycelium of a strain caused senescence of the transformant,i.e., the transformant began dying. Thus the use of senescence as the sole selection marker severely limited the usefulness of the senescence plasmid-based vector. Although initial studies using vector pSP17, which contained pBR322 and two copies of the senescence plasmid suggested that this vector replicated autonomously in P. anserina(247); however the Southern blot data could not rule out excision tandemly repeated integration into the chromosome followed by precise excision as discussed above for A. nidulans.

Further studies were done with two additional vectors each containing a different senescence plasmid. The su4-1 gene(which could suppress the leu1-1 mutant) was cloned into α-pBR325(pBR325 with the α senescence plasmid), and 221-pBR325(pBR325 containing the β senescence plasmid) to create vectors pBαsu4 and pB211su4, respectively. It was shown that transformation with each of these vectors was exclusively by integration into the chromosome, in which the transforming phenotype was passed through mitotic and meiotic divisions in a stable Mendelian fashion(248). In addition these investigators concluded that the prescence

of the senescence DNA on the vector did not cause senescence the transformants. This difference in results of different investigators could be due to the different genotypic backgrounds of P. anserina used.

The ura5 gene(orotidylic acid pyrophosphorylase) of P. anserina was cloned by complementation of a corresponding E. coli mutant and the resulting vector, pPAura5(the ura5 gene in pBR322) was used to transform P. anserina. The types of integration events observed in the transformants were varied. Genetic analyses indicated that unlinked insertion events are predominant, although linked insertion events were seen(249,250). Transformants with up to 10 tandemly linked copies of pPAura5 were found and an increase in the expression of the orotidylic acid pyrophosphorylase proportional to the number of copies of pPAura5 was observed in the transformants. Two of the transformants with multiple copies of pPAura5 were crossed to create recombinant strains with even greater specific activity of this enzyme(251). Another vector containing a suppressor tRNA(su4-1; see above) cloned into pBR328 was used to transform P. anserina. Genetic analyses showed that most of the individual transformants were likely due to unlinked insertions, and a number of hotspots for integration in different chromosomes were found(252). Transformation of P. anserina to benomyl resistance using pBT6, which contains the tubulin-2 gene of N. crassa has been accomplished in which primarily single site unlinked insertions into the chromosome are observed(253). A cosmid

library of P. anserina was constructed starting from a transformant which contained an integrated vector linked to the mating type locus. A series of cosmids encompassing the chromosomal region surrounding the mating type locus(254). Transformation of P. anserina with these several of the cosmid clones, which contained the su8 gene(suppressor tRNA) as a selection marker, resulted in a high proportion of linked insertion transformation events in transformants obtained from all cosmid clones used. Genetic analysis of the transformants with linked and unlinked insertion showed that the unlinked integration events were stable through meiosis while those carrying linked insertion events were unstable. Although the reason for the instability was not examined, the unstability seemed to be due to a RIP-like event. Attempts to isolate a centromere from P. anserina for use in the development of a potential autonomous vector were not successful(255). However, this cosmid library has been used to clone two alleles(s and S) involved in heterokaryon incompatibility(256). A strain with a neutral incompatibility allele was used as a recipient for a cosmid library from an s allele in transformation. A process of sib selection was used to identify transformants which could then cause incompatibilty to an S strain to identify an s allele containing transformant. This allele was then used as a DNA probe to a cosmid library of an S allele strain of P. anserina to clone the corresponding S allele.

Recently, an autonomous vector for P. anserina was

constructed(257). Telomeres from Tetrahymena thermophila were linked to a linearized P. anserina integration vector, pPAura5(see above), resulting in vector pPATura2. Linearized pPATura2 was used to tranform P. anserina and over 50 % of the ura+ transformants were found to be unstable by during non-selective culturing. This is in contrast to those transformants arising from transformation with pPATura2(lacking telomeres), which were all stable. This suggested the potential presence of an autonomous vector. Southern analysis of the unstable pPATura2 transformants showed the presence of an extrachromosomal band indicative of an autonomously maintained vector. The pPATura2 transformants which were mitotically stable showed chromosomal integration of the vector only. One interesting phenomenon was that the copy level of the linear plasmid was very low, one per 5-10 nuclei, indicating that the few nuclei with the plasmid are enough to sustain the positive phenotype of the transformants. It was reasoned that this type of low copy number maintenance is possible in coenocytic multinucleate fungi with a common cytoplasm, as opposed to single celled organisms, where each each cell must maintain the phenotype to survive. The basis for the low copy maintenance of transforming vectors in P. anserina is not known at this time.

B. *Phycomyces blakesleeanus*

P. blakesleeanus is a fungus of considerable interest since it is strongly dependent on sensory responses, including light intensity, gravity, and pressure, and as a result many behavioral mutants of this organism have been isolated(258). Both spheroplast(259-261) and liposome mediated fusion(262) transformation systems have been developed for *P. blakesleeanus* . The transformation vectors used have been based on G418 resistance using the Tn903 *kan*^{r} gene as a selection marker without the addition of fungal transcription signals(260-262). Transformation of *P. blakesleeanus* to G418 resistance using the *kan*^{r} marker of Tn5 was not successful, unless a fungal promoter was inserted(259). In this case, random genomic fragments of *P. blakesleeanus* were ligated to the vector pVB32 at an *Eco*R1 site upstream of the promoterless *kan*^{r} gene and used to tranform *E. coli*. DNA from thirty four independent transformants was then used transform *P. blakesleeanus* to G418 resistance. A range in transformation frequencies from 20 to 2909 tranformants per μ g vector, indicating that some likely contained promoters functional in *P. blakesleeanus*. Analysis of conidially purified transformants from several of these plasmids indicated that the vectors were maintained in a stable form in the individual transformants, although chromosomal integration was not tested by Southern analysis.

Autonomous maintenance of transforming vectors in P. blakesleeanus has been reported by two different groups, lending support to the idea that non-integrative transformation occurs in P. blakesleeanus. In the first report, pJL2, a vector in which a P. blakesleeanus genomic DNA fragment capable of ars activity in S. cerevisiae(arspy) was used with the Tn903 kan^r gene to select for G418 resistance(260). Autonomous replication of the vector in the tranformants was suggested by the fact that intact pJL2 could be recovered from the total DNA of the transformants via E. coli transformation. Additonal evidence supporting autonomous replication of pJL2 was that a deletion analysis of the P. balakesleeanus arspy element identified a specific region within the arspy which resulted in high frequency transformation of P. blakesleeanus in comparison to the deletions of pJL2 which did not have this region. Southern blot data to support this was not given, and whether this site in the arspy promoted autonomous replication or more efficient integration into the chromosome was not determined. Another ars based vector, designated pPS11, was also shown to tranform P. blakesleeanus and the intact vector could also be recovered in E. coli(261). Southern blot analysis of genomic digests of pPS11 transformants indicated the presence of monomer length plasmids in the transformants. It was not determined, however, whether these vector sequences were maintained autonomously or integrated into the chromosome in tandem repeats, which could also account

for the monomer length vectors in the hybridization analyses done. Only 2-10% of the conidia isolated from selectively grown transformants maintained the G418 resistant phenotype whereas none of the spores isolated from non-selectively grown transformants were found to be G418 resistant indicating a loss of vector consistent with autonomous replication of pPS11(260).

C. Schizophyllum commune

S. commune is probably the most well characterized basidiomycete in terms of its' genetics and several important genetic concepts have resulted from studies with this organism, including the concept of genetic incompatibility(263). S. commune was initially transformed using the homologous TRP1 gene(indole-3-glycerol phosphate synthetase) in pRK9 as the selection marker(264). The TRP1 gene was isolated due to its ability to complement a trpC mutant of E. coli. Transformation was shown to be by integration into the chromosome. Ullrich and coworkers used this transformation to isolate many structural genes of S. commune by complementing selected mutants with an S. commune gene library in pRK9(265) and have made many improvements in the optimization of transformation(266). Southern blotting data indicated that unlinked insertion events were common when these homologous genes were used for transformation. Genetic analysis indicated that the transforming sequences were integrated

into the chromosome and stably maintained through both mitosis and meiosis. This transformation system was also used to isolate two mating type alleles of S. commune(267). A cosmid containing the TRP1 gene for selection was constructed(pTC20) and cosmid libraries from two strains differing in Aα mating type alleles(Aα4 or Aα1) were made. A series of cosmid clones from each cosmid library, spanning a region of DNA 50 kb were isolated in a chromosome walk which began from the PAB1 gene(closely linked to the mating locus). This series of cosmids from both strains was used to transform a trp1 strain to prototrophy. Transformants were then tested in a mating assay to confirm the presence of those cosmids containing Aα alleles.

D. Coprinus cinereus

C. cinereus, along with S. commune, has been used as a model genetic system for basidiomycetes and is a particularly interesting model for the study of genetic compatibility considering the complexity of the C. cinereus incompatibility system(268). In one transformation system, using the homologous trp-1 gene(tryptophan synthase; 269), isolated by cross hybridization with the TRP5 gene of S. cerevisiae, was used as the selection marker. The vector used, pCc1001, contained this trp-1 gene cloned into pUC9.

Either mycelial fragments or oidia were used as a source for the generation of spheroplasts for transformation. Unlinked integration into the chromosome was the predominant event. Meiotic analysis of linked insertion transformants indicated that they were stable, and no RIP like phenomenon was detected. This system was also used to confirm that a gene cloned by cross hybridization to the heterologous *acuD* gene(isocitrate lyase) from *A. nidulans* was a functional *acu-7* gene in *S. commune*. The vector used, pHIONA1, contained the putative *acu-7* gene cloned into pUC13. Transformation to acu^+ was done by cotransformation with pCc1001 and trp^+ transformants were selected since selection for acu^+ was toxic to the spheroplasts. Cotransformation of the *acu-7* gene was found in 31 to 47% of the transformants, depending on the experiment. Transformation has been shown to be by integration and predominantly by unlinked integration events(270). Insertion of multiple copies of unlinked selection markers have been shown to occur, resulting in an increase in enzymatic activity in some of the transformants(271), depending on the integration site of the transforming vector.

C. cinereus transformation was also used to examine the function of heterologous genes from other fungi in cotransformation experiments(272). It was found that the *trp1* gene of *S. commune* and the *trpC* gene of *Phanerochaete chrysosporium*, both basidiomycetes like *C. cinereus*, could complement *trp-2* mutants of the latter fungus. Seperate

cotransformation experiments in which the transformation vector pAK3(containing the trp-2 gene of C. cinereus) was used along with plasmids containing the trpC and acuD genes of the ascomycete A. nidulans to transform C. cinereus to TRP^+. It was found that the trpC and acuD genes of the ascomycete A. nidulans could be cotransformed with pAK3, but not expressed. In addition, the plasmid pHL1, containing the hph gene of E. coli fused to a promoter of the hemibasidiomycete Ustilago maydis was also shown to be non-functional in C. cinereus. These results indicated that the range of applicability of fungal genes and/or promoters as selection markers may be restricted. Coprinus bilanatus has also been transformed to tryptophan prototrophy using plasmid pAK3 containing the trp-2 gene of C. cinereus(273)

E. Ascobolus immersus

The study of the genetics of A. immersus has contributed greatly to the understanding of the process of meiotic recombination(274). Transformation in this fungus has been achieved using an homologous homoserine o-transacetylase gene to complement a $met2^-$ auxotroph(275,276). When vector M13CG1, containing the A. immersus met2 gene cloned into M13, was used for transformation, the plasmid was shown to integrate either at the homologous locus(gene replacement and linked

insertions) or at heterologous sites in the chromosome. The gene replacements resulted in a stable wild type phenotype through both mitosis and meiosis. The linked and unlinked insertions were unstable through meiosis, while being mitotically stable. This same pattern of stability was also found when the met2 gene was cloned into pUC19 and pBR329(to create vectors pCG5 and pCG6, respectively) and was used to transform A. immersus. These results suggested that the instability of the individual transformants containing a gene duplicated during transformation(linked and unlinked insertions) through meiosis may due to an RIP-like effect methylation such duplications(276).

The physical state of the transforming vector also appeared to affect the type of integration event observed in A. immersus. A comparison was made between the type of integrative events observed using circular single stranded DNA and circular double stranded DNA using vector M13CG1(276). It was found that using single stranded M13CG1 increased the frequency of gene replacement transformation events to 65% from the 5% observed with the double stranded vector. To examine if linearizing a vector within the A. immersus met2 gene similarly increased the probability of gene replacement, transformants of circular pCG5 were compared to transformants obtained with linearized pCG5. It was found that with the circular vector, only 10% of the transformation events were due to gene replacement while 85% of the transformants were of the gene replacement variety when the linearized vector was

used. These results suggest that gene targeting is possible in A. immersus and this can be enhanced by providing either homologous double stranded DNA or a single stranded form of DNA, either of which may be intermediates in the process of homologous recombination.

A further transformation study tested this methylation phenomenon using the heterologous marker amdS on either p3SR2 or pAMD21 to transform A. immersus transformed to amdS^{+}(277). It was found that 50% of the meiotic progeny containing two unlinked copies of amdS from the unlinked transformants, and up to 90% of the meiotic progeny of transformants containing tandemly linked amdS genes had both copies of the transforming gene inactivated. It was found that only the duplicated amdS genes, not adjacent bacterial sequences of the tranforming vector, were methylated. This indicated that the inactivation was specific to the duplicated sequences. The methylation was found to be due to a de novo cytosine methylation in the haploid dikaryon just prior to the meiotic cycle which inactivated both the duplicated genes in such transformants. In contrast the the RIP effect of N. crassa, this methylation was reversable, as subsequent vegetative growth resulted in the loss of the methylation and the recovery of the complementing activity.

6. Fungi of Industrial Importance

A. *Penicillium chrysogenum*

This fungus produces the antibiotic penicillin during secondary metabolism. Classical mutagenesis and strain improvement techniques have resulted in strains producing extremely high levels of this antibiotic(278). Further attempts to increase production may require a better understanding of the secondary metabolic regulation of genes involved in penicillin production, which would be facilitated by the development of a transformation system. Transformation of *P. chrysogenum* has been achieved by complementation of appropriate auxotrophs with vectors containing the heterologous *pyr4* gene of *N. crassa*(pJDB2; 279), and the *niaD* genes of both *A. nidulans*(vector lambdaAN8a; 280) and *A. niger*(vector pSTA10; 280). Transformation was by integration into different sites of the chromosome with each of these vectors. Interestingly, the results of transformation with the heterologous *niaD* genes indicated that the regulation of expression of these genes in *P. chrysogenum* was similar to the wild type gene, indicating that the control signals of these heterologous genes were recognized in *Penicillium*(279). The homologous *trpC* gene of *P. chrysogenum* was isolated by two groups using a genomic library in to complement the *trpE* mutation of *E. coli*. The vector pPctrpC1(*trpC* gene in pUC13) was

found to integrate into the chromosome of transformants primarily by unlinked insertion(133), while the vector pPC-31(*trpC* gene in pBR328) was found to integrate primarily by linked insertion(281). Whether this difference is due to the strains used or the different vectors is not certain. Vector p3SR2(Table 6; Fig. 3) was used to transform *P. chrysogenum* to acetamide utilization(282,283) and an homologous resistance marker(oligomycin, pPOL1, containing the oligomycin resistance marker in pUC9; 284). Transformation appears to be by chromosomal integration in all cases. Unlinked insertions were shown to occur in the above cases, with multiple, often tandemly repeating integrations being common. Co-transformation was found to be efficient. When p3SR2, containing the *amdS* gene as the selection marker was cotransformed with pAN5-41B(see above and Fig. 3) and amd^+ transformants were selected, 90% of these transformants showed cotransformation of pAN5-41B and expression of β-galactosidase(282). Different transformants showed varying levels of β-galactosidase enzyme activity indicating either a position effect due to the site of integration of pAN5-41B or differing copies of the vector integrated into the chromosome in different transformants.

Two bacterial antibiotic resistance markers have been successfully used to transform *P. chrysogenum*. The phleomycin resistance gene of *E. coli*(*ble*) was fused to the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene of *A. nidulans* and the terminator of the *CYC1*

terminator of S. cerevisiae in pUC8 to create pHS103(282) and used for transformation. Integrative transformation at various sites of the chromosome was found. Sulfonamide resistance has also been used as a selection marker for transformation of P. chrysogenum (285). P. chrysogenum transcription signals from the trpC gene were fused to the bacterial sulfonamide resistance gene cloned into pUC13. The resulting vectors, pML1 and pML2(pML1 with the entire trpC gene as an additional selection marker) were used for transformation. The results showed integration into the chromosome at various sites in both cases. Dominant fungal resistance markers have also been used.

The feasability of developing an autonomous vector for P. chrysogenum was tested by constructing two vectors containing mitochrondrial DNA fragments of P. chrysogenum which allowed high frequency transformation of S. cerevisiae, indicating the putative presence of ars elements in the mitochondrial sequences utilized. These vectors, pSP530 and pSP533(along with pSP525, the parental form containing a fusion of pBR325 and pACYC177 with a bacterial kan^r gene as a selection marker for P. chrysogenum transformation) were used to transform the fungus to G418 resistance. The results lead to the conclusion that pSP530 an pSP533 are maintained autonomously based on the fact that they were recovered intact by E. coli transformation. Not unexpectedly, the parental vector pSP525 could not be recovered by E. coli transformation and based on Southern blot analysis appeared

to be integrated into the chromosome of transformants. However, not all pSP533 and pSP530 transformants of P. chrysogenum gave recovery of the vectors by E. coli transformation. Furthermore, Southern analysis of transformant DNA of pSP533 and pSP530 transformants apparently could not distinguish between integrative and autonomous maintenance of these vectors so the conclusion of autonomous replication may be preliminary(286).

P. chrysogenum cannot produce cephalosporins. It would be useful to develop a strain which may be able to convert a naturally produced penicillin to a useful cephalosporin, 7-ADCA(7-aminodeacetoxycephalosporanic acid). A gene capable of this chemical modification, penicillin N expandase(cefE), has been cloned from Streptomyces clavuligerus. This was attached to the promoter from the P. chrysogenum pcbC gene(isopenicillin N synthetase) on pPS65 which contains the amdS gene from A. nidulans for selection. Transformants were found which expressed both high levels of endogenous penicillin and expandase activity were found, but it was suggested that the substrate specificity of the expandase may need to be altered before production of high levels of 7-ADCA are possible(287).

B. Cephalosporium acremonium

C. acremonium produces the antibiotic cephalosporin C(288) which can be chemically modified to produce many clinically important antibiotics. C. acremonium was

tranformed to hygromycin B resistance using the hygromycin B phosphotransferase gene of E. coli linked to the C. acremonium isopenicillin N synthetase gene promoter to make the vector pPS25(289). Both single and multiple integration of pPS25 into the chromosome were observed. When vector DNA was linearized within the C. acremonium sequences, an increase in transformation frequency of two to three fold was obtained. Whether the increase in frequency was associated with an increased proportion of linked or gene replacement type events was not discussed.

To determine whether a mitochondrial DNA fragment containing ars activity in S. cerevisiae promoted high frequency transformation of C. acremonium, two plasmid vectors, pPS29(no mitochondrial insert) and pPS21(with mitochondrial insert) were used for transformation. It was found that the mitochondrial insert did not promote a high frequency of transformation when compared to the vector without the mitochondrial insert and that both vectors transformed C. acremonium by integration into the chromosome(289).

To determine whether this integrative transformation system can be used to increase expression of a transformed gene, the vector pPS56 containing the cefEF gene encoding a bifunctional protein with deacetoxycephalosporin C synthase and deacetylcephalosporin C synthase, two enzyems involved in the biosynthesis of the antibiotic cephalosporin C, was used to transform C. acremonium to hygromycin resistance. An increase in the levels of activity of these

two enzymes was observed in some of the transformants while no increase in these activities could be observed in control transformations of C. acremonium using a vector lacking the cefEF gene(290).

C. Mucor circinelloides(syn. M. racemosus)

M. circinelloides is industrially useful as a source of extracellular protease,lipases, amylases, and cellulases. In addition, this organism is of basic scientific interest since it is considered a model system for the study of dimorphic fungi(291). The yeast replicative vector YRp17, containing a M. circinelloides genomic DNA library, was used to complement a M. circinelloides leu$^-$ auxotroph(292). Two plasmids, pMCL1302 and pMCL1647, were found which complemented the leu$^-$ mutant. Southern analysis of restriction digests of DNA from individual transformants containing these plasmids showed DNA bands of hybridization corresponding to the presence of plasmid length monomers. This together with the recovery of the intact plasmid from transformant DNA by E. coli transformation suggested autonomous replication. Based on results seen with the other fungi discussed above, these data are also consistent with the presence of tandem insertions of the vector into the chromosome.

The question of autonomous maintenance of vectors in M. circinelloides was examined further with vector pMCL1302(293). Undigested transformant DNA was found to

have monomer length circular vectors and no hybridization to the chromosome was observed. Two different vectors, pMCL1302 and pMCL002(containing the *leu* gene of *M. circinelloides* cloned into pBR322) appeared by Southern analysis of total undigested DNA of individual transformants to be present only extrachromosomally. This indicated that there should be *ars*-like sequences on the chromosomal fragment containing the *leu* gene were responsible for this mode of maintenance. Both vectors could be recovered intact by *E. coli* transformation and the mitotic stability of the transformants was found to be low, also indicative of extrachromosomal maintenance of the vectors. Subcloning of the genomic insert in pMCL1302 found that the region responsible for this autonomous mainetenance could be delineated to a 4.4 kb fragment of the 10.5 kb *M. circinelloides* insert in pMCL1302. This 4.4 kb fragment was found to contain both the *leuA* gene and the *ars* function, which was further localized to a 222 bp fragment upstream of the gene. Some sequence similarity in this region to the *ars* consensus of *S. cerevisiae* was observed, but this region did not function as an *ars* in *S. cerevisiae*(294).

D. *Trichoderma reesei*

T. reesei has been studied extensively as a model system for the analysis of fungal cellulases(295) and many of these have been cloned and sequenced(see ref. 296).

Transformation has been shown with both the amdS(p3SR2) and argB(pSal43) genes of A. nidulans(297). Integration of the transforming sequences into the chromosome was observed at multiple locations and occasionally in multiple copies. When pAN5-41B was cotransformed with p3SR2, β-galactosidase was found to be expressed in 19 to 47% of the amd^+ transformants. β-galactosidase expression in these transformants was found to be independent of the carbon source, indicating that some of the regulatory signals of the gpd promoter of A. nidulans on pAN5-41B were not recognized in T. reesei. The T. reesei transformation system has also been used for expression of a heterologous protein, calf chymosin(298). The cDNA of calf chymosin was fused to promoter and termination signals of the cellobiohydrolase I(cbhI) gene of T. reesei in a series of vectors differing in the length of the 5′ end of the cbhI gene attached to the coding region of the calf chymosin cDNA. These vectors were then cotransformed with p3SR2 and amd^+ transformants were isolated. Active calf chymosin protein was found to be expressed in all constructions, indicating that T. reesei might be a viable system for the expression of heterologous genes. This is especially important since T. reesei is a basidiomycete, whereas the other fungi used for expression of heterologous proteins(mainly the various Aspergillus species) are ascomycetes, and expression of genes across these lines is not always certain(272). As a further demonstration of this usefulness, a cDNA of a lignin peroxidase gene from Phlebia radiata was fused to

the cbhI promoter on plasmid pMS16 for use in T. reesei tranformants. Plasmid pMS16 was cotransformed with p3SR2 and acetamidase producing transformants were selected. The lignin peroxidase gene was found to be expressed as an RNA transcript, although an antibody study found no lignin peroxidase protein either within the cell, or in the extracellular medium(94).

E. Phanerochaete chrysosporium

P. chyrsosporium is the most extensively studied fungal model system for the physiological, biochemical, and molecular genetic analysis of lignin degradation(8). There have been several reports on transformation of P. chrysosporium. An heterologous ade2 gene from S. commune cloned into pRK9 and designated pADE2, was used to complement an ade^- auxotroph(299). Similarly, the ade5 gene from S. commune was found to transform an ade^- auxotroph of P. chrysosporium to prototrophy(300). Since neither S. commune gene had homology to a corresponding P. chrysosporium gene, integration of the vectors by non-homologous recombination into various sites of the chromosome were seen exclusively. A different approach used the kan^r gene of Tn903 to transform P. chrysosporium to G418 resistance(301). A vector containing a genomic fragment of P. chrysosporium cloned into YIp5 showing ars activity in S. cerevisiae(302) was modified by the addition of the kan^r gene and used to transform P. chrysosporium.

In this case no integration events into the chromosome were observed and the intact vector was recoverable by E. coli transformation indicating a possible extrachromosomal maintenance of the vector.

7. Fungal Plant Pathogens

A. Ustilago maydis

U. maydis is a corn pathogen which has been studied extensively at the genetic level for a number of years as a model for other fungal plant pathogens. It has well developed systems for both mitotic and meiotic genetic analysis(303). A PEG-$CaCl_2$ transformation system has been developed for this fungus based on hygromycin B resistance. A heat shock promoter of U. maydis was fused to the coding region of the E. coli hygromycin B resistance gene cloned into pUC12(pHL1; Fig. 3) and was used for transformation(304). Southern blot analysis of the transformants indicated that the vector integrated primarily at a single site in the chromosome, although some tandem repeats were observed. There were two types of integration events seen in the transformants. The predominant type of integration seen was unlinked insertion, but some linked insertion events at the heat shock promoter locus were also observed. No gene replacements were detected. Linearizing the vector inproved the transformation frequency of pHL1 20 fold. The

transformants were both mitotically and meiotically stable. A transformation system using the homologous PYR3 gene has also been described(305). The pyr3 gene was isolated by transforming an E coli pyrC mutant with a genomic library of U. maydis cloned into YEp13. Further subcloning of the pyr3 gene into pBR322 resulted in the vectors pMH2002 and pMH2003. These vectors were linearized within the U. maydis chromosomal DNA and were used to transform U. maydis to prototrophy. Gene replacements were observed in up to 60% of the transformants using linearized pMH2003 whereas linked insertions were observed in 53% of the transformants using linearized pMH2002.

A different study analyzing the effect of DNA conformation on the type of transformation events seen has also been made(306). Vector pCM124, which contains the U. maydis leu1 gene in pUC12 was used to transform U. maydis either as a circular molecule or after linearization within the leu1 gene. With the circular vector two thirds of the transformants indicated integration at sites unlinked to the leu1 gene, while the rest appeared to be gene replacements. U. maydis transformed with the linearized pCM124 showed a much different pattern of events. While a small fraction of the tranformants showed linked integration events, estriction digests of most of the individual tranformants(20 of 23) indicated that bands of monomer length pCM124 were present. When probed with the leu1 gene, these bands were found to be, on the average 25 times more intense than the endogenous copy of this gene.

Since no fast migrating species of DNA corresponding to an autonomous pCM124 was seen, it was concluded that the vectors were integrated in a large tandem repeat at non-homologous sites in the chromosome, although Southern hybridization analyses did not detect any chromosomal junction fragments expected from such an integration. The first indication that this might be an extrachromosomal concatamerization of vector DNA was that the mitotic stability of these such transformants was always much less than for those which clearly demonstrated chromosomal integration. To confirm the extrachromosomal nature of this concatamer, contour-clamped homogeneous electric field(CHEF) gels were run to seperate the U. maydis chromosomes in both transformants containing integrated pCM124, and those containing the putative concatamers. With integrated transformants, vector sequences could be shown by Southern analysis of the CHEF blots to associate with one of chromosome bands, whereas the concatamer transformants showed bands which did not correspond to any of the chromosomes, but to lower molecular weight regions of the gel. E. coli transformation showed that only monomer length pCM124 could be recovered, even in recA$^-$ and recBC sbcB recF strains of E. coli. No identification of any sequences of pCM124 responsible for this unique extrachromosomal maintenance were idenfified.

A one step gene disruption was shown with the homologous pyr6 gene in which the coding region of the pyr6 gene was disrupted with the hygromycin marker discussed

above. The linear DNA fragment containing the disrupted pyr6 gene was then used to transform U. maydis and 70% of the resulting transformants were found to contain a disrupted pyr6 gene(307). Disruption of a heat shock gene, ums2 was also reported by a similar procedure and this disruption was found to be lethal, indicating that the ums2 gene is essential for growth(308). In addition, this transformation system has been used to clone several alleles of the b locus involved in both pathogenicity and sexual development in U. maydis(309). An analysis of one of these alleles, b2, by constructing various deletions and frameshift mutations and using these to replace the wild type allele in U. maydis by transformation, has shown that this locus is necessary for tumor induction by this fungus(310).

Recently, an ars element which apears to function similar to the yeast ars elements was described(311). A genomic library of U. maydis DNA was made in pHL1(see above; Fig. 3) was used to transform U. maydis to hygromycin resistance. DNA from a pool of transformants was used to transform E. coli and several of the plasmids which were recovered were used to retransform U. maydis. One plasmid containing a 3.4 kb U. maydis DNA fragment which gave the highest transformation frequency was used for further analysis(pCM43). Southern analysis of individual transformants showed that pCM43 was maintained as a monomer extrachromosomal element, was maintained at approximately 25 copies per cell and was unstable during non selective

growth. A deletion analysis of pCM43 localized the minimal *ars* activity to a 383 bp fragment. Consensus sequences found in either *S. cerevisiae* or *S. pombe ars* elements were not seen in this *U. maydis ars*-like element.

In a further demonstration of autonomous plasmid maintenance in *U. maydis*, a terminal inverted repeat(TIR) from a linear plasmid from *Nectria haematococca*(pFSC1) was inserted into pIC19RLH, containing the hygromycin selection marker described above inserted into pIC19 to result in vector pTIR1. This vector, when transformed into *U. maydis*, was found to be maintained as an autonomous element by Southen hybridization, although some transformants also contained vector integrated into the chromosome. Circular pICRLH, and linearized pTIR1 also resulted in chromosomal integration in transformants studied(312). An exact identification of the putative element in the TIR allowing for autonomous maintenance was not made.

Both *Ustilago hordei* and *Ustilago nigra*(313), and *Ustilago violacea*(314) have recently been transformed to hygromycin resistance; in each case chromosomal integration at various sites was observed.

B. *Fusarium* species

F. solani is an invasive pea pathogen which is of interest as a model for the penetration of plant cell wall by a pathogenic fungus(315). A transformation system based on G418 resistance has been developed for this fungus. The

vector used was pEMT2, which contains a promoter from cauliflower mosaic virus(35Sp) fused to a bacterial kanamycin resistance gene of Tn5 in pBR322. A spheroplast transformation system was used and all the transformants were shown to be by integration of the vector at various sites in the chromosome(316). A three fold increase in transformation frequency was observed if pEMT2 was linearized.

Kolattakudy and co-workers(317) cloned the cutinase gene which is involved in the initial penetration by the fungus of the plant cell wall. The promoter region was delineated and the promoter was then fused to the bacterial hygromycin resistance gene in pUC19 to result in pCT43 which was used in both spheroplast and LiCl transformation protocols to transform F. solani to hygromycin resistance(318). All transformants were of the integrative type. Vector pCT43 was also successfully used to transform Colletrichium capsica to hygromycin resistance(318). In further studies, the cutinase gene was cloned into the hygromycin vector to result in pU5-11. This vector was used to transform a noninvasive pathogen, Mycosphaerella, which then allowed hygromycin resistant transformants to become invasive, demonstrating the importance of the cutinase gene in plant cell wall penetration by the pathogen(319).

Two other species of Fusarium have been transformed. Protoplasts from a cycloheximide-sensitive, non zearalonone-producing strain of Fusarium culmorum was

incubated with total DNA from a cycloheximide-resistant, zearalonone- producing strain of Fusarium graminearum in a vectorless transformation(320). Some cycloheximide resistant transformants were found to produce zearalonone. Fusarium oxysporum was transformed to hygromycin resistance with pDH25(Fig. 3). Integration of the vector into the chromosome at various sites was observed in all cases(321). Attempts to optimize transformation frequencies were made using the vector pAN301(chlorate resistance; see Fig. 3). While using original protocols, frequencies of 1-10 transformants per μg were obtained, optimizing the DNA/protoplast ratio improved this to 1-200 transformants per μg(322). An electroporation procedure was also developed for pAN301 and comparable frequencies were obtained(323). An extrachromosomally maintained transfomration vector has also been developed for F. oxysporum. A circular plasmid containing pUC12, a linear endogenous Fusarium plasmid, and a hygromycin marker(pFT1) was found to transform at a low frequency(1-10 transformants per μg). Analysis of transformant DNA found that in some cases the vector was present extrachromosomally as a linear DNA element(323). Analysis of this linear vector found that it had acquired telomere sequences from the Fusarium chromosome. Thus it was concluded that this extrachromosomal vector was the product of an in vivo rearrangement of the original vector. A similar rearrangement was found with a vector which contained no endogenous plasmid sequences. These

rearranged vectors were found to be able to transform F. oxysporum and be maintained stably as a linear extrachrosomal element with no further rearrangements. This extrachromosomal vector(pFOLT4R4) was found to be lost quickly during non-selective growth. Further experiments found that pFOLT4R4 could transform both Nectria haematococca and Cryphonectria parasitica and could be maintained extrachromosomally in these fungi also.

8. Other Fungal Transformation Systems

Transformation systems have recently been described for a number of other filamentous fungi which are not as well characterized as those discussed above. These are outlined in Table 5. One fact that became clear with the development of an increasing number of transformation systems for filamentous fungi. is that, barring a few exceptions, promoter signals can be recognized across a broad range of filamentous fungi. Vectors designed to transform an ascomycete such as A. nidulans can in many cases transform basidiomycetes or other classes of fungi. The reverse is also true, i.e. some basidiomycete genes can be expressed in ascomycetes(324). In contrast to this, some genes seem only to function within a specific class of fungi(272).

The fact that promoter signals of certain genes are broadly recognized has also allowed the construction of fusions between these fungal promoters and bacterial

TABLE 5

Transformation markers used in recently described fungal transformation systems[a]

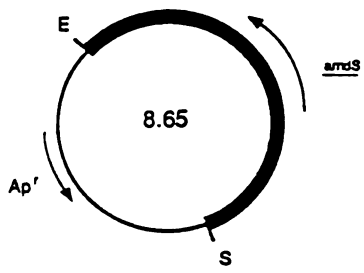
Fungus	Marker	Reference
Integrative		
Cochliobolus heterostrophus	amdS (p3SR2)	342
" "	hph (pHIS)	329
Magnaporthe grisea	argB (pMA2)	343
Fulvia fulva	hph (pAN7-1)	344
Glomerella cingulata	amdS (p3SR2)	345
" "	hph (pHIS)	345
Leptosphaeria maculans	hph (pAN7-1)	346
Gaeumannomyces graminis	ben (pBT3)	347
Septoria nodorum	hph (pAN7-1)	348
Achlya ambisexualis	G418 (pSV2neo-MKαG)	349
Colletotrichum trifolii	hph (pBHM-1)	350
" "	ben (pSV50)	350
Gliocladium roseum	hph (pHIS)	351
Gliocladium vireus	hph (pHIS)	351
Pseudocercosporella herpotrichoides	hph (pAN7-1)	352
" "	ben (pBT3)	352
Botryotinia squamosa	hph (pDH25)	353
Metarhizium anisopliae	ben (pSV50)	354
" "	hph (pBENA3)	355
Sordaria macrospora	ura5 (pLLC810)	356
Curvularia lunata	hph (pAN7-1)	357
Colletotrichum lindemuthianum	niaD (pAN301)	358
Beauveria bassiana	"	358
Aphanocladium album	"	358
Nectria haematococca	"	358
Pyricularia oryzae	"	358
Cryphonectria parasitica	hph (pHIB,pH1S, pUCH1, pH2S, pDH25, pAN7-1, pHL1, pPS29) ben (pSV50) G418 (pEMT2)	359
Absidia glauca	neo (pAmpN1, pAmpN5)	360
Extrachromosomal		
Absidia glauca	neo (pAmN61)	330
Scytalium flavo-brunneum	aza (pSFB-1)	359
Nectria haematococca	hph (pFOLT4R4)	323
Cryphonectria parasitica	"	323

[a] hph = hygromycin B; ben = benomyl; cyh = cycloheximide; neo = neomycin; amdS = acetamide utilization; aza = 15-azasterol; G418 = G418(geneticin); argB = ornithine transcarbamylase; niaD = nitrate reductase

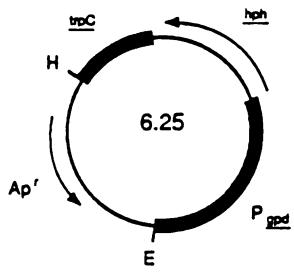
[b] H = gene replacement or linked insertion; N = unlinked insertion; S = single copy; M = multiple copies; ND = data not given

antibiotic selection markers, as an alternative to the use of auxotrophic markers. There are several vectors in particular which have found a broad use in the tranformation of many species of fungi. These are outlined in Table 6 and shown in Figure 3. The hygromycin gene of E. coli has been fused to promoter signals from A. nidulans(pAN7-1, 210; pDH25, 227), U. maydis(pHL1; 304), and Cochliobolus heterostrophus(pHIS; 342) and have been used extensively in the development of new fungal transformation systems(see Table 5 for complete list of references). Three N. crassa vectors containing the benomyl resistant β-tubulin gene(pBT3, 142; pBT6, 144; pSV50, 161) have been used to transform various fungi to benomyl resistance(Table 5). Similarly, two A. nidulans dominant markers(amdS and niaD) have been used widely. The amdS gene, in p3SR2(188) has been used to select for scetamide utilization in those fungi which do not normally grow on acetamide as a sole nitrogen source(Table 6). Vector pAN301(325), containing the nitrate reductase(niaD) gene of A. nidulans has been used to transform many fungi to chlorate resistance(Table 6). The most extreme case of a heterologous promoter function has been the kanamycin determinant of Tn903(326) which has been used without the addition of any eukaryotic signals to tranform a range of filamentous fungi and yeasts. This gene has previously been shown to function unaltered in the yeasts S. cerevisiae(327) and Kluyveromyces lactis(328) and in filamentous fungi(Table 6) such as N. crassa(143),

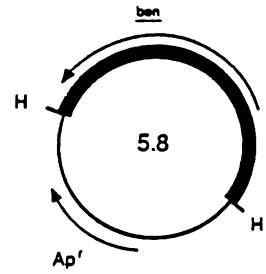
Figure 3. Common transformation vectors used across species lines. The diagram is adapted from the original references describing each vector. The thick lines in each vector represent fungal DNA while the thin lines represent E. coli vector DNA(pUC18,pBR322, etc.). The location of the selection markers for both bacterial and fungal transformation are given for each vector and the approximate location is noted as a line outside of the plasmid figure. Where known, the transcriptional orientation is also denoted with an arrowhead. The number within each circle indicates the size of each vector in kilobase pairs. For the genes representing bacterial coding regions fused to fungal transcription signals the promoter(P) and terminator(T) regions are given if present(see below for abbreviations). The restriction sites noted are only those relevant to the delineation of the major functional domains of each vector and the maps shown are not complete for these enzymes. Gene abbreviations: Ap^r, ampicillin; hph, hygromycin phosphotransferase; amdS, acetamidase; gpd, glucose-6-phosphate dehydrogenase; trpC tryptophan biosynthetic gene cluster; argB, ornithine transcarbamylase; niaD, nitrate reductase; lacZ, β-galactosidase; uidA, β-glucuronidase; ben, benomyl; p1, promoter 1 of Cochliobolus heterostrophus. Restriction site abbreviations: B, BamHI; C, Cla1; E, EcoRI; H, HindIII; N, Nde1; S, Sal1; X, Xho1.



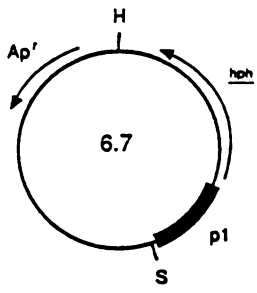
p3RS2



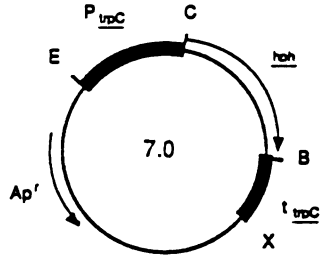
pAN7-1



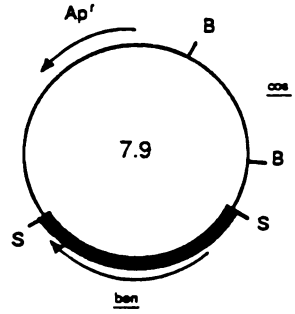
pBT3



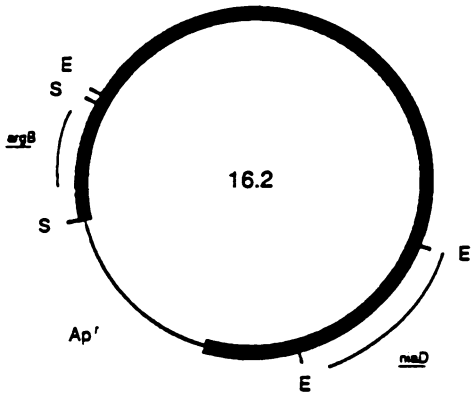
pH1S



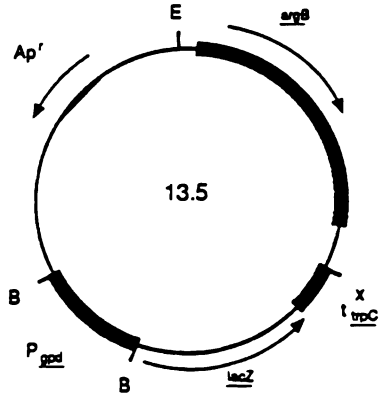
pDH25



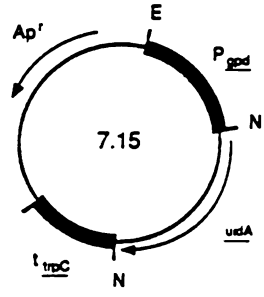
pSV50



pAN301



pAN5-41B



pNOM102

TABLE 6

Vectors and selection markers used across genus/species lines for selection of transformants

A) **Benomyl resistance using a benomyl resistant form of β-tubulin (*ben*^{r}) from Neurospora crassa**

Organism	Vector	Reference
Neurospora crassa	pBT3 (Fig. 3)	142
"	pSV50 (Fig. 3)	161
"	pBT6, pJD21, pJD71, pJD81, pJD82, pRM111 (modified forms of pBT3)	144
Aspergillus oryzae	pBT3	237
Gaeumannomyces grammis	pBT3	347
Podospora anserina	pBT6	256
Aspergillus niger	pBT6	233
Metarhizium anisopliae	pSV50	354
Colletotrichium trifolii	pSV50	349
Cryphonectria parasitica	pSV50	359
Pseudocercosporella herpotrichoides	pBT3	352

B) **Hygromycin resistance using the hygromycin phosphotransferase gene or E. coli (*hph*) fused to appropriate fungal promoters**

Organism	Vector	Reference
1. *Aspergillus nidulans*	pAN7-1 (Fig. 3)	210
Aspergillus ficuum	pAN7-1	242
Fulvia fulva	pAN7-1	344
Leptosphaeria maculans	pAN7-1	346
Septoria nodorum	pAN7-1	348
Curvularia lunata	pAN7-1	357
Cryphonectria parasitica	pAN7-1	359
Aspergillus giganteus	pAN7-1	243
Pseudocercosporella herpotrichoides	pAN7-1	352
Aspergillus nidulans	pDH25 (Fig. 3)	227
Fusarium oxysporum	pDH25	321
Botryotinia squamosa	pDH25	353
Cryphonectria parasitica	pDH25	359
3. *Ustilago maydis*	pHL1 (Fig. 3)	304
Ustilago hordei	pHL1	313
Ustilago nigra	pHL1	313
Cryphonectria parasitica	pHL1	359
4. *Cochliobolus heterostrophus*	pHIS (Fig. 3)	342
Colletotrichium trifolii	pHIS	350
Glomerella cingulata	pHIS	345

Organism	Vector	Reference
Gliocladium roseum	pHIS	351
Gliocladium virens	pHIS	351
Cryphonectria parasitica	pHIS	359
Ustilago violacea	pUCH1	314
Cryphonectria parasitica	pUCH1	359
5. *Fusarium solani* pCT43 (*F. solani* promoter)		318
Colletotrichium capsici	pCT45	318

C) Acetamidase utilization using the amdS gene of Aspergillus nidulans

Organism	Vector	Reference
Aspergillus nidulans	p3SR2 (Fig. 3)	188
Aspergillus niger	p3SR2	211
Aspergillus oryzae	p3SR2	181
Aspergillus ficuum	p3SR2	242
Cochliobolus heterostrophus	p3SR2	329
Trichoderma reesei	p3SR2	296
Penicillium chrysogenum	p3SR2	282
Penicillium chrysogenum	p3SR2	283
Glomerella cingulata	p3SR2	345

D) Nitrate reductase (niaD) gene allowing for chlorate resistance

Organism	Vector	Reference
1. *Aspergillus nidulans*	pAN301 (Fig. 3)	325
Fusarium oxysporum	pAN301	358
Beauveria bassiana	pAN301	358
Penicillium chrysogenum	pAN301	358
Aphanocladium album	pAN301	358
Nectria haematococca	pAN301	358
Pyricularia oryzae	pAN301	358
Penicillium chrysogenum	lambdaAN8a	280
Colletotrichium lindemuthianum	pAN301	358
2. *Aspergillus niger*	pSTA10 (pUC8 + *A. niger* niaD gene)	232
Penicillium chrysogenum	pSTA10, pSTA12	280

3. *Aspergillus oryzae*	pSTA14 (pUC18 + *A. oryzae* niaD gene)	237
Aspergillus nidulans	pSTA14	237
Aspergillus niger	pSTA14	237
Penicillium chrysogenum	pSTA14	237

E) **Orotidine-5'-monophosphate decarboxylase (pyr)**

Organism	Vector	Reference
1. *Neurospora crassa*	pFB6 (pBR322 + *N. crassa* pyr4)	130
Aspergillus nidulans	pFB6	186
Penicillium chrysogenum	pFB6,pDJB1,pDJB2	279
2. *Aspergillus niger*	pAB4-1 (pUC19 + *A. niger* pyrG)	231
Aspergillus niger	pGW613 (pUN121 + *A. niger* pyrG)	230
Aspergillus oryzae	pAB4-1	235
Aspergillus awamori	pANG1	239

F) **Ornithine carbamoyl transferase (argB of Aspergillus nidulans)**

Organism	Vector	Reference
Aspergillus nidulans	pSal43,pMA2,pMT201	183,214,215
	pA1,pAA1,pM159	219,336
Aspergillus niger	pDG3	229
Aspergillus oryzae	pILJ16	238
"	-	181
Aspergillus awamori	pUC4AP-arg	240
Trichoderma reesei	pSal43	296
Magnaporthe grisea	pMA2	343

G) **Isocitrate lyase (acu7 of Coprinus cinereus)**

Organism	Vector	Reference
Coprinus cinereus	pIONA-1 (pUC13 + *C. cinereus* acu7)	270
Aspergillus nidulans	pIONA-1	324

H) Indole-3-glycerol phosphate synthetase (trp1 of Schizophyllum commune)

Organism	Vector	Reference
Schizophyllum commune	pAM1 (pRK9 + *S. commune* trp1)	265
Coprinus cinereus	pSc1	272

I) G418 resistance (kan^r gene of Tn903)

Organism	Vector	Reference
Neurospora crassa	pBC10 pEB10	143
Phycomyces blakesleeanus	pJL2	260
" "	pPS11	261
	pPS11	262
Penicillium chrysogenum	pSP525,530,533	285
Phanerochaete chrysosporium	pRR12	301

Phycomyces blakesleeanus(260-262), Penicillium chrysogenum(286), and Phanerochaete chrysosporium(302). This particular kan^r determinant appears to be unique among prokaryotic resistance markers in that it functions unaltered in many lower eukaryotes. The sequence(s) functioning as a eukaryotic promoter in this gene has not yet been identified. In other instances, the hygromycin marker(318,329) and the kanamycin marker(259,316,330) have used to screen for fungal promoters which will drive their transcription. In this type of fusion, gene libraries of the appropriate fungus are made by inserting random fragments just upstream of a promoterless selection marker. The library is then used to transform the fungus and those transformants which arise are presumed to have promoter fragments upstream of the promoterless gene allowing for their transcription. The vector containing teh promoter funcion must then be recovered from the chromosome of an individual transformant. The promoter can then be used as a basis for the development of a vector for further transformation analyses.

Two types of gene fusions between fungal promoters and bacterial reporter genes have been constructed for the analysis of regulatory signals in fungi(Fig. 3, Table 7). The glyceraldehyde-3-phosphate dehydrogenase(gpd) promoter and the trpC termination signal of A. nidulans have been fused to the lacZ gene of E. coli and this construction(pAN5-41B; Fig. 3) has been used to detect β-galactosidase activity in

TABLE 7

Transformation vectors for expressing reporter genes from Escherichia coli

A) β-galactosidase (lacZ) gene fused to promoter and termination signals of Aspergillus nidulans[a]

Organism for expression	Vector	Reference
Aspergillus nidulans	pAN5-41B (Fig. 3)	202
Aspergillus nidulans	pAN923 series (derivatives of pAN5-41B)	203
Aspergillus oryzae	pAN5-41B	236
Aspergillus ficuum	pAN5-41B	243
Penicillium chrysogenum	pAN5-41B	281
Trichoderma reesei	pAN5-41B	296
Aspergillus nidulans	pCSC5-4 (amdS promoter)	206
A. niger	pCSC5-4	206

[a]promoter from glycerol-3-phosphate dehydrogenase gene except where indicated

B) β-glucuronidase (uidA) gene fused to gpd[a] promoter and trpC[b] terminator of A. nidulans

Organism for expression	Vector	Reference
A. nidulans	pNOM102 (Fig. 3)	331
A. *niger*	pNOM102	331
"	pNOM102	233
A. *oryzae*	pNOM102	236
"	pNOM102	237
Fulvia fulva	pNOM102	331

[a]glyceraldehyde-3-phosphate dehydrogenase
[b]phosphoribosylanthranilate somerase(PRAI); indoleglycerolphosphate synthase (IGPS); glutamine aminotransferase(GAT)

TABLE 8

Heterologous expression of genes of scientific, commercial and medical interest expressed in filamentous fungi

Organism	Gene	Vector(Promoter)	Reference
Aspergillus nidulans			
	nit-2 (*N. crassa*)	pNIT2 (endogenous)	192
	pla[c]	pNhT9 (endogenous)	212
	LTB[d]; *E. coli*	pL2 (amdS[e])	184
	endoglucanase[f]	pGL2CENDO (gla[g])	182
	"	pALCAISENDO (alcA[h])	182
	interferon α2 (human)	pGL2BIFN (gla[g])	182
	"	pALCAISIFN (alcA[h])	182
	tpa[i] (human)	pM159 (tpiA[j])	183
Other Aspergillus species			
	ACVS-IPNS-ACT[k]	pCX3.2 (endogenous)	363
	aspartic proteinase[l]	pBoel-777 (α-amylase)	181
	bovine chymosin	pGAMpR (gla[g])	362
Neurospora crassa			
	ACVS-IPNS-ACT[k]	pCX3.2 (endogenous)	185
Trichoderma reesei			
	bovine chymosin	pAMH101-3 (cbh 1[m])	298
		pAMH106 (cbh 1[m])	
	lignin peroxidase[n]	pMS16	94
Cephalosporium acremonium			
	cefEF[o] (*C. acremonium*)	pPS56 (endogenous)	290

[c] pisatin demethylating activity of *Nectria haematococca*
[d] enterotoxin subunit B
[e] acetamidase
[f] from Cellulomonas fimi
[g] glucoamylase
[h] alcohol dehydrogenase I
[i] tissue plasminogen activator
[j] triosephosphate isomerase
[k] penicillin biosynthetic cluster; δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS); isopenicillin N synthetase (IPNS); acyl coenzyme A:6-aminopenicillanic acid acyltransferase (ACT)
[l] from *Rhizomucor miehei*
[m] cellobiohydrolase I
[n] from *Phlebia radiata*
[o] deacetoxycephalosporin C synthetase(DAOCS); deaceytylcephalosporin C synthase(DACS)

a variety of fungi(Table 7). Similarly, the β-glucuronidase(uidA) gene of E. coli has been fused to the gpd promoter of A. nidulans(pNOM102; Fig. 3) has been and used to detect β-glucuronidase enzyme activity in various fungi(Table 7). These are, of course, a prelude to the analysis of endogenous promoter elements from these fungi(331). The lacZ fusion system has already been used to anaylze the regulatory regions of A. nidulans genes(203-205). A series of vectors for this type of promoter study have also been constructed(203,204) which allow translational fusions in all three reading frames to the lacZ gene. The vectors(Fig. 3) also contained the argB gene of A. nidulans for selection.

There is a widespread interest in the use of fungal systems for the expression of heterologous proteins, as an alternative to S. cerevisiae(Table 8). The reasons for this are several. Fungi often recognize intron splice sites of genes from heterologous organisms(188) and therefore whole genes can be used for the expression of a foreign protein. High level expression with some fungi is obtainable due to a long history of selection for strains which optimize secretion of proteins(278). With the expansion of the analysis of fungal genes, many strong promoters from filamentous fungal genes have been characterized and some of these(gpd and amdS of A. nidulans) can be used in the construction of the necessary gene fusions for the expression of heterologous proteins.

9. Conclusions

Rapid advances have been made in the last ten years in developing transformation systems for many yeasts and fungi of importance in medicine, agriculture, food and fermentation, and basic scientific research. Fundamental differences between yeast and fungal transformation systems are apparent in terms of the fate of the transforming vectors introduced into the organisms. In yeast there is a clear distinction between vectors that replicate autonomously and those which integrate into the chromosome. The type of transformation event also dictates the stability and recoverability of the vector. In *S. cerevisiae* *ars* elements have been identified which, if not origins of replication *in situ*, at least mimic their action and allow vectors to be maintained autonomously Autonomous vectors are generally quite unstable, transform at high frequencies, and can be easily recovered by *E. coli* transformation. With integrative transformation, the selection vector is stably integrated(generally at homologous sites) into the chromosome and conforms to classical Mendelian inheritance patterns. These integration vectors are unrecoverable, except by artificial manipulations.

Attempts to translate the principles which govern yeast transformation vectors to the development of vectors for filamentous fungi have given mixed results. There are two primary differences between yeast and filamentous fungal

systems. The first is that extrachromosomal replicative vectors, similar to those described for yeasts(such as the 2μ or *ars*-based vectors), have been difficult to construct in fungi. Even in fungi with endogenous plasmids, it is not always possible to construct reliable extrachromosomal shuttle vectors, although isolated cases of extrachromosomal maintenance was observed. The very presence of the endogenous plasmids in filamentous fungi suggests that construction and stable maintenance in fungi of such vectors should be possible in fungi, however, investigations to this end have not been very extensive or successful to date. This may be due in part to the mitochondrial origin of most plasmids of filamentous fungi; they may not be stable in a nuclear environment.

A second major difference between transformation systems of yeasts and filamentous fungi is that the integrative transformation in the latter is often by non-homologous recombination whereas in yeast homologous recombination is the most prevalent event and heterologous recombination is relatively rare. In fungi, homologous recombination events are infrequently observed even when vectors containing a homologous selection marker are employed. The types of integration events are also marker and strain dependent in many cases. This suggests that the transforming mechanisms of fungi are closer to that seen in higher eukaryotes, in which viral vectors are often seen to integrate by unlinked insertion into the chromosome(332). In some cases, such as with SV40, this may not be

completely random since small homologies(4-5 bp) between the vector and the chromosome have been seen at integration sites(333,334). Such a detailed analysis of integration sites in fungi has not been done. The phenomenon of head to tail tandem integration of vectors in fungi is also quite similar to the mammalian systems studied(335).

The development of the molecular genetics of filamentous fungi, including the development of transformation systems for many of the most well characterized fungi has been quite rapid over the past several years. This has already been exploited in the analysis of several very interesting systems in A. nidulans(179,180). If one looks at A. nidulans as a prototype of the potential for the molecular analysis of filamentous fungi using transformation systems, one can find that the development of the types of manipulations possible parallels that of S. cerevisiae in many cases. The developments in N. crassa and U. maydis are equally impressive and offer hope that such in depth molecular genetic analysis may be possible in other systems. Construction of useful recombinant strains by creating specific gene disruptions and gene fusions, and using such strains for the detailed analysis of gene function and regulation is possible. The use of A. nidulans and A. niger as hosts for cloning and expressing foreign genes of interest has been limited to date. However, considering the close similarity between the gene structure of filamentous fungi and that of higher eukaryotic systems, this is likely

to become more common in the future. Based on recent investigations, filamentous fungi appear to be ideal hosts for the expression and production of medically and industrially important heterologous proteins.

Transformation in filamentous fungi has presented some interesting problems, such as the difficulty in directing or controlling chromosomal integration events, the difficulty in construction of true autonomous vectors discussed above, and the RIP phenomenon in N. crassa(177) similar related phenomena in other fungi(254,276a). These studies, none the less, have led to a better understanding of the genetics of filamentous fungi and the problems encountered do not appear to be insurmountable. In fact, some very interesting and useful properties of fungal integrative transformations have been exploited. The commonly encountered multi-copy integration of vectors in chromosomes of transformants can in part overcome the inability to develop multi-copy autonomous transformation vectors and offer some of the same advantages in increasing expression a desired gene. Such integration systems also offers the advantage of higher mitotic stability due to chromosomal integration. The RIP phenomenon can even be useful in that it may be possible to use this type of event to construct specific mutants of any cloned sequence as an alternative to gene disruptions. Further analysis of fungal transformation systems will undoubtedly uncover more scientifically interesting and biotechnologically useful properties which can be exploited in the molecular genetic

analysis of individual fungal systems.

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CHAPTER 1

A Novel Extrachromosomally Maintained Transformation Vector for the Lignin Degrading Basidiomycete *Phanerochaete chrysosporium*

in review, Molecular and Cellular Biology

ABSTRACT

A stable extrachromosomally maintained transformation vector (pG12-1) for the lignin-degrading filamentous fungus Phanerochaete chrysosporium is described. The vector is 6.3 kb and contains a kan^r marker, pBR322 ori, and a 2.2 kb fragment (ME-1) derived from an endogenous extrachromosomal DNA element of P. chrysosporium. Vector pG12-1 was able to transform P. chrysosporium to G418 resistance and was readily and consistently recoverable from the total DNA of transformants via Escherichia coli transformation. Southern blot analyses indicated that pG12-1 is maintained at a low copy number in the fungal transformants. The vector is demonstrable in the total DNA of individual G418-resistant basidiospore progeny of the transformants only after amplification by polymerase chain reaction. Dam methylation and exonuclease III analyses, respectively, indicated that pG12-1 undergoes replication in P. chrysosporium and that it is maintained extrachromosomally in a circular form. The vector is stably maintained in the transformants even after long term non-selective growth. There is no evidence for integration of the vector into the chromosome at any stage.

INTRODUCTION

In recent years, research has intensified worldwide on the ligninolytic fungus Phanerochaete chrysosporium because of the industrial potential of this organism in biopulping, in the conversion of lignin and lignocellulosic materials to feeds, fuels, and chemicals(22,47), and in detoxifying recalcitrant environmental pollutants such as dioxins, PCBs, and benzo(a)pyrenes(10,11). Lignin peroxidases and manganese peroxidases, two families of extracellular, glycosylated, heme-proteins involved in lignin degradation by P. chrysosporium, have been isolated and characterized(16,22, 47,48). Genetic studies with this organism have been limited, but auxotrophic mutants(15,26,27) and mutants lacking various secondary metabolic activities have been isolated(7,21,24). Both cDNA(13,49,53,54) and genomic clones(3,5,8,20,41,42,52, Y.Z. Zhang, and C.A. Reddy, 1988. Abstr.Annu.Mtg.Am.Soc. Microbiol. H51) for lignin peroxidases and cDNA clones for manganese peroxidases(31,34) have been isolated and sequenced. We previously described vector pRR12 which was shown to transform P. chrysosporium to G418 resistance and was rescued in E. coli from the total DNA of pRR12 transformants(35). Alic et al.(1,2) have described an integrative transformation system in which a plasmid vector carrying either an heterologous ADE2 or ADE5 gene from Schizophyllum commune was used to complement the appropriate adenine auxotroph of P.

chrysosporium . We describe here a novel tranformation vector for P. chrysosporium that is maintained stably in the transformants in an extrachromosomal circular form and is readily recoverable from the total DNA of transformants.

MATERIALS AND METHODS

Strains and Media. P. chrysosporium strain ME446(ATCC 34541) was maintained as previously described(21). E. coli DH5α (F^- endA1 hsdR17[r_k^- m_k^+] supE44 thi-1 recA1 gyrA96 relA1[argF$^-$ lacZYA]U169 80μlacZ-15 lambda$^-$), was used for the maintenance of plasmid vectors. Gold's medium B(14) supplemented with 10% sorbose and 3% agar (Difco, Detroit, MI) was used for the regeneration of P. chrysosporium spheroplasts. Golds' medium B, without sorbose or deoxycholate and supplemented with 100 μg per ml G418 (Gibco, Grand Island, NY) was used for the mitotic stability assay .

DNA Isolations and Manipulations. Plasmid DNA was isolated from E. coli by the method of Birnboim and Doly(6). Molecular biological techniques were performed according to Maniatis et al.(25), except where indicated. Restriction enzymes were used per manufacturers' specifications. DNA probes were labeled according to specifications in the Random Primer labeling kit (BMB Biochemicals, Indianapolis, IN). P. chrysosporium DNA isolation and the high stringency Southern hybridization conditions have been outlined previously(36).

Transformations. Transformation of P. chrysosporium was carried out according to previously described methods(35). E. coli transformation was done by the method of Hanahan(18).

Polymerase Chain Reaction(PCR) analysis. Amplification by PCR was carried out as follows: 2 μg of AvaII digested genomic DNA was resuspended in 10 μl of TE(10 mM Tris pH 7.4, 1 mM EDTA). Individual reactions were performed using a GeneAmp DNA Amplification Kit (Perkin Elmer Cetus, Norwalk, Ct.) using Taq polymerase. 20 mer primers (5'-ATGTGGTGATTTTGAACTTT-3' and 5'-GTTGGTGATTTTGAACTTTT-3' from Research Genetics, Inc., Huntsville, Ala.) corresponding to the terminal ends of the kan^r determinant as defined by the AvaII sites at positions 945 and 2146 of the published DNA sequence(29) were used to prime the synthesis of a 1201 bp DNA fragment at 100 pmol per reaction. To determine the optimum amplification conditions, a Mg^{++} titration was carried out and a final concentration of 2.1 mM $MgCl_2$ was found to be optimal. Individual reactions were carried out using a Perkin Elmer Cetus DNA Thermal Cycler (source as given above). After an initial denaturation by boiling for 5 min, 30 amplification cycles were done as follows: 1 min at 94°C (denaturation); 2 min at 55°C (annealing); and 2 min at 72°C (extension). Individual samples were then phenol/ chloroform-isoamyl alcohol extracted, ethanol precipitated and resuspended in 20 μl TE for agarose gel electrophoresis and Southern blotting(25). A 520 bp HindIII-Xho1 DNA fragment of the kan^r determinant internal to the amplified region was used as the hybridization probe.

Exonuclease III Analysis. Total DNA (5 μg) from fungal transformants was incubated with 4 U of Exonuclease III according to the manufacturers' specifications (Bethesda Research Laboratories, Bethesda, Md.) at 37°C for 2 hrs. The DNA was then phenol-chloroform-isoamyl alcohol extracted, ethanol precipitated, and incubated in 20 μl of S1 buffer with 10 U of S1 nuclease at 37°C for 2 hrs. This DNA along with an equal aliquot of untreated transformant DNA was electrophoresed on a 0.7% agarose gel and stained with ethidium bromide. A linear DNA sample (lambda HindIII DNA marker, BRL, Gaithersburg, Md.) and a circular plasmid (pUC18) served, respectively, as positive and negative controls for the Exonuclease III and S1 nuclease treatments.

Isolation of basidiospores. A mycelial plug from individual pG12-1 transformants was placed across from a similar plug from P. chrysosporium ME446 on an agar plate containing Kirks low N medium supplemented with 2% agar. Growth and anastomosis of hyphae was allowed to occur, and after 7-10 days basidiospores were ejected to the lid of the inverted Petri dish. Basidiospores were collected in H_2O, diluted on Golds medium B and transferred to the same medium containing 100 μg/ml G418 for selection of G418 resistant progeny which were used for further analyses.

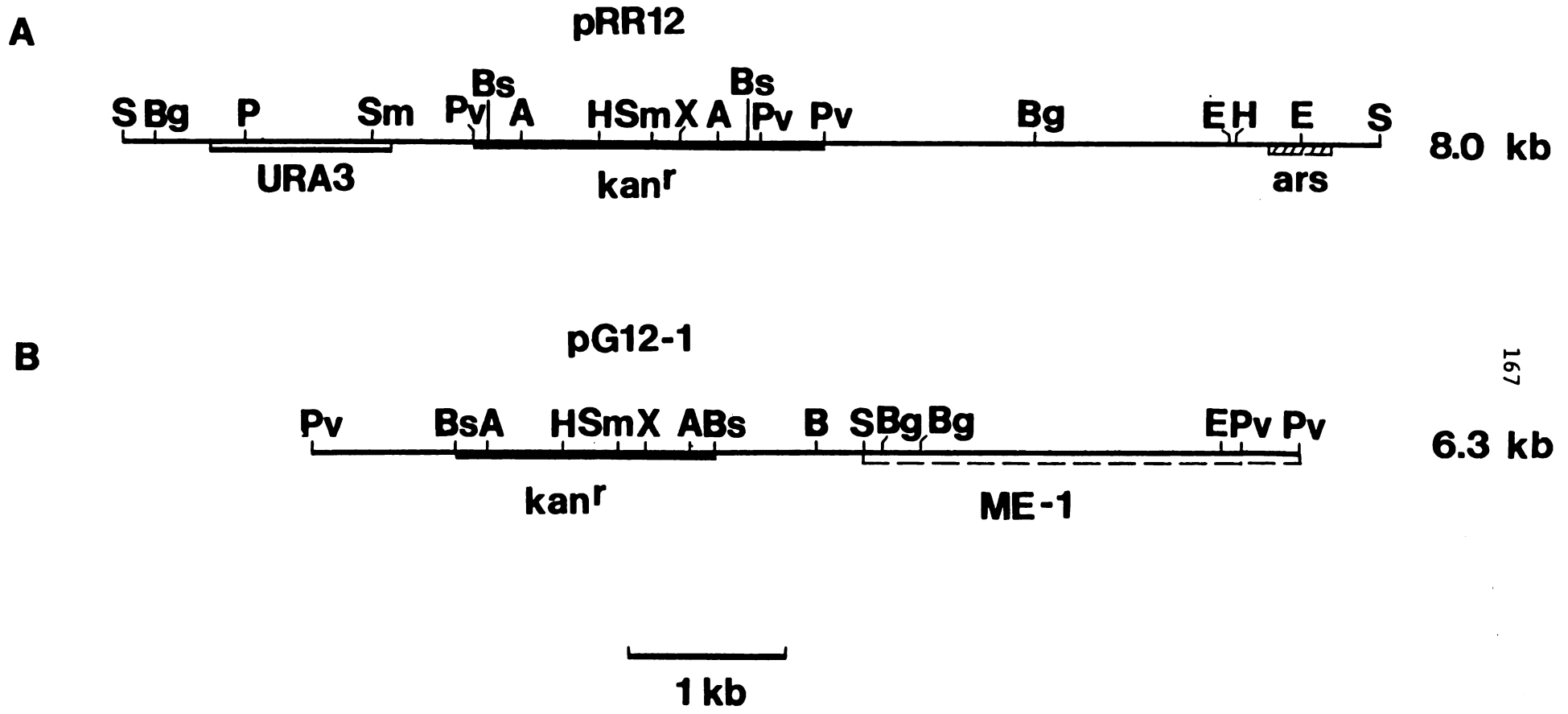
Stability analyses. To determine the stability of vector pG12-1 during vegetative growth without G418-selection, fungal transformants were subcultured on malt extract medium (2% malt extract. 2% glucose, 0.1% peptone, pH 4.5) without G418. For

each subculture, a small mycelial plug was transferred to a malt extract slant and was incubated at 37°C for 2-3 days when a dense, white growth of new mycelium covered the slant. A mycelial plug from the latter was used to inoculate a new slant and the process was repeated for 10 such transfers. After 1, 5, and 10 such non-selective transfers, a mycelial plug was transferred to malt extract slants containing 200 μg/ml G418 to determine if the transformants were still resistant to G418. Total DNA from each of the three above mentioned non-selectively maintained cultures, grown in malt extract broth was isolated and was subjected to PCR analysis as described above to detect the presence of vector sequences. This DNA was also used for recovery of pG12-1 by *E. coli* transformation as further confirmations for the presence of pG12-1 in transformants maintained under non-selective conditions.

RESULTS

Isolation and characterization of plasmid pG12-1. Vector pRR12 (Fig. 1A), previously used for transformation of *P. chrysosporium* (35; Appendix 1), consists of the plasmid YIp5(46) into which the *kan*^{r} determinant of Tn903(17) and a genomic fragment of *P. chrysosporium* having *ars* activity in *Saccharomyces cerevisiae*(36) were inserted. In the course of characterizing pRR12 transformants, a derivative of pRR12, designated pG12-

Figure 1. A schematic illustration of plasmid vectors pRR12(A) and pG12-1(B). The vectors are presented in a linear form for ease of comparison. Symbols: thin line, pBR322 sequences, including origin of replication (ori), and ampicillin resistance (amp^r) marker; thick line, kan^r determinant; cross hatched box, ars of P. chrysosporium(36); double line, URA3 sequences of S. cerevisiae; the dashed box (designated ME-1), the region of DNA that hybridizes to extrachromosomal DNA of P. chrysosporium ME-446(see text, and Fig. 2). Restriction site abbreviations: A; AvaII, B; BamH1, Bg; Bgl1, Bs; BssHII; E; EcoR1, H; HindIII, P; Pst1, Pv; PvuII, S; Sal1; Sm; Sma1; X; Xho1.


A
pRR12
S Bg P Sm Pv Bs A HSmX A Bs Pv Pv Bg E H E S
8.0 kb
URA3
kan^r
ars
B
pG12-1
Pv BsA HSmX ABs B SBg Bg EPv Pv
6.3 kb
kan^r
ME-1
1 kb

1 (Fig. 1B), was recovered by E. coli transformation from the total DNA of one of the transformants. A comparison of the restriction map of pG12-1 to that of pRR12 (Fig. 1) and Southern blot analyses showed that pG12-1 is an extensively rearranged version of pRR12. Vector pG12-1 contains homology to all the major domains of pRR12 except that the URA3 gene and the ars of P. chrysosporium appear to have been deleted. The intact coding region of the kan^r gene (internal to the BssHII fragment) was present. Furthermore, pG12-1 contained a new DNA fragment designated ME-1 (Fig. 1B) that is not originally a part of pRR12 and appears to have been acquired from an endogenous extrachromosomal element of P. chrysosporium (see below). To determine the origin of ME-1, a Southern blot of total DNA of P. chrysosporium was probed with ^{32}P-labeled ME-1(Bgl 1-Pvu II fragment of pG12-1 as shown in Fig. 2A (lane a). To determine the position of the chromosomal DNA, the same blot was stripped of ME-1 and reprobed with lignin peroxidase cDNA CLG5 (Fig. 2A, lane b; 53), a chromosome specific probe, to show the relative migration of the chromosomal DNA. The results show that ME-1 hybridizes to extrachromosomal bands of DNA presumably representing open circular and covalently closed circular forms of a circular DNA element. To purify this endogenous plasmid with ME-1 homology, hereafter designated pME, we ran large samples of P. chrysosporium genomic DNA ($\sim$ 1 mg) in a two step CsCl-ethidium bromide gradient(25). We were not able to see a plasmid band

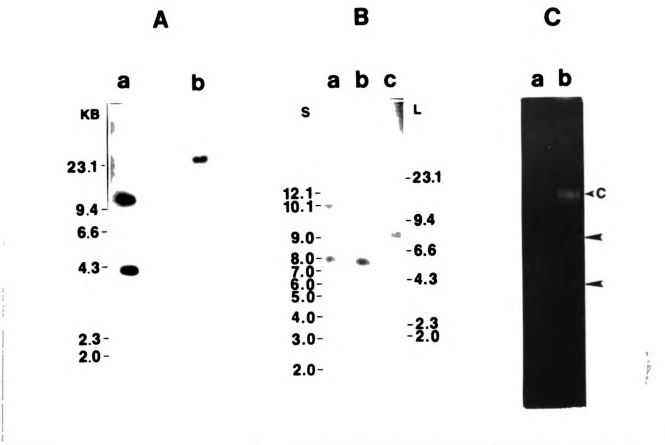


Figure 2. Southern hybridization of P. chrysosporium total genomic DNA to the ME-1 fragment of pG12-1. In Fig. 2A, total DNA was electrophoresed through a 0.7% agarose gel and transferred to a nitrocellulose filter. Lane a shows the hybridization pattern when probed with a ^{32}P-labeled Bgl1-PvuII fragment of pG12-1 containing ME-1 (see Fig. 1). The ME-1 probe was then stripped off and this filter was re-probed with a chromosome-specific lignin peroxidase cDNA (CLG5;53) and this is shown in lane b. Fig. 2B, lane a contains an exonuclease III digest of pME; lane b contains a lambda exonuclease digest of pME, and lane c contains an EcoR1 digest of pME. This Southern blot was probed with a ^{32}P-labeled Hind III digest of pME. The "S" on the left refers to supercoiled plasmid DNA marker and the "L" refers to the linear DNA marker. The sizes of the markers are in kilobases. Fig. 2C is a photograph of a 0.7% agarose gel after staining with ethidium bromide. Lane a contains plasmid DNA isolated from a CsCl-EtBr gradient containing P. chrysosporium ME446 genomic DNA and lane b contains plasmid DNA isolated from a CsCl-EtBr gradient containing P. chrysosporium der 8-5 genomic DNA. The arrows indicate the migration of the plasmid bands.

below the main chromosomal band, but were able to isolate a circular plasmid from a fraction below the main chromosomal band (Fig. 2B and C). The plasmid isolated (Fig. 2B) was digested with either exonuclease III or lambda-exonuclease, Southern blotted, and probed with total pME. The results showed that neither exonuclease III nor lambda exonuclease affected the migration of the plasmid DNA, indicating that it was circular (Fig. 2B, lanes a and b). The EcoR1 digest of the plasmid (Fig. 2B, lane c) indicated that this circular element was resolved to a linear form of 8.5 kb. This extrachromosomal circular DNA element, which is the source of ME-1 sequences, is present at a relatively low copy number since it is not visible on ethidium bromide stained agarose gels. Furthermore, from 500 μg - 1 mg of P. chrysosporium genomic DNA, we can purify only about 0.5 - 1.0 μg of pME. Only after purification of such a quantity of DNA through a CsCl-EtBr gradient can enough plasmid be isolated to observe on an agarose gel (Fig. 2C). A further characterization of pME, including a basic restriction map, cloning, and transcript analysis is underway.

PCR analysis of pG12-1 transformant DNA. To demonstrate the presence of transforming DNA, total DNA from individual G418 resistant meiotic progeny (basidiospores) of pG12-1 transformants of P chrysosporium was analyzed. When this DNA was probed with the labeled kan^r marker (Fig.3) no band(s) of hybridization were detected (Fig. 3, lanes b,d,f, and h) suggesting that the

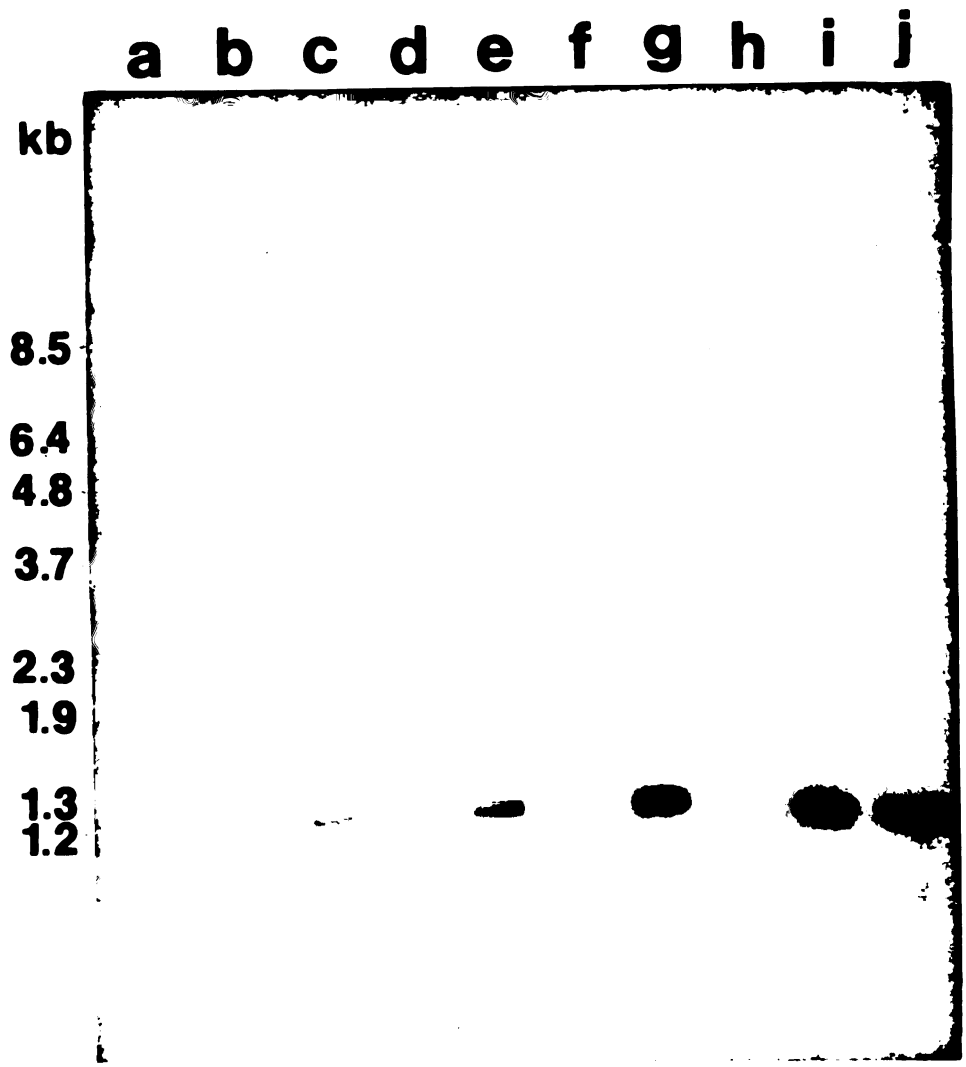


Figure 3. Southern blot analysis of polymerase chain reaction (PCR)-amplified and unamplified DNA from G418 resistant basidiospore progeny of pG12-1 transformants. Lane a contained AvaII digested, genomic DNA from wild type P. chrysosporium ME446 amplified by PCR. Lanes b,d,f, and h contained unamplified AvaII-digested DNA while lanes c,e,g, and i contained PCR amplified DNA. Lane j contained 50 ng of the 1.2 kb AvaII fragment of the kan^r determinant (positive control). The blot was probed with the ^{32}P-labeled 0.5 kb Hind III-Xho 1 DNA fragment internal to the amplified AvaII kan^r determinant.

vector sequences are maintained at a low concentration, below the normal detectability limit (usually 0.1 pg of linear or circular DNA) of the Southern hybridization procedure.

Amplification by polymerase chain reaction (PCR; 40) is a sensitive procedure for visualizing DNA present at a very low concentration. For this analysis, we used total DNA from G418 resistant basidiospore progeny of pG12-1 transformants and amplified the *kan*^{r} sequences by using the appropriate primers (see Materials and Methods). Positive hybridization of the *kan*^{r} probe to this amplified DNA from the G418 resistant progeny of pG12-1 transformants (Fig. 3, lanes c, e, g, and i), but not to amplified DNA from the wild type *P. chrysosporium* strain (lane a) or to that from unamplified total DNA from the same G418 resistant basidiospore progeny (lanes b, d, f and h). These results indicate that the vector is indeed present in a low copy number in the fungal transformants and that amplifying the vector sequences using the PCR procedure is necessary for detecting the vector in *P. chrysosporium* transformants by Southern analysis. The above data also indicate that recovery of pG12-1 from the total DNA of fungal transformants does not result from excision of pG12-1 from the chromosome. If excision from the chromosome were the case, hybridization of the *kan*^{r} probe to the unamplified genomic DNA of the basidiospore progeny of the G418 resistant transformants would have been observed.

Similar amplification by PCR was needed to detect pG12-1 in conidial progeny also (data not shown). Furthermore, pG12-1

could be rescued by E. coli transformation from the genomic DNA of G418 resistant basidiospore or conidial progeny while no vector was rescued from the total DNA of G418 sensitive conidial progeny, indicating a positive correlation between the presence of pG12-1 and G418 resistance. When conidia were isolated from the G418-resistant meiotic progeny tested in Fig. 3, 14 to 27% of these conidia were G418-resistant. These data showing a low transmission of the G418-resistance marker through mitosis also indicate an extrachromosomal maintenance of pG12-1.

Low copy maintenance of the pG12-1 is further supported by the fact that pG12-1 can be recovered from the DNA of fungal transformants by E. coli transformation only when a high transformation frequency (>1x10^8/μg DNA) is obtained. We routinely obtain transformation frequencies of 4-9 x 10^8 transformants per ug of pUC18 plasmid DNA. Ideally, at these transformation frequencies, one should be able to rescue in E. coli as little as 1-10 fg of transforming DNA. Such high sensitivity, which is generally beyond the detectability range of Southern hybridization analysis, appears to be necessary to detect a transformation vector that is maintained at a low copy number.

Replication and exonuclease III assays of pG12-1 transformants. A dam methylation assay(19,23) was used to confirm that pG12-1 undergoes replication in P. chrysosporium transformants. The restriction enzyme Mbo 1 cuts at the sequence GATC when the adenine residue in this sequence is

unmethylated at the N^6 position while Dpn 1 cuts at this site only when the adenine is methylated. Plasmids which pass through *dam*$^+$ *E. coli* strains acquire an N^6 methylated adenine at GATC sites due to *dam* methylation(12) and are resistant to digestion by Mbo 1. *P. chrysosporium* has no comparable *dam* methylation activity and its DNA has previously been shown to be susceptible to Mbo 1 digestion(34) and resistant to Dpn 1 digestion. Hence, pG12-1 DNA recovered from *P. chrysosporium* should be unmethylated and completely digestible by Mbo 1 if it replicates in the fungus. Our results showed this to be the case since pG12-1 could not be recovered by *E. coli* transformation (Table 1) if the total DNA from primary pG12-1 transformants or their G418 resistant basidiospore progeny was digested with Mbo 1 whereas pG12-1 could be rescued from Dpn 1-digested DNA of the same fungal transformants. These results indicate that pG12-1 undergoes replication in *P. chrysosporium*.

An exonuclease assay was used to confirm that pG12-1 is maintained as an extrachromosomal circular DNA. Exonuclease III, which digests linear DNA but not circular DNA, completely degraded *P. chrysosporium* chromosomal DNA (Fig. 4). An equal aliquot of the undigested and Exo III digested transformant DNA samples used in Fig. 4 were employed to transform *E. coli* DH5α to kan^r and individual colonies arising on the plates were counted; miniprep DNA was then prepared from selected kanamycin resistant colonies of *E. coli* to confirm the presence of pG12-1 (Table 2). The recovery of pG12-1 in *E.*

TABLE 1. Transformation of *E. coli* with *Dpn*1 and *Mbo*1 digested DNA from G418-resistant pG12-1 transformants of *P. chrysosporium*[a]

No.	Treatment	DNA(μg)	Total No. of Transformants	Transformation Frequency(#/ug)
1	*Dpn*1	2.0	63	31.5
	*Mbo*1	2.0	0	0
2	*Dpn*1	2.0	51	25.5
	*Mbo*1	2.0	0	0
3	*Dpn*1	2.0	112	56
	*Mbo*1	2.0	0	0

[a]Aliquots of *Dpn* 1 and *Mbo* 1 digested DNA from four individual G418 resistant pG12-1 transformants of *P. chrysosporium* was used to transform *E. coli* to kanamycin resistance. 1 and 2 were DNA from primary pG12-1 transformants, while 3 was from a G418 resistant basidiospore purified progeny of 1. The total number of transformants obtained as well as the no. of transformants per μg DNA are indicated. The *E. coli* transformation frequency in this experiment was 2.7×10^8 transformants per μg of pUC19 (positive control) DNA.

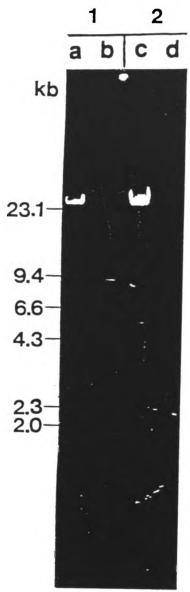


Figure 4. Exonuclease-S1 nuclease digestion of total DNA from two individual P. chrysosporium transformants. DNA(2.5 μg) from two G418 resistant pG12-1 transformants(1 and 2 of Table 1) was digested with exonuclease III and S1 nuclease as described in Materials and Methods. Lanes a and c contained undigested DNA while lanes b and d contained DNA digested with exonuclease III and S1.

TABLE 2. Transformation of *E. coli* with exonuclease III-S1 nuclease treated genomic DNA of G418-resistant pG12-1 transformants of *P. chrysosporium*[a].

	Number of *E. coli* transformants recovered	
Transformant	undigested DNA	ExoIII-S1 digested DNA
1	41	60
2	25	34

[a] 2.5 μg of DNA from two individual G418 resistant pG12-1 transformants of *P. chrysosporium*, either undigested or digested with exonuclease III and S1 nuclease, was used for transforming *E. coli* DH5α to kanamycin resistance. The total number of *E. coli* transformants obtained with 2.5 μg of transformant DNA are presented.

coli transformed with exonuclease III-digested total DNA of individual pG12-1 transformants of P. chrysosporium was comparable to that obtained with undigested total DNA from the fungal transformants indicating that pG12-1 is maintained in P. chrysosporium transformants in a circular, extrachromosomal form.

Maintenance of pG12-1 in transformants through non-selective growth. One means of examining the mitotic stability of the transforming vector is to monitor its maintenance through vegetative growth in non-selective media. For those fungal systems for which extrachromosomally maintained vectors have been described, such a vector is generally lost quickly during non-selective growth(50,51). In the case of single celled fungi, such as S. cerevisiae this is easily measured by determining the doubling time of the organism and examining the stability as a function of the number of generations of growth(28). This approach is impractical for filamentous fungi and stability can be expressed as a function of the number of transfers to new slants of solid media, although each transfer actually encompases many nuclear divisions. We transferred several of our pG12-1 transformants through ten transfers and examined whether or not pG12-1 could be demonstrated in the vegetative mycelium after 1, 5, and 10 such non-selective transfers.

The results showed the presence of pG12-1 in the transformants after 1,5, and 10 subcultures in the non-

selective medium based on three lines of evidence: growth when transferred to selective slants (G418-supplemented malt extract slants); rescue of pG12-1 from the total DNA of the non-selective subcultures by *E. coli* transformation; and demonstration of vector sequences by PCR analysis of the total DNA samples from the non-selectively grown subcultures (Table 3). A representative PCR analysis (Fig. 5) confirmed the presence of pG12-1 after 10 subcultures in non-selective medium. The results show that the *kan*^{r} marker, as defined by the 1.2 kb *Ava*II restriction fragment, was present in the amplified DNA of these cultures (lane d), and was identical to the *kan*^{r} containing *Ava*II fragment of the same pG12-1 (lane e). The *kan*^{r} marker was not detected in unamplified DNA from the 10th non-selective transfer of this pG12-1 transformant (lane c) nor in unamplified or amplified DNA from untransformed *P. chrysosporium* (lanes a and b, respectively). These data indicate that pG12-1 is maintained at a low copy level in transformants after long term non-selective growth.

TABLE 3. Stability of pG12-1 through vegetative growth under non-selective conditions.

Subcultures [a]	Recovery in [b] *E. coli*	$G418^r$ [c]	PCR detection of pG12-1 [d]
1	79	+	+
5	62	+	n.d.
10	23	+	+

[a] A G418-resistant pG12-1 transformant of *P. chrysosporium* was subcultured non-selectively as described in Materials and Methods.

[b] DNA isolated from the transformant after 1,5, and 10 transfers on non-selective medium was used to transform *E. coli* and kan^r colonies were counted. The *E. coli* transformation frequency in this experiment was 2.2×10^8 transformants per μg of pUC19 (positive control) DNA.

[c] A fragment of mycelium was inoculated onto a malt extract(ME) slant containing G418 and growth or no growth was indicated as "+" or "-" respectively. Untransformed *P. chrysosporium* did not grow in these ME+G418 slants.

[d] PCR analysis was carried out as described in Materials and Methods. n.d. = not determined.

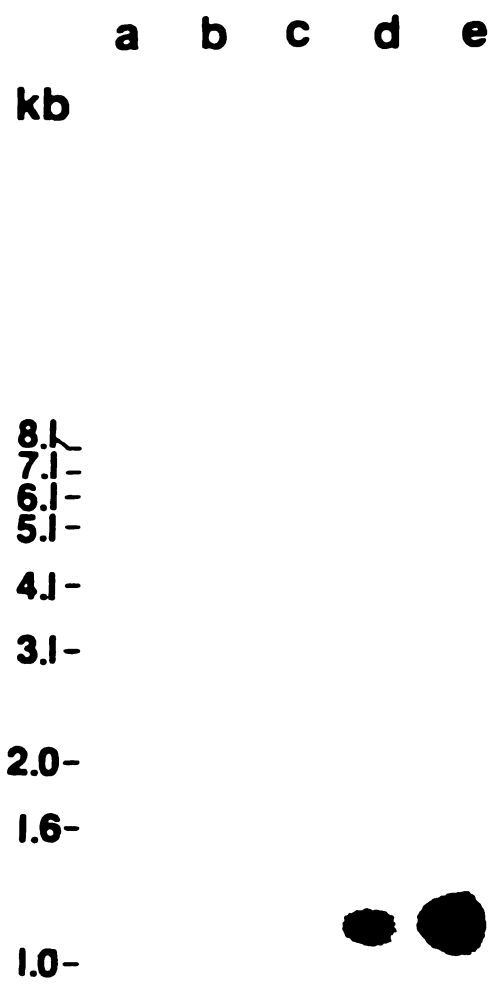


Figure 5. Southern blot analysis of PCR amplified DNA from G418-resistant pG12-1 transformants of P. chrysosporium after 10 subcultures on non-selective medium. DNA samples were electrophoresed in a 0.7% agarose gel and transferred to nitrocellulose. Lanes a and b contained total DNA from untransformed P. chrysosporium ME446 digested with AvaII. Lanes c and d contained total DNA from a non-selectively grown G418-resistant pG12-1 transformant digested with AvaII. The DNA in lanes a and c was unamplified while that in lanes b and d was subjected to the PCR amplification protocol described in Materials and Methods. Lane e contains gel purified kan^r fragment. The blot was probed with a ^{32}P-labeled HindIII-XhoI kan^r gene fragment.

DISCUSSION

The results of this study show that pG12-1 is stably maintained in a low copy, extrachromosomal form in P. chrysosporium transformants even under non-selective conditions and that the amplification by PCR is necessary to visualize the vector sequences in the total DNA of the transformants. The vector can be rescued from the DNA of individual fungal tranformants by E. coli transformation. Treatment of the total DNA of fungal transformants with exonuclease III-S1 nuclease resulted in complete digestion of the chromosomal DNA whereas intact pG12-1 was rescued from such treated DNA by E. coli transformation, indicating that the vector is maintained in a circular form in the transformants (Table 2). Sensitivity of the transforming DNA to Mbo 1 digestion but not to Dpn 1 digestion indicates that the vector pG12-1 replicates in the fungus (Table 1). PCR analysis of the DNA from the basidiospore progeny of the individual G418-resistant fungal transformants confirmed that pG12-1 is present in a low copy extrachromosomal form (Fig. 3). These results, plus the fact that no hybridization of pG12-1 DNA with chromosomal DNA (unamplified or amplified) of individual basidiospore progeny was ever observed argues against the possibility that pG12-1 integrates into the chromosome and that the recovery of the vector is due to its precise excision from the chromosome.

No evidence for integration of the vector sequences into the

chromosome was found even after two years of maintenance of pG12-1 transformants under selective conditions. Furthermore, the low copy extrachromosomal maintenance of pG12-1 in P. chrysosporium transformants was observed even after 10 successive transfers of these transformants during non-selective conditions. This stable maintenance of pG12-1 in transformants at a low copy level, and during non-selective growth conditions is quite novel and distinguishes it from previously described fungal extrachromosomal vectors(32,39,44,45,50,51). Extrachromosomal maintenance of pG12-1 in P. chrysosporium appears to be unique to this vector since Alic et al.(1,2) recently demonstrated an integrative transformation system for P. chrysosporium in which a plasmid vector carrying either an heterologous ADE2 gene or ADE5 gene from Schizophyllum commune was used to complement different adenine auxotrophies.

The results indicate that the ME-1 component of pG12-1 originated from a low copy extrachromosomal plasmid (pME) of P. chrysosporium. As the ME-1 component is common to both pG12-1 and pME, it appears possible that the ME-1 sequences acquired by pG12-1 might be responsible for the stability of this vector and its low copy maintenance, which is similar to that of pME. Further studies are needed to do a detailed characterization of pME and to determine the mechanism allowing for the stability and extrachromosomal maintenance of pG12-1 in P. chrysosporium. A similar report of an autonomously replicating vector which underwent an in vivo

rearrangement was recently reported for Fusarium oxysporum(33). In this case, a circular vector containing sequences from a Fusarium linear plasmid, pUC12, and a hygromycin selection marker was found in some instances to acquire chromosomal telomere sequences which allowed the resulting rearrangement to be maintained autonomously as a linear plasmid.

Previous reports on circular, extrachromosomal vectors in other fungi indicated that such vectors were either transiently circular, preceding integration into the chromosome(30), or were due to excision of either intact or altered vectors from the chromosome(23,37). . Stohl and Lambowitz(41,42) described a transforming vector containing mitochondrial plasmid sequences of Neurospora crassa and showed that the original vector, along with a consistently recovered deletion derivative, is maintained extrachromosomally in N. crassa, and is recovered by E. coli transformation. In Podospora anserina, on the other hand, the attachment of telomeres from Tetrahymena to the ends of a linear plasmid resulted in extrachromosomal maintenance of this plasmid(32) as an unstable extrachromosomal element in approximately half of the transformants studied. High copy autonomous vectors for Ustilago maydis(50) and Mucor circinelloides(39,50) with features similar to those of Saccharomyces cerevisiae autonomous vectors have also been described.

It is of interest that pG12-1, even at a low copy number,

confers adequate G418 resistance to P. chrysosporium transformants. Low copy level maintenance of extrachromosomal vectors (one copy per 5-10 nuclei) has also been reported for P. anserina(32). It is possible that the coenocytial nature of filamentous fungi allows for sufficient cross-feeding to allow survival of transformants under selective conditions. The kan^r determinant of Tn903 (encoding an aminoglycoside phosphotransferase which confers resistance to kanamycin and G418; 17), was chosen as a selection marker in this study as it has been shown to be expressed in several filamentous fungal systems without the addition of eukaryotic promoter and termination signals(9,35,38,43). This property appears to be unique to the kan^r from Tn903 as other kan^r determinants, such as that from Tn5, require appropriate eukaryotic transcription signals for similar function(4).

The pG12-1 based transformation system described here should be of considerable value for molecular genetic analysis of the lignin biodegradation system and for studying the regulation of secondary metabolism in P. chrysosporium, if we can improve the copy number of this vector. Furthermore, this system should serve as a useful model for the analysis of low copy extrachromosomal vectors in filamentous fungi.

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CHAPTER 2

The Nature of Extrachromosomal Maintenance of Transformation Vectors in the Filamentous Basidiomycete *Phanerochaete chrysosporium*

Summary. The nature of extrachromosomal maintenance of the transforming vector p12-6 in Phanerochaete chrysosporium was studied. Our results indicate that the transforming vector is maintained in the fungal transformants extrachromosomally as part of a larger endogenous plasmid of P. chrysosporium. Using total DNA of p12-6 fungal transformants, not only p12-6, but also a larger plasmid, p511, were recovered in recA$^-$ E. coli strains while only p12-6 was recovered in recA$^+$ E. coli strains. The lacZ gene cloned into p12-6 was shuttled through P. chrysosporium transformants.

Introduction

In view of the ecological and industrial importance of lignin biodegradation there has been a great increase in research on the lignin degrading white rot basidiomycete, Phanerochaete chrysosporium (Kirk and Farrell 1987; Tien 1987). This organism is known to degrade lignin more rapidly and completely than most other organisms. Integrative DNA transformation systems using heterologous adenine genes from Schizophyllum commune to complement appropriate adenine auxotrophs of P. chrysosporium have been described recently (Alic et al. 1989; Alic et al. 1990). A vector, pG12-1, which transforms P. chrysosporium to G418-resistance has also been described recently by

Randall et al.(1989 and 1990). In contrast to the integrative system of Alic et al., (1989 and 1990), vector pG12-1 is maintained in the fungal transformants extrachromosomally in a circular form in both primary transformants and their basidiospore progeny. The plasmid occurs in a low copy number in both cases and polymerase chain reaction (PCR) amplification of the vector sequences is necessary to demonstrate its presence in the total DNA of transformants.

Extrachromosomal maintenance of transforming plasmids is unusual in filamentous fungi (Fincham 1989), and so far appears unique to a few filamentous fungi (Tsukuda et al., 1988; Roncero, et al., 1989; Randall et al., 1990) We were, therefore, interested in studying further the nature of extrachromosomal maintenance of transforming plasmid(s) in P. chrysosporium. In this study, we investigated the following questions: 1) Are the transforming plasmids (pG12-1 and p12-6) maintained in a true autonomous form in P. chrysosporium or are they maintained as part of a larger circular element? 2) Does the recA allele of the E. coli strain which is used to rescue the transforming plasmid from the total DNA of fungal transformants have an effect on the structure of the plasmid recovered in E. coli? and 3) Is plasmid p12-6 potentially useful as a shuttle vector?

Materials and methods

Strains and media. P. chrysosporium strain ME446 (ATCC 34541) was used in this study. The various E. coli strains used, and their source, are listed in Table 1. P. chrysosporium and E. coli were maintained as previously described (Kelley et al. 1986, Maniatis et al. 1982).

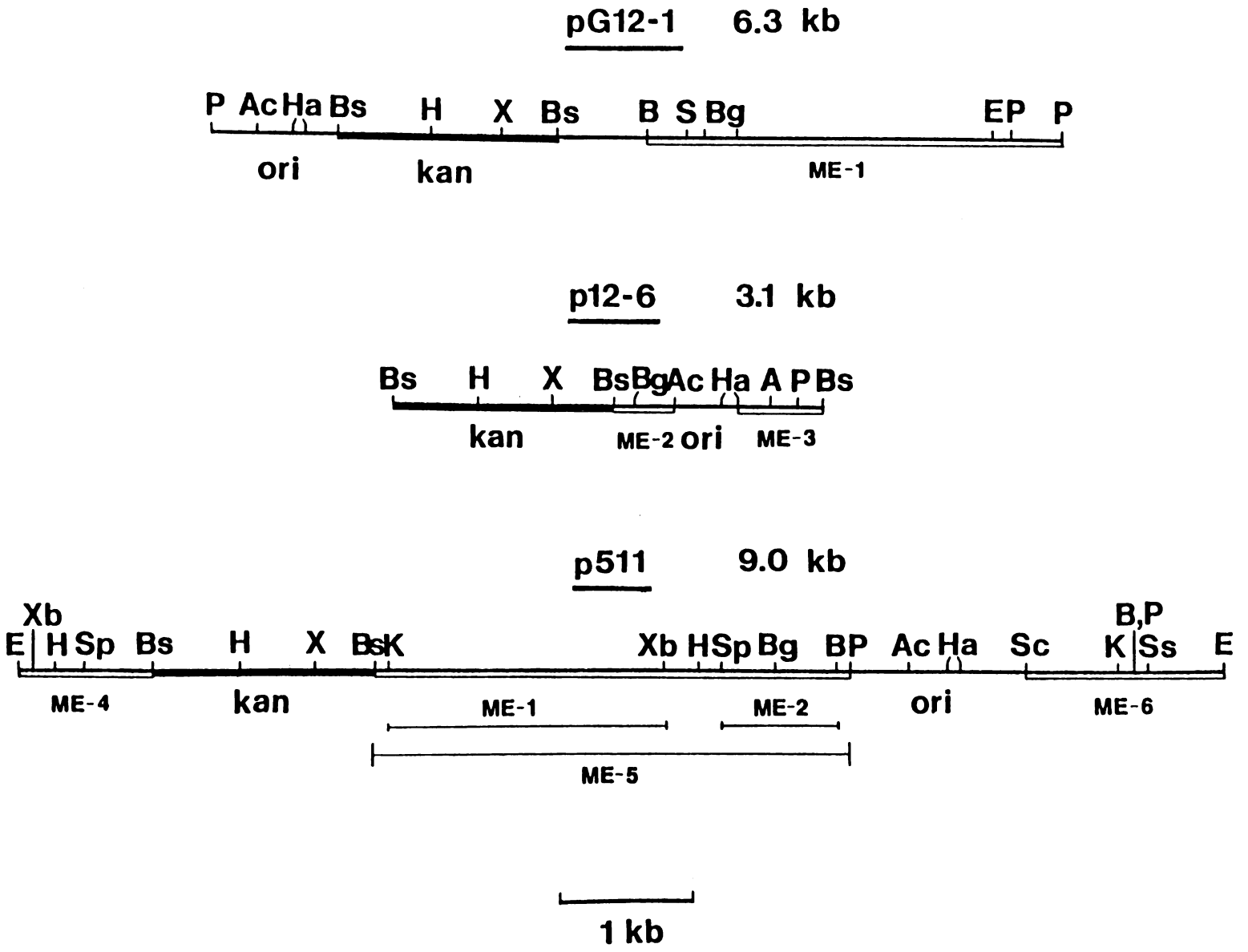
DNA Isolations and Manipulations. P. chrysosporium DNA was isolated by the method of Rao and Reddy(1984) and the transformation of P. chrysosporium spheroplasts was performed as described by Randall et al. (1989). Plasmid DNA isolation and all other molecular biological techniques were performed according to Maniatis et al.(1982) unless specified otherwise. All DNA fragments used as probes were twice purified through low melting agarose (SeaPlaque, FMC Bioproducts, Rockland, ME) and were labeled using the Random Primer labelling kit(BMB Biochemicals, Indianapolis, IN). E. coli transformation was done according to the method of Hanahan(1983) and the transformants were plated either on LM medium (Hanahan 1983) supplemented with 50 μg/ml kanamycin or ampicillin. LM medium was supplemented with 40 μg/ml x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; BRL, Gaithersburg, MD).

Results

Isolation of p12-6

We have previously shown that plasmid pG12-1 (6.3 kb; Fig. 1A) transformed *P. chrysosporium*, was maintained in a low copy, circular, extrachromosomal form, and that it could be readily rescued from fungal DNA by *E. coli* transformation (Randall et al. 1990). Further studies showed that in addition to pG12-1 a second smaller class (3.1 kb) of plasmids was also rescued from pG12-1 transformants. Generally, 10 to 20% of plasmids rescued in *E. coli* from pG12-1 transformants were of this class. The fact that these smaller plasmids had identical restriction maps suggested that they were probably generated by some consistent deletion or alteration of the parental pG12-1 vector. A comparison of the restriction map (Fig. 1A) of a representive of this class of small plasmids, designated p12-6, to the parental pG12-1 showed that p12-6 has major domains of homology to pBR322, the *kan*^{r} selection marker, and an endogenous plasmid of *P. chrysosporium* ME446 designated pME (Fig. 1B; Randall et al. 1990). Plasmid p12-6 showed homology to the *ori* (origin of replication) of pBR322, as defined by a 473 bp *Acc*1-*Hae*II-*Hae*II region flanked by pME sequences designated ME-2 and ME-3. Southern hybridization of ME-2 to total DNA from

Figure 1. A) Restriction map of plasmids pG12-1, p12-6, and p511. The plasmids are presented in a linear form for ease of comparison. The designation of all sequences is based on Southern analysis with the appropriate fragments prepared as described in Materials and Methods. Sequences hybridizing to the *kan*^{r} gene are indicated by a thick line; The open boxes indicate the regions of DNA in each plasmid with pME homology. These are designated ME-1 to ME-6. Regions of ME-5 in p511 which showed hybridization to either ME-1 or ME-2 are also shown. Other fragments of pME (ME-1 to ME-6) have no apparent cross-hybridization to each other. The thin line in each plasmid designates sequences of pBR322, with the region containing the *E. coli ori* shown in each case. B) Southern blot of undigested total DNA of *P. chrysosporium* ME446. Panel 1 is probed with labeled *Bss*HII-*Acc*1 fragment of p12-6, designated ME-2 (Fig. 1A). In panel 2 the same blot is reprobed with a lignin peroxidase cDNA to show the position of the chromosomal DNA band. Restriciton site abbreviations: A; *Ava*II, Ac; *Acc*1 B; *Bam*H1, Bg; *Bgl*1, Bs; *Bss*HII, E; *Eco*R1, H; *Hin*d III, Ha; *Hae*II, K; *Kpn*1, P; *Pvu*II, S; *Sal*1, Sc; *Sca*1, Sp; *Sph*1, Ss; *Sst*II, X; *Xho*1, Xb; *Xba*1.


pG12-1
6.3 kb
P Ac Ha Bs H X Bs B S Bg EP P
ori
kan
ME-1
p12-6
3.1 kb
Bs H X Bs Bg Ac Ha A P Bs
kan
ME-2
ori
ME-3
p511
9.0 kb
Xb
E H Sp Bs H X Bs K Xb H Sp Bg BP Ac Ha Sc B,P K Ss E
ME-4
kan
ME-1
ME-2
ori
ME-6
ME-5
1 kb

untransformed P. chrysosporium showed that ME-1 originated from pME (Fig. 1B). Similar results were obtained when ME-3 was employed as the probe. Both ME-2 and ME-3 hybridized to the same endogenous plasmid (pME) described previously (Randall et al. 1990) and exhibited homology to pG12-1. Additional Southern blot experiments showed that neither ME-2 nor ME-3 of p12-6 hybridized to pG12-1, and, conversely, the ME-1 sequence of pG12-1 did not hybridize to p12-6. These results suggested that p12-6 is not a simple deletion product of pG12-1.

Rearrangements of pG12-1, even consistent ones such as those that resulted in the formation of p12-6, might limit the use of pG12-1 as a shuttle vector for molecular genetic analysis of P. chrysosporium. To better understand the nature of maintenance of extrachromosomal transforming plasmids in P. chrysosporium, we decided to analyze transformations using p12-6 as a model. Vector p12-6 is a useful model for studying transformation in P. chrysosporium for several other reasons. First, p12-6 appears to be maintained in a higher copy number relative to pG12-1 (see below). Second, as p12-6 was recovered as the only rearranged version of pG12-1, it appeared that p12-6 may have the stability desired in a transformation vector. Also, for use as a cloning vector, p12-6 appeared to have certain potential advantages over pG12-1 in that it is smaller in size, and has unique restriction sites which would facilitate its use as the basis for a shuttle

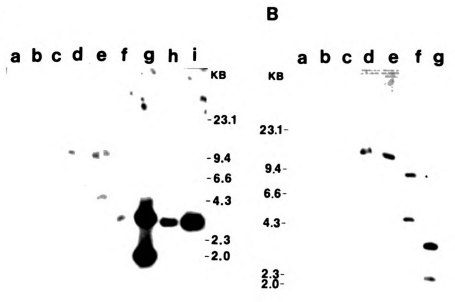
vector (see below).

Transformation of P. chrysosporium with p12-6

Plasmid p12-6 was able to transform P. chrysosporium to G418 resistance at about the same frequency as pG12-1 (5-10 transformants per μg DNA). However, p12-6 is detectable in the total DNA of transformants by Southern analysis, using the labeled kan^r gene as the probe (Fig. 2A, lanes d and e). This is a distinct advantage of p12-6 over pG12-1, because the latter is maintained in fungal transformants in too low a copy number and is not detected by normal Southern analysis (lanes b and c) unless the vector sequences are amplified by PCR (Randall et al. 1990). Analysis of the total DNA of transformants also showed that p12-6 is maintained in the transformants in an extrachromosomal form, but that the size of the plasmid with p12-6 homology is larger than the parental p12-6 (Fig. 2A, lanes f and g). These data showed that p12-6 occurs as part of a larger plasmid, although these data do not rule out the possibility that a small proportion of p12-6 may be present in an autonomous form but at too low a copy number to be detected by normal Southern analysis.

When total DNA from individual p12-6 transformants was used to transform E. coli DH5α to kanamycin resistance two types of plasmids (p12-6 and a larger plasmid p511; Fig. 1A) were consistently recovered in approximately a 1:1 ratio from these p12-6 transformants. Restriction mapping and

Figure 2. Southern blot analysis of total DNA of pG12-1 and p12-6 transformants of P. chrysosporium. A) 5 μg of genomic DNA was electrophoresed in a 0.7% agarose gel, stained with ethidium bromide, and transferred to nitrocellulose. The 1.7 kb PvuII fragment of the kan^r gene from Tn903 was purified from a low melting agarose gel and labeled with ^{32}P-dCTP using the random primer labeling method and was used to probe the blot. Lane a contained total undigested DNA from P. chrysosporium ME446; lanes b-c and d-e contained undigested total DNA from two pG12-1 and p12-6 transformants, respectively; lanes f-g contain, respectively, 0.1 and 1.0 pg undigested p12-6 DNA; lanes h-i contain, respectively, 0.1 and 1.0 pg of linearized p12-6 DNA. B) 10 μg of total DNA of p12-6 and pG12-1 transformants was electrophoresed in a 0.7% agarose gel, stained with ethidium bromide, transferred to nitrocellulose and probed as above with the labeled kan^r gene. Lane a contained total undigested DNA from P. chrysosporium ME446; lanes b-c and d-e contained undigested total DNA from two pG12-1 and p12-6 transformants, respectively; lanes f and g contained, respectively, 1 pg uncut p511 and p12-6 plasmid DNA. C) In this panel the same blot is reprobed with a lignin peroxidase cDNA to show the position of the chromosomal DNA band.


A
a b c d e f g h i
KB
23.1
9.4
6.6
4.3
2.3
2.0
B
a b c d e f g
KB
23.1
9.4
6.6
4.3
2.3
2.0
C
a b c d e
KB
23.1
9.4
6.6
4.3
2.3
2.0

Southern analysis showed that p511, in addition to having homology to pBR322 and the *kan*^{r} sequences, had three major regions of homology to the endogenous plasmid, pME; these are designated ME-4, ME-5, and ME-6. Part of ME-5 showed homology to the previously identified regions of pME designated ME-1 and ME-2 (see Fig. 1A) while ME-4 and ME-6 did not, and represent new portions of pME. Data presented in Fig. 2B show that p511 is not identical to the large plasmid form present in p12-6 transformants (Fig. 2B, lanes d and e). The unequal migration of the extrachromosomal element in lanes d and e of Fig. 2B is most likely an artifact of the agarose gel as the data in Fig. 2A show that the larger plasmid in the p12-6 transformants is very similar in size (Fig. 2A). Reprobing the blot in Fig. 2B with CLG5, a cDNA for a lignin peroxidase gene (de Boer et al. 1987), showed the migration of the chromosomal DNA band (Fig. 2C) relative to the migration of the extrachromosomal element in Fig. 2B, confirming extrachromosomal maintenance of p12-6 in the transformants. The results also show that the plasmid seen in the total DNA of p12-6 transformants is larger than p511 suggesting that both p511 and p12-6 may be deleted forms of this larger plasmid.

Plasmid recovery from p12-6 transformants is dependent on E. coli recA allele

To investigate the possibility that the E. coli strain used has an effect on the type of plasmids recovered from P. chrysosporium transformants, we used E. coli CES200 (Table 1) as a host to recover plasmids from the total DNA of p12-6 transformants because this strain contains mutations in the recBC and sbcB genes. The latter encode exonuclease functions, and mutations in these have been shown to stabilize DNA structures such as palindromes and inverted repeats which occasionally get rearranged during cloning (Leach and Stahl 1983; Nader et al. 1985). Our results showed that only the p12-6 class of plasmids, but not the p511 class of plasmids, were recovered in CES200 (Table 2). The fact that only p12-6 type plasmids were recovered in CES200 in contrast to the recovery of both p12-6 and p511 type plasmids in E. coli DH5α, which primarily differs from CES200 in its recA allele, suggested that the recA protein might be involved in the resolution of p511 to p12-6. To further test the effect of the recA protein on the type of plasmids recovered from p12-6 transformants, the total DNA of the latter was used to transform both E. coli HB101 and RR1 strains, which are isogenic, except that HB101 is recA$^-$ and RR1 is recA$^+$ (Table 1). The results showed that both p511 and p12-6 are

Table 1. Genotypes of *E. coli* strains used in this study

Strain	Genotype	Reference
E. coli DH5α	F-, *rec*A1,*hsd*R17(r_k-,m_k+), *sup*E44,*thi*-1,*end*A1,*gyr*A96, *rel*A1,(*arg*F-*lac*ZYA)U169,P80d*lac*Z M15, lambda	(BRL[a])
E. coli DH5αMCRF-	*rec*A1,*mcr*A,*mcr*B,*mrr*, *hsd*R17(r_k-,m_k+),*sup*E44,*thi*-1, *end*A1,*gyr*A96,*rel*A1,(*arg*F-*lac*ZYA)U169,P80d*lac*Z M15, lambda$^-$	(BRL[a])
E. coli HB101	F-, *rec*A13,*hsd*S20(r_k-,m_k-), *ara*-14,*pro*A2,*lac*Y1,*gal*K2, *rsp*L20(Sm^r),*xyl*-5,*mtl*-1, *sup*E44,lambda	(Maniatis et al. 1982)
E. coli RR1	F-, *hsd*S20(r_k-,m_k-),*ara*-14, *pro*A2,*lac*Y1,*gal*K2,*rsp*L20(Sm^r), *xyl*-5,*mtl*-1,*sup*E44,lambda	(Maniatis et al. 1982)
E. coli CES200	F-,*rec*B21,*rec*C22,*sbc*B15,*his*-4, *thr*-1,*leu*-6,*thi*-1,*pro*A2,*arg*E2, thr::Tn10,*lac*Y1,*gal*K2,*ara*-14, *xyl*-15,*tsx*-33,*hsd*R(r_k-,m_k+)	(Nader et al. 1985)

[a] Grant et al. 1990; purchased from Bethesda Research Laboratories, Gaithersburg, MD

Table 2. Recovery of plasmids from total DNA of p12-6 transformants of *P. chrysosporium* in *recA*$^+$ and *recA*$^-$ *E. coli* strains

Strain	*recA* allele	Type of plasmid recovered	
		p12-6	p511
DH5α	*recA*$_+$	14	19
CES200	*recA*$_-$	42	0
HB101	*recA*$_+$	10	7
RR1	*recA*$^+$	24	0

Total DNA from G418-resistant p12-6 transformants of *P. chrysosporium* was used to transform a *kan*^{r} given *E. coli* strain and plasmid DNA was prepared from *kan*^{r} resistant colonies and examined by electrophoresis on a 0.7% agarose gel in comparison to p12-6 and p511 standards. Selected minipreps were also compared by restriction analysis to confirm the identity of the plasmids

recovered from HB101, while only p12-6 is recovered from RR1 (Table 2). This further supports the idea that *recA*$^+$ strains favor the recovery of the p12-6 class of plasmids whereas *recA*$^-$ strains allow recovery of both classes of plasmids (i.e. p12-6 and p511). The *rec*A protein is known to mediate homologous recombination between DNA sequences (Smith 1987). It is likely that the pME portion of the transforming vector seen in *P. chrysosporium* p12-6 transformants contains sequences which are a substrate for such a recombination event mediated by *rec*A.

The exact relationship between pG12-1, p12-6 and p511 is not apparent from an examination of the restriction maps, although they do have elements of the endogenous plasmid in common. Further analyses of these plasmids and pME will be necessary before a more complete understanding of how extrachromosomal, transformed sequences are maintained in *P. chrysosporium*.

A functional methylcytosine restriction system of *E. coli* has been shown to inhibit recovery of incoming eukaryotic DNA which has cytosine methylation (Orbach et al. 1988; Grant et al. 1990). We therefore tested whether the products of the *mcr* (cytosine methylation restriction deficient) and *mrr* (adenine methylation restriction deficient) loci of *E. coli* might also be causing the alteration of the larger plasmid (Fig. 2A, lanes d and e) in the total DNA of p12-6 transformants of *P. chrysosporium* to p511 and p12-6. However, we found no difference in the

recovery of either p511 or p12-6 from the DNA of P. chrysosporium transformants in either E. coli DH5α (wild type methylcytosine restriction) or E. coli DH5α-MCR[mcrA⁻ mcrB⁻mrr⁻]; data not shown). These results show that the cytosine methylation system is not important in the observed strain dependent recovery of p12-6 and p511 from the p12-6 transformants of P. chrysosporium.

The use of p12-6 as a Shuttle Vector

To test the potential usefulness of p12-6 as a P. chrysosporium-E. coli shuttle vector, we cloned the lacZ gene from pUC19 (Yanisch Perron et al. 1985), which encodes the α peptide of β-galactosidase and complements appropriate lac deletion strains such as E. coli DH5α, into the Nde 1 site of p12-6 (Fig. 3). The recombinant plasmid, pSV7, was then used to transform P. chrysosporium to G418 resistance. The presence of pSV7 (with a functional lacZ gene) in the total DNA of these fungal transformants was demonstrated by transforming E. coli DH5α and plating the transformants on selective medium containing x-gal and kanamycin. Both white and blue colonies were obtained (Table 3), although blue colonies appeared in only 3.8 to 11.7% of the cases. All of the blue colonies contained intact pSV7, while each white colony tested contained a plasmid which was identical to either p12-6 or p511. These results indicated that the lacZ gene could be passed intact through P. chrysosporium. Furthermore, it is

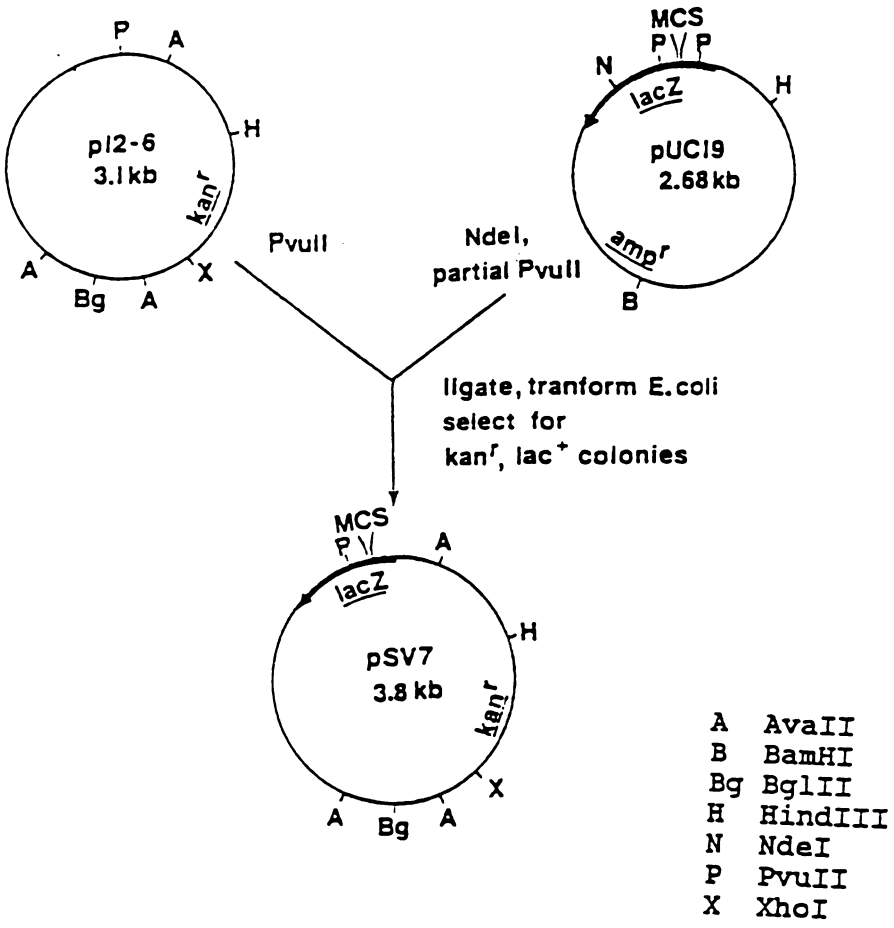


Figure 3. Construction of plasmid pSV7. Plasmid pUC19 was linearized with Nde1 and partially digested with PvuII using conditions to maximize the level of the large Nde1-PvuII fragment in the population. The 5′ overhang created by Nde1 digestion was filled in with the Klenow fragment of DNA polymerase I to create a completely blunt ended fragment. p12-6 was linearized at the single PvuII site and was dephosphorylated. The linearized p12-6 was then incubated in a ligation reaction with the entire population of a pUC19 Nde1-PvuII digestion. The ligation mixture was then used to transform E. coli DH5α to kanamycin resistance and blue colonies on x-gal plates were screened for the correct plasmid(pSV7) containing the lacZ gene inserted at the PvuII site of p12-6. Restriction site abbreviations: A; AvaII, B; BamH1, Bg; Bgl1, H; Hind III, N; Nde1, P; PvuII, X; Xho1.

Table 3. Recovery of plasmids from total DNA of pSV7 transformants of *P. chrysosporium* in *E. coli* DH5α

Transformant	Colony color	
	white	blue
SV2	145	17
SV3	948	45
SV4	269	22
SV6	181	7

Plasmid pSV7 was used to transform *P. chrysosporium* ME446 to G418 resistance and 10 μg of total DNA from individual fungal transformants was used to transform *E. coli* to kan^r on LM plates containing x-gal. Figures on the number of *E. coli* colonies examined and the blue colonies (all yielding intact pSV7) is presented.

also of interest that the pSV7 transformants can be rearranged after rescue in E. coli in a consistent manner to yield p12-6 and p511. The actual process of identifying a true complementing insert using this vector may require a screening of a larger number of E. coli transformants and elimination of those containing p12-6 or p511 followed by a comparison of the restriction map of the cloned insert to that of the actual chromosomal copy to confirm that no rearrangements have occurred.

Discussion

The purpose of this study was twofold. The first was to study the nature of extrachromosomal maintenance of low copy transformation vectors in P. chrysosporium and to determine the potential of the low copy, extrachromosomal transformation vector(s) to be used as P. chrysosporium/E. coli shuttle vectors.

Two lines of evidence suggest that the vectors used to transform P. chrysosporium are maintained as part of a larger plasmid (i.e. pG12-1 + pME or p12-6 + pME). First, two types of plasmids (pG12-1; 6.3 kb and p12-6; 3.1 kb) are recovered from total DNA of pG12-1 transformants of P. chrysosporium. Both pG12-1 and p12-6 contain sequences derived from pME (Fig. 1A and 1B). However, the pME sequences in pG12-1 are different from those in p12-6 suggesting that p12-6 is not a simple deletion product of

pG12-1. Since this putative larger plasmid form is not directly visible by Southern hybridization in pG12-1 transformants (Fig. 2A and 2B) the exact structure of the larger plasmid remains undetermined. Secondly, Southern blot data using total DNA of p12-6 transformants of P. chrysosporium indicate that p12-6 is maintained predominantly as part of a larger plasmid (Fig. 2A and 2B, lanes d and e).

A review of the literature shows that low copy, extrachromosomally maintained transforming vectors are quite rare in filamentous fungi in which the bulk of the transformation systems described to date are of the integrative type (Fincham 1989). We have described here several vectors which are maintained at a relatively low copy number and which undergo apparently predictable rearrangements. Similar rearrangements of transforming plasmids have been shown in other fungi. In Neurospora crassa, a transformation vector (containing sequences of a mitochondrial plasmid) was observed to be extrachromosomally maintained (Stohl and Lambowitz 1983; Stohl et al. 1984). In contrast to our results, a specific rearrangement of this N. crassa plasmid rescued in E. coli resulted in the complete loss of the mitochondrial plasmid sequences. The fact that the parental vector as well as its deletion product was recovered consistently by E. coli transformation (Stohl et al. 1984) indicates parallels to the events which occur in P. chrysosporium, although the

cause(s) of the rearrangement observed with this vector was not discussed in these reports.

Several studies of Neurospora crassa integrative transformation systems have also indicated that altered plasmids (i.e. different from the parental transforming vector) can be recovered in E. coli (Kuiper and de Vries 1985; Fehér et al. 1986). In each case distinct classes of plasmids which lost part of the parental vector sequences but contained additional sequences from the chromosome that were not a part of the original vector were rescued in E. coli. What is particularly unusual about the P. chrysosporium transformation system is that there is no evidence for integration of pG12-1 or p12-6 into the chromosome; instead, there is strong evidence for extrachromosomal maintenance of pG12-1 and pí2-6 in this organism, presumably due to recombination of these plasmids with the endogenous plasmid, pME.

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CHAPTER 3

Rearrangement of Transformation Vectors in *Phanerochaete chrysosporium* via Recombination With an Endogenous Plasmid

Summary. Phanerochaete chrysosporium ME446 was transformed with two plasmids, YIp5-kan^r and pAN7-1, respectively, to G418 and hygromycin resistance. PCR analysis indicated that, with both, the vector sequences are maintained in low copy number in P. chrysosporium transformants. Transformation of Escherichia coli with total DNA of YIp5-kan^r or pAN7-1 transformants resulted in the recovery of two altered classes of plasmids for both of these vectors. Each of the altered plasmid classes recovered contained sequences from an endogenous plasmid(pME) of P. chrysosporium. The results indicated that both YIp5-kan^r and pAN7-1 recombined with pME and were rearranged.

Introduction

There has been much recent interest in lignin biodegradation by filamentous fungi due to the ecological and industrial importance of this process (Kirk and Farrell 1987; Tien 1987). P. chrysosporium has been intensively studied from both a physiological and genetic standpoint in recent years due to its ability to degrade lignin completely and rapidly (Kirk and Farrell 1987; Tien 1987). We have recently described a vector (pG12-1) which transforms P. chrysosporium to G418 resistance (Randall et al. 1989; Randall et al. 1990a) and is maintained in the

transformants as a low copy, circular, plasmid. It is demonstrable in the total DNA of the transformants only after amplification of the vector sequences by polymerase chain reaction (PCR; Randall et al. 1990). Further studies of pG12-1 (and a pG12-1 derivative, p12-6) showed that both were maintained in P. chrysosporium transformants as part of a larger extrachromosomal element (Randall and Reddy 1990) and that both had sequences derived from an endogenous plasmid (pME) of P. chrysosporium (Randall et al. 1990; Randall and Reddy 1990). In this study we have tested the nature of maintenance of two plasmid vectors, YIp5-kan^r (Rao and Reddy 1986) and pAN7-1 (Punt et al. 1987) in P. chrysosporium transformants. The previously tested transformation vectors all contained P. chrysosporium chromosomal(pRR12) or endogenous plasmid DNA sequences which we thought might account for the ability of these vectors to replicate and be maintained extrachromosomally. YIp5-kan^r (Rao and Reddy, 1986) does not contain sequences homologous to the P. chrysosporium genome. Therefore, it appeared that it might escape recombination with pME and integrate into the P. chryosporium chromosome. Vector pAN7-1 is known to transform a wide range of filamentous fungi by integration into the chromosome. Hence, it appeared reasonable that this vector might promote integrative transformation in P. chrysosporium. Our results, however, indicate that both pAN7-1 and YIp5-kan^r, respectively, transform P. chrysosporium to G418 and

hygromycin resistance, and that both vectors recombine with pME of P. chrysosporium and are maintained in low copy number. Furthermore, altered classes of pAN7-1 and YIp5-kan^r which were recovered from the total DNA of fungal transformants by E. coli contained sequences homologous to pME suggesting that both vectors recombine with pME and undergo rearrangement. All the plasmids recovered in E. coli showed homology to the endogenous plasmid.

Materials and methods

Strains and media. P. chrysosporium strain ME446 (ATCC 34541; Kelley et al. 1986) and E. coli DH5α (Maniatis et al. 1982) were maintained as previously described.

DNA Isolations and Manipulations. P. chrysosporium DNA was isolated by the method of Rao and Reddy(1984) and the transformation of P. chrysosporium spheroplasts was performed as previously described (Randall et al. 1989). G418 selection was at 100 μg/ml and hygromycin selection was at 50 μg/ml. Plasmid DNA isolation from E. coli by the alkaline lysis method and all other molecular biological procedures were performed according to Maniatis et al.(1982). DNA fragments used as probes were prepared as previously described (Randall et al. 1990). E. coli transformation was done according to the method of Hanahan(1983) and the transformants were plated on LM media

supplemented with 50 μg/ml kanamycin or ampicillin. PCR amplification of the 1.2 kb AvaII fragment of the kan^r determinant (Grindley and Joyce 1981) was performed as described by Randall et al. (1990). The amplification of the 0.9 kb ScaI-EcoRI fragment of the hph gene was performed using the following 17 mers: 5′-CTTCTACAC AGCCATCG-3′ and 5′-AGAGCCTGACCTATTGC-3′ and the amplification conditions for PCR were as previously described (Randall et al. 1990).

Results and discussion

Most studies with fungal transformation systems to date have shown that the transforming DNA in a majority of cases undergoes integration with chromosomal DNA of the transformant. However, previous studies with P. chrysosporium showed that transformation vectors pG12-1 (Randall et al. 1990) and p12-6 (Randall and Reddy 1990), both of which contain sequences that have homology to the endogenous plasmid (pME) of P. chrysosporium, were maintained in transformants of this organism in an extrachromosomal, low copy form. The results also suggested that vectors pG12-1 and p12-6 undergo recombination with pME and that the pME sequences acquired by the vectors confers on them the ability to replicate and be maintained extrachromosomally in P. chrysosporium. To determine if other vectors transformed into P.

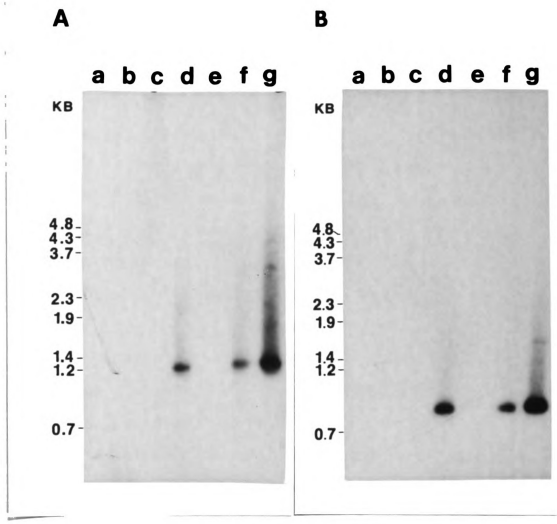


Figure 1. PCR analysis of YIp5-*kan*^{r} and pAN7-1 transformants of *P. chrysosporium*. Panel A contains a Southern blot of genomic DNA probed with the *kan*^{r} determinant. Lanes a and b contained *P. chrysosporium* genomic DNA, lanes c and d contained genomic DNA from a YIp5-*kan*^{r} transformant and lanes e and f contained genomic DNA from a different YIp5-*kan*^{r} transformant. All DNA samples were digested with AvaII and the DNA from lanes b, d and f were amplified with *kan*^{r} specific primers in a PCR reaction (see methods). Lane g contained approximately 50 ng of the gel purified 1.2 kb AvaII *kan*^{r} fragment. Panel B contains a Southern blot of genomic DNA probed with the *hph* determinant. Lanes a and b contained *P. chrysosporium* genomic DNA, lanes c and d contained genomic DNA from a pAN7-1 transformant and lanes e and f contained

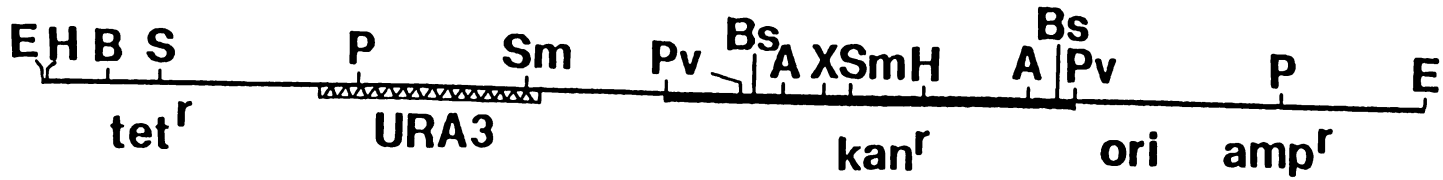
chrysosporium also behave the same way (i.e., recombine with pME) or undergo integration into the host chromosome (Fincham 1989), we tested the nature of maintenance of two other quite different plasmids (pAN7-1 and YIp5-kan^r) in P. chrysosporium transformants.

Plasmid pAN7-1 (Punt et al. 1987), which contains an E. coli hygromycin phosphotransferase gene fused to promoter and termination signals of Aspergillus nidulans and pUC9 (Fig. 3), was used to transform P. chrysosporium to hygromycin resistance. Plasmid YIp5-kan^r, which contains the kan^r determinant of Tn903 (Grindley and Joyce 1980) in YIp5 (Fig. 2; Rao and Reddy 1986) was used to transform P. chrysosporium to G418 resistance. Prior to using these vectors we established that neither of the plasmids contained homology to either pME or chromosomal DNA of P. chrysosporium (also see Fig. 1, see below).

The transformation frequency of P. chrysosporium with YIp5-kan^r and pAN7-1 was 4 and 12.5 transformants per μg DNA, respectively. When total DNA from YIp5-kan^r and pAN7-1 transformants of P. chrysosporium was probed with YIp5-kan^r and pAN7-1, respectively, no hybridization bands were observed (Fig. 1A, lanes c and e, Fig. 1B, lanes c and e). However, after PCR amplification of either the kan^r (a 1.2 kb AvaII fragment) or hph (0.9 kb Sca1-EcoR1 fragment) DNA in the transformants, bands of hybridization characteristic of the vector sequences could be seen (lanes d and f in Fig.

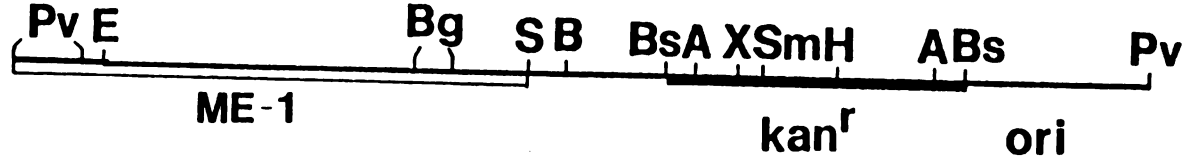
Figure 2. Restriction map of YIp5-*kan*^{r} and its derivatives rescued in *E. coli* from the total DNA of YIp5-*kan*^{r} transformants. The plasmids are presented in a linear form for ease of comparison. The designation of all sequences is based on Southern analysis with the appropriate fragments prepared as described in Materials and Methods. The single thin line represents sequences of pBR322; the thick line defines sequences hybridizing to the *kan*^{r} gene; the double cross-hatched line represents *URA3* sequences of *Saccharomyces cerevisiae*; the open boxes represent sequences hybridizing to the endogenous plasmid of *P. chrysosporium* ME446. The designation of ME-1 to ME-3 is based on comparison and cross hybridization to plasmids pG12-1 and p12-6 which have been described (Randall and Reddy, 1990). The restriction sites defining to the bacterial *ori* are not shown in YIp5-*kan*^{r} and pYK5-7 to improve clarity. Restriction site abbreviations: A; *Ava*II, B; *Bam*H1, Bg; *Bgl*1, Bs; *Bss*HII, E; *Eco*R1, H; *Hin*d III, P; *Pst*1, Pv; *Pvu*II, S; *Sal*1, Sm; *Sma*1, X; *Xho*1.

YIp5-kan^r



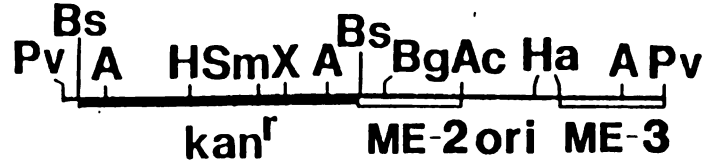
7.05 kb

pYK5-7



6.3 kb

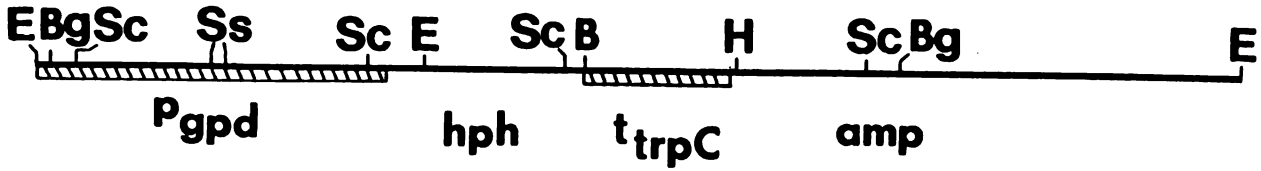
pYK5-4



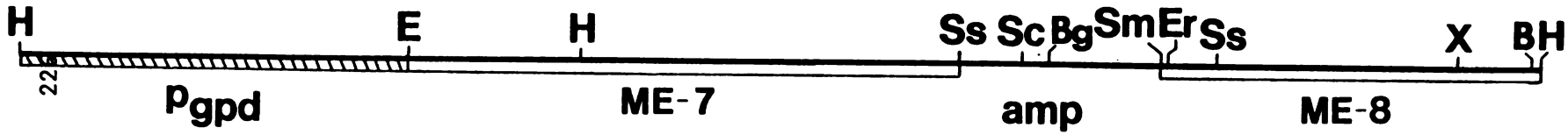
3.1 kb

Figure 3. Restriction map of pAN7-1 and its derivatives rescued in E. coli from the total DNA of pAN7-1 transformants. The plasmids are presented in a linear form for ease of comparison. The designation of all sequences is based on Southern analysis with the appropriate fragments prepared as described in Materials and Methods. The single thin line represents sequences of pBR322; the single cross-hatched boxes indicate sequences from A. nidulans and open boxes indicate regions containing homology to pME of P. chrysosporium. Restriction site abbreviations: A; AvaII, B; BamH1, Bg; Bgl1, E; EcoR1, Er; EcoRV, H; Hind III, N; Nde1, P; Pst1, Pv; PvuII, Sc; Sca1, Sm; Sma1, Ss; Sst1, Ss2; SstII, X; Xho1. P_{gpd} is the promoter from the glycerol-3-phosphate dehydrogenase gene of A. nidulans and T_{trpC} is the terminator from the trpC gene cluster of A. nidulans.

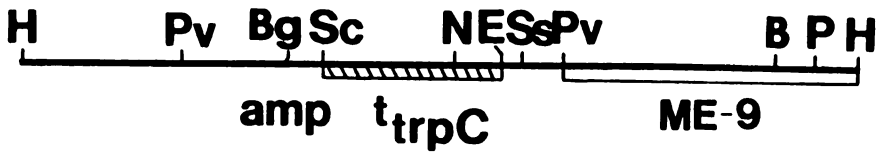
pAN7-1



pAN1



pAN2



1A and B). These results suggested that the vector sequences are maintained at a low copy number in the transformants
similar to pG12-1 described by Randall et al. (1990).

In an attempt to recover pAN7-1 from P. chrysosporium transformants, total DNA from the latter was used to transform E. coli. Two classes of plasmids (pAN1 and pAN2) were recovered from these E. coli transformants, neither of which was identical to pAN7-1 (Fig. 3). Each of the two plasmid classes, however, contained some residual pAN7-1 sequences. Plasmid pAN1 had homology to the gpd region of pAN7-1 while pAN2 had homology to the trpC region. The restriction maps of even these two regions did not correspond to those of the original pAN7-1, suggesting that these regions may have also been rearranged. Neither pAN1 nor pAN2 contained any homology to the hph marker but both contained sequences homologous to pME (not shown). Vector pAN2 contained sequences which cross-hybridized to ME-1 (Randall et al. 1990) whereas pAN1 had homology to regions designated ME-7 and ME-8 (Randall and Reddy 1990).

Total DNA of YIp5-kan^r transformants yielded in E. coli two classes of plasmids both of which are different from YIp5-kan^r and were found to be identical to pG12-1 and p12-6 previously described (Randall et al. 1990; Randall and Reddy 1990). The recovery of plasmids identical to pG12-1 and p12-6, though unexpected, may be explained by the fact

that pG12-1 and YIp5-*kan*^{r} are both based on the yeast vector YIp5 (Struhl et al. 1979). The fact that identical types of plasmids weere rescued in *E. coli* from the total DNA of either pG12-1 or YIp5-*kan*^{r} transformants of *P. chrysosporium* suggested that sequences common to both YIp5-*kan*^{r} and pG12-1 are involved in recombination with pME.

The PCR data (Fig. 1B) showed that the *hph* selection marker(identified by the amplified *Sca*1-*Eco*R1 fragment) is present in the total DNA from pAN7-1 transformants. However, pAN1 and pAN2, the plasmids rescued in *E. coli* from the total DNA of pAN7-1 transformants, did not carry the hygromycin selection marker, suggesting that they are altered forms of the pAN7-1 vector. This is analagous to the situation found with p12-6 transformation of *P. chrysosporium* (Randall and Reddy 1990). We found that we could recover two types of plasmids in *E. coli*, p12-6 and p511. Both of these plasmids were smaller than the circular vector which we could see in Southern blots of p12-6 transformants, suggesting that p12-6 and p511 were altered forms of that plasmid which we could observe in the genomic DNA of p12-6 transformants.

Both YIp5-*kan*^{r} and pAN7-1 were maintained in *P. chrysosporium* transformants at a low copy level. With both YIp5-*kan*^{r} and pAN7-1 *P. chrysosporium* transformants, we found the recovery in *E. coli* of rearranged forms of these two vectors each with pME sequences. This indicated that each original vector recombined with the pME. These results

lead us to conclude that the recombination event with pME is a general phenomenon common to a number of plasmids that have been used to transform P. chrysosporium.

Acknowledgements. This work was supported in part by the Agricultural Experimental Station of Michigan State University, grants DE-FG02-85ER 13369 from the U.S. Department of Energy, 1-RO1-GM39032-01A1 from NIH and a REF grant from Michigan State University.

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CHAPTER 4

An Improved Transformation Vector for the Lignin-degrading White Rot Basidiomycete *Phanerochaete chrysosporium*

submitted to Gene

SUMMARY

In this study, a lignin peroxidase(LIP) gene of Phanerochaete chrysosporium was disrupted by inserting into its coding region the Km^R determinant from Tn903. The resulting recombinant plasmid, pUGLG1:kan, was transformed into P. chrysosporium with the expectation that the disrupted gene might replace the homologous LIP gene in the chromosome. However, the results showed that pUGLG1:kan sequences do not integrate into the chromosome; instead, the plasmid is maintained intact in the transformants in an extrachromosomal state. Our data also show that pUGLG1:kan undergoes replication in P. chrysosporium, is maintainted as a circular element, is stable through meiosis, and can be recovered intact by Escherichia coli transformation. These results indicate that the genomic clone GLG1 contains as yet unidentified sequences that allow autonomous replication of pUGLG1:kan in P. chrysosporium transformants. These sequences should potentially be useful for constructing a stable high copy number tranformation vector for P. chrysosporium.

INTRODUCTION

There has been much recent interest in lignin biodegradation by fungi because of the ecological and industrial importance of this process(Kirk and Farrell, 1987; Tien, 1987). Most of these studies focused on the filamentous basidiomycete Phanerochaete chrysosporium since it has been shown to degrade lignin more rapidly and completely than most other organisms(Kirk and Farrell, 1987). P. chrysosporium produces two families of important extracellular, glycosylated H_2O_2 requiring heme proteins, designated lignin peroxidases(LIP) and manganese peroxidases(MNP) that appear to play a key role in lignin degradation(Tien and Kirk, 1984; Wariishi, et al., 1988). These enzymes are produced in response to nutrient starvation only during secondary metabolism. At least six major LIP isozymes (arbitrarily designated H1, H2, H6, H7, H8, and H10) and four MNP isozymes have been described(Kirk and Farrell, 1987; Tien, 1987; Wariishii, et al. 1988). Both cDNA and genomic clones of LIP isozymes have been isolated and sequenced (Tien and Tu, 1987; de Boer, et al., 1987; Brown, et al., 1988; Walther, et al., 1988; Schalch, et al., 1989; Zhang, et al., 1990). We have recently described a DNA transformation system for P. chrysosporium in which the circular DNA vector was shown to be maintained in an extrachromosomal, low copy form which

can only be observed after amplification by polymerase chain reaction. The vector can be recovered by E. coli transformation (Randall, et al. 1989; 1990). Integrative DNA transformation systems (Alic et al., 1989; 1990) in which adenine auxotrophs of P. chrysosporium was complemented with the corresponding heterologous adenine genes of Schizophyllum commune have also been described.

Because of the presence of multiple LIPs in P. chrysosporium, the isolation of a mutant lacking a specific LIP isoenzyme using traditional mutagenic approaches has been difficult (Boominathan, et al., 1990). Mutants lacking specific LIP isozymes will be useful in determining the relative contributions of each LIP isozyme to lignin degradation as well as to other biological activities known to be catalyzed by LIP isozymes. Hence, we initiated this study to construct mutants lacking H_2, a major LIP isozyme, by employing a gene disruption technique. To accomplish this, we transformed P. chrysosporium with a vector carrying a disrupted LIP2 gene that encodes H_2. However, we did not observe either the expected disruption of the LIP gene or the integration of the vector sequences into the chromosome. Instead, we made the important finding that the transforming vector is maintained extrachromosomally in a circular form at a relatively high copy number, as compared to the pG12-1 vector previously described by us (Randall, et al., 1990).

MATERIALS AND METHODS

(a) Strains and media

P. chrysosporium strain BKM-F (ATCC 24725) was maintained as previously described (Kelley, et al., 1986). *E. coli* DH5α (F_, *end*A1, *hsd*R17[r_k^-,m_k^+], *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1,[*arg*F$^-$, *lac*ZYA]U169, 80μ*lac*Z-15, $^-$), was used for the maintenance of plasmid vectors(Maniatis, et al., 1982). Gold's medium B (Gold, et al, 1978) supplemented with 10% sorbose and 3% agar (Difco, Detroit, MI) was used for the regeneration of *P. chrysosporium* spheroplasts. Golds medium B, without sorbose or deoxycholate and supplemented with 100 μ g per ml of G418 was used for the mitotic stability assay (see below).

(b) DNA Isolations and other manipulations

Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Maniatis et al.,(1982). Most molecular biological techniques were performed according to Maniatis et.al, (1982). Restriction enzymes were used per manufacturers' specifications. DNA probes were labelled according to specifications in the Random Primer labelling kit (Boeringer Mannheim Biochemicals, Indianapolis, IN). *P. chrysosporium* DNA was isolated by the method of Rao and

Reddy(1984). DNA fragments used as probes were purified twice through low melting agarose (SeaPlaque, FMC Bioproducts, Rockland, ME) before labelling. Transformation of P. chrysosporium spheroplasts was performed as previously described (Randall, et al., 1989). E. coli transformation was done according to the method of Hanahan(1983).

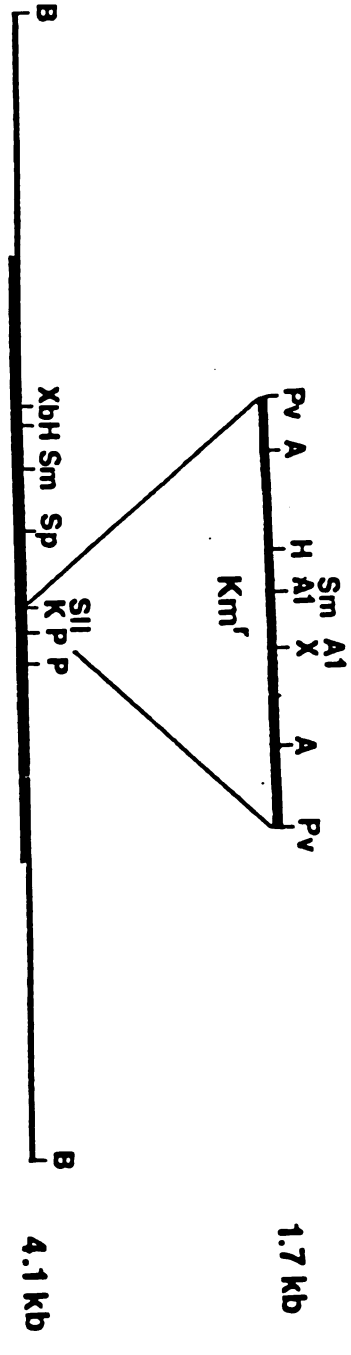
RESULTS AND DISCUSSION

(a) Insertion of the Km^R determinant into the LIP2 gene in clone GLG1.

Our initial objective was to inactivate a specific LIP gene of P. chrysosporium and construct strain lacking that specific LIP enzyme so that the relative contribution of each of the LIP enzymes to overall lignin degradation by P. chrysosporium could be evaluated. For this, we first needed to determine whether a disrupted LIP gene introduced into P. chrysosporium would replace the endogenous LIP gene by integrating into a homologous and/or heterologous site(s) in the chromosome as is common in a number of fungi, including P. chrysosporium (Fincham, 1989; Alic et al., 1989).

The P. chrysosporium BKM-F genomic insert in clone GLG1 (a 4.1 kb Bam H1 genomic fragment) contains the LIP2

Figure 1. Restriction map of pUGLG1:kan. The LIP2 gene was carried on a 4.1 kb BamH1 fragment designated GLG1 isolated from a P. chrysosporium BKM-F genomic library in YRp12(Zhang, et al., 1990). GLG1 was recloned into pUC19 and designated pUGLG1. The Km^R gene of Tn903 was inserted as a 1.7 kb PvuII fragment into the single, blunt ended SstI site within the coding region of the LIP2 gene. The resulting recombinant plasmid is designated pUGLG1:kan. Only the GLG1:kan segment of pUGLG1:kan is shown in detail. The thick line represents the approximate coding region of the LIP2 gene and the arrow indicates its transcriptional orientation(Zhang, et al., 1990). The thin line represents chromosomal DNA flanking the LIP2 gene. Restriction site abbreviations: A; AvaII, A1; Ava1; B; BamH1, H; HindIII, K; Kpn1, P; Pst1; Pv; PvuII, Sm; Sma1, Sp; Sph1, SII; SstII, X; Xho1; Xb; Xba1.



pUGLG1:kan (8.5 kb)

gene based on the fact that the sequence of the coding region of this gene is identical to that of the cDNA clone CLG4 which is known to encode LIP protein H_2 (de Boer, et al., 1987; Naidu and Reddy, 1989). The choice of LIP2 is based on the fact that the enzyme encoded by this gene is readily identifiable in FPLC profile of extracellular fluid (Dass and Reddy, 1990; Tien and Kirk, 1988) and that LIP2 shows only faint hybridization with the other LIP genomic clones of P. chrysosporium (Zhang, et al., 1990). The GLG1 genomic fragment contains the LIP2 gene flanked by 1.3 kb of genomic DNA upstream on the 5′ end and 1.05 kb of genomic DNA on the 3′ end (Fig. 1). The Km^R determinant from Tn903 (Grindley and Joyce, 1980) was inserted as a blunt ended, 1.7 kb PvuII fragment into an unique SstI site within the LIP2 coding region to disrupt the LIP2 gene. The resulting plasmid (i.e. pUC19, plus GLG1 with the inserted Km^R fragment) was designated pUGLG1:kan and was used to transform P. chrysosporium BKM-F to G418 resistance.

(b) Analysis of the DNA of transformants

Undigested total genomic DNA from individual G418-resistant fungal transformants was electrophoresed through an agarose gel and the Southern blot was probed with a HindIII-Xho1 fragment internal to the Km^R gene (see Fig. 1)

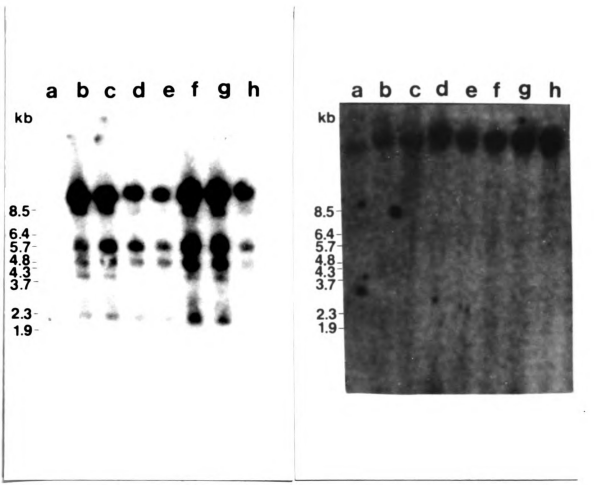


Figure 2. Southern analysis of genomic DNA from pUGLG1:kan transformants. Uncut genomic DNA (10 μg/lane) was electrophoresed on a 0.7% agarose gel, stained with ethidium bromide and transferred to nitrocellulose. In the left panel, the Southern blot is probed with the 1.7 kb PvuII Km^R fragment. In the right panel, this same blot was then stripped of the Km^R probe and reprobed with the LIP6 gene the LIP gene to determine the position of the chromosomal DNA in this blot. Lane a contained genomic DNA from untransformed P. chrysosporium BKM-F and lanes b through h contained total genomic DNA from individual GLG1:kan transformants of BKM-F.

to determine if the transforming sequences integrated into the P. chrysosporium chromosome (Fig. 2A). Our results showed that the probe exhibited intense hybridization with extrachromosomal DNA but not to the chromosomal DNA of P. chrysosporium. In addition, undigested genomic DNA isolated from seven different G418 resistant transformants exhibited identical hybridization patterns with the Km^R probe (Fig. 2A, lanes b-h). In contrast, total DNA from untransformed P. chrysosporium gave no chromosomal or extrachromosomal hybridization with the Km^R probe (Fig. 2A, lane a). This blot was then stripped of the Km^R probe and reprobed with the chromosome-specific LIP cDNA CLG5 (de Boer, et al., 1987) to demonstrate the position of the chromosomal DNA in both transformants and wild type P. chrysosporium (Fig. 2B). These results indicate that the Km^R probe is hybridizing to extrachromosomal DNA of the transformants.

c. Intact transforming plasmid(pUGLG1:kan) is maintained extrachromosomally

Southern blot analyses indicated that the extrachromosomal DNA seen in Fig. 2 contained intact pUGLG1:kan plasmid. Southern blots of undigested and BamH1 digested total DNA from three of the transformants were probed with the 4.1 kb GLG1 insert (Fig. 3A). Identical patterns of hybridization were observed with the DNA of all

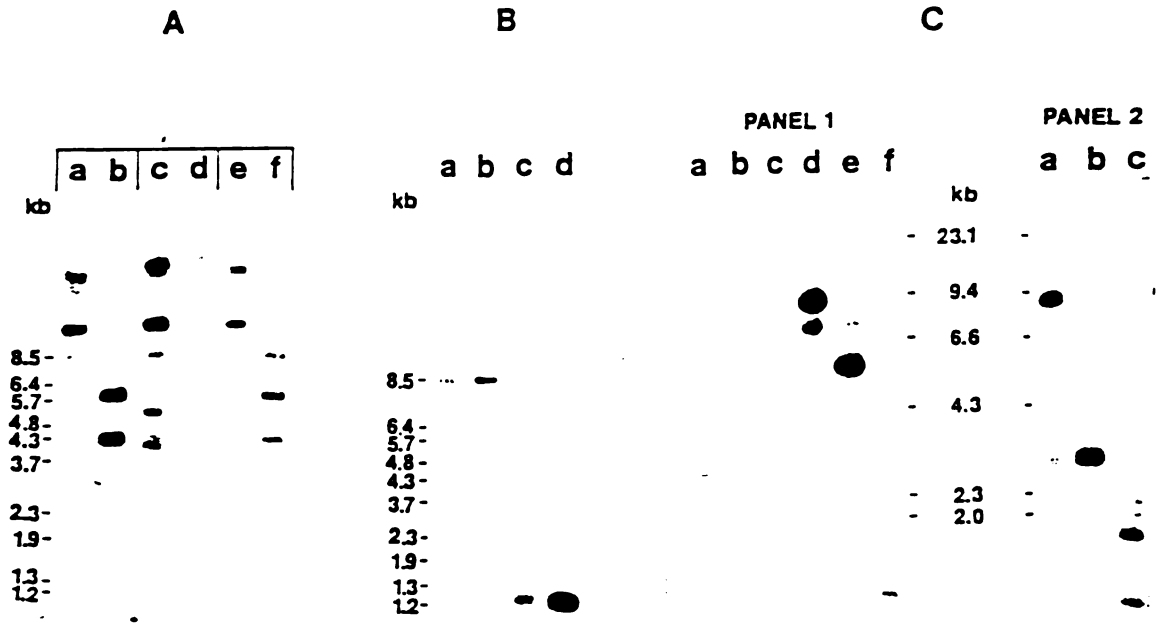


Figure 3. Restriction and Southern blot analysis of transformant DNA. (A) Total DNA from three transformants(labelled 1, 2, and 3) were electrophoresed on a 0.7% agarose gel and transferred onto nitrocellulose. Lanes a,c, and e contain uncut DNA; lanes b,d, and f contain BamH1 digests of total DNA. (B) Total DNA from one G418-resistant transformant(#1 from Fig. 3A) was probed with the HindIII-Xho1 fragment of the Km^R fragment. Lane a contains 3 μg uncut DNA; lanes b and c contain, respectively, Xho1 and AvaII digests of the same transformant DNA. Lane d contained 50 ng AvaII digested pUGLG1:kan DNA. (C) Panel 1. Total DNA from untransformed P. chrysosporium and a G418 resistant transformant was probed with Km^R fragment. Lanes a-c in panel 1 contained P. chrysosporium DNA, uncut, BamH1 cut, and AvaII cut, respectively. Lanes d-f in panel 1 contain genomic DNA from a G418-resistant transformant, uncut, BamH1 cut, and AvaII cut, respectively. The Southern blot of panel 1 was stripped and reprobed with pUC19 for panel 2. Lanes a-c of panel 2 shows the part of the blot in panel 1(lanes d-f) containing the transformant DNA.

the three transformants. In the lanes containing digested DNA (b,d, and f) a 5.8 kb band of hybridization (corresponding to the sum of the 4.1 kb BamH1 fragment containing the GLG1 insert, and the 1.7 kb Km^R fragment) and a 4.1 kb band (corresponding to the endogenous GLG1 locus of P. chrysosporium; see also Fig. 5, lane a) were observed. The faint 8.5 kb band in lane f is due to partial digestion of pUGLG1:kan. In the lanes containing undigested transformant DNA (Fig. 3A, lanes a, c, and e) the largest band corresponds to the position of the chromosomal DNA, while the smaller extrachromosomal bands of hybridization are due to the transforming plasmid, pUGLG1:kan. Additional analyses of these data also indicated that approximately one or two copies of pUGLG1:kan can be maintained per haploid nucleus in P. chrysosporium.

Xho1 digestion of the total DNA from one of the transformants (Fig. 3B, lane b) showed that the size of the extrachromosomal element (8.5 kb) is identical to that of the original pUGLG1:kan vector. An AvaII digest of total transformant DNA (Fig. 3B, lane c) resulted in a 1.2 kb band of hybridization, identical to that of the 1.2 kb Ava II Km^R fragment of pUGLG1:kan (Fig. 3B, lane d) showing that the Km^R fragment is intact. Our results also showed that both the GLG1:kan and the pUC19 portions of pUGLG1:kan are present intact in the transformants. The Km^R probe showed no hybridization to the total DNA from untransformed P. chrysosporium (Fig. 3C, panel 1, lane a-c), while the BamH1

and AvaII digests of the transformant DNA, respectively, showed 5.8 kb (representing the GLG1:kan insert in pUGLG1:kan; panel 1, lane e) and 1.2 kb (the 1.2 kb AvaII fragment of Km^R in pUGLG1:kan; panel 1, lane f) bands of hybridization. When the same blot was reprobed with pUC19, the undigested DNA (Fig. 3C, panel 2, lane a) showed bands of hybridization representing the extrachromosomal element, while in lane b a 2.7 kb band of hybridization (representing the size of the pUC19 portion of pUGLG1:kan) was seen indicating that pUC19 is a part of the extrachromosomal element in the transformant. Two small AvaII bands (Fig. 3C, lane c) are observed which represent AvaII fragments of pUGLG1:kan containing both pUC19 and GLG1 homology. In summary the above data suggest that the intact pUGLG1:kan is being maintained extrachromosomally in the P. chrysosporium transformants.

(d) pUGLG1:kan is maintained in a circular form

An exonuclease III (Exo III) assay was used to demonstrate that the vector DNA is maintained in a circular form in the transformant. Exo III digests linear DNA but leaves circular DNA intact and has been used to assay the physical state of plasmid elements (Kistler and Leong, 1986). Exo III treated transformant DNA showed extrachromosomal bands of hybridization to the Km^R probe (Fig. 4, lane c) identical to the extrachromosomal bands

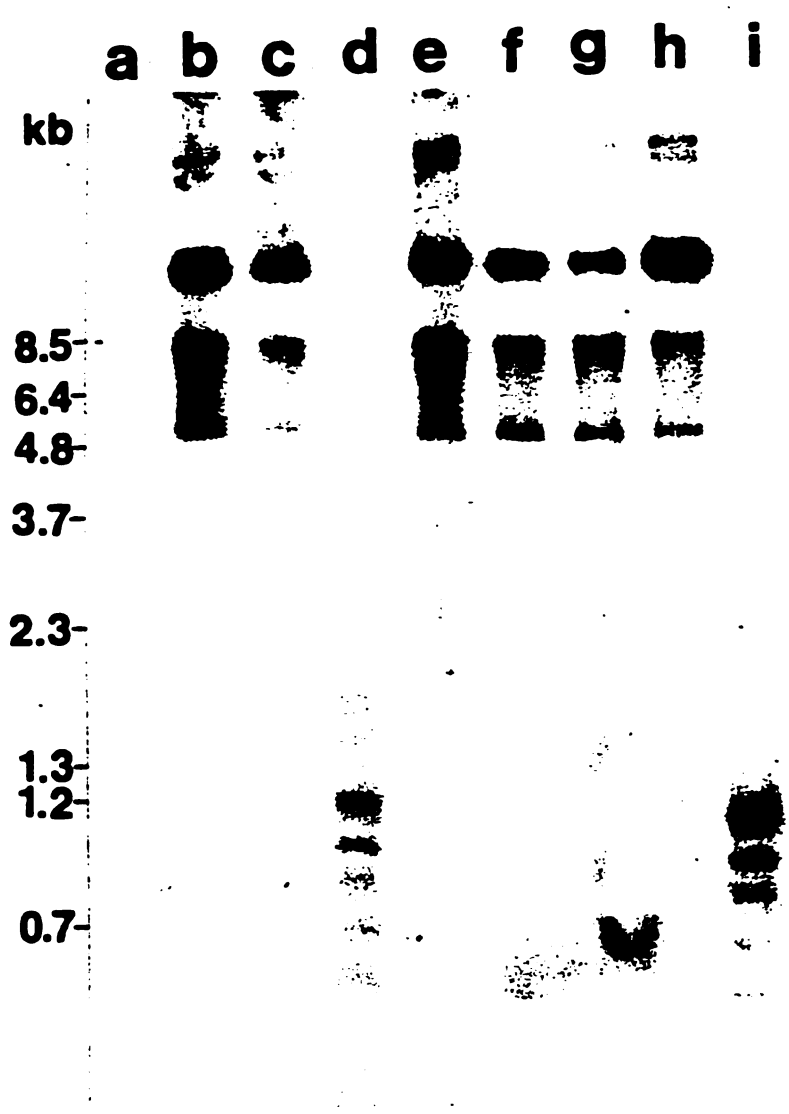


Figure 4. Confirmation of circular and fungal origin of the transforming plasmid in a G418-resistant P. chrysosporium transformant. Fragment purified Km^R gene was used as hybridization probe. Lane a contained 3 μg of untransformed BKM-F DNA; lane b-e contained 3 μg, respectively, undigested, exonuclease III digested, Mbo1 digested, and Dpn1 digested transformant DNA; lanes f-i contained, respectively, 10 ng of undigested, exonuclease III digested, Mbo1 digested, and Dpn1 digested pUGLG1:kan plasmid DNA. Restriction digestions were performed per manufacturers specifications.

observed with the undigested transformant DNA (lane b) as well as with the undigested and Exo III digested pUGLG1:kan DNA isolated from E. coli (lanes f and g). These results indicate that pUGLG1:kan exists in a circular form in the transformant DNA.

(e) pUGLG1:kan replicates in P. chrysosporium transformants

A Dam methylation assay was performed to confirm that the pUGLG:kan sequences undergo replication in the fungus. Mbo1 and Dpn1 are isoschizomers which recognize GATC sites. Dpn1 specifically digests DNA that is methylated at the N^6 position of the adenine residue, while Mbo1 digests only when the adenine residue at these sites is unmethylated (Holst, et al., 1988). DNA which has passed through a dam^+ strain of E. coli will have a N^6-methylated adenine at GATC sites in the plasmid whereas DNA which has replicated in P. chrysosporium is not known to have the methylated adenine at GATC site(s). The Km^R gene of Tn903 contains a number of Mbo 1 sites which digest the gene into fragments of several hundred base pairs. Therefore, if pUGLG1:kan replicates in P. chrysosporium it will be digested by Mbo1 but not by Dpn1. The data in Fig. 4 also illustrates the specificity of Mbo1 and Dpn1. The pUGLG1:kan DNA isolated from E. coli DH5α (dam^+) was resistant to digestion with Mbo1 (lane h) but was

susceptible to digestion by Dpn1 (lane i). In contrast, when P. chrysosporium transformant DNA was digested with Mbo1 (lane d), pUGLG1:kan bands characteristic of complete digestion at GATC sites were seen to hybridize to the Km^R probe as in Fig. 4, lane i. When the same transformant DNA was digested with Dpn1 (lane e), the pUGLG1:kan bands characteristic of undigested plasmid were observed (see lanes b and c). These results indicate that pUGLG1:kan has undergone replication in P. chrysosporium transformants.

(f) pUGLG1:kan is passed intact through meiosis and mitosis in P. chrysosporium transformants

Total DNA from basidiospore progeny of a pUGLG1:kan transformant was analyzed by Southern blot hybridization to determine if pUGLG1:kan is stably inherited by basidiospore progeny. Using the 4.1 kb GLG1 fragment as the probe, two of the basidiospore progeny (Fig. 5, lanes 2 and 3) showed a 5.8 kb band of hybridization (representing the GLG1:kan insert of pUGLG1:kan) indicating the presence of pUGLG1:kan in these progeny. Reprobing this blot with the Km^R gene also showed the same 5.8 kb band in lanes 2 and 3, whereas reprobing with pUC19 showed a 2.7 kb band representing the linear size of pUC19 (similar to Fig. 3C, panel 2, lane b; data not shown). The above two basidiospore progeny also grow well on G418 containing media. In contrast, three of the basidiospore progeny

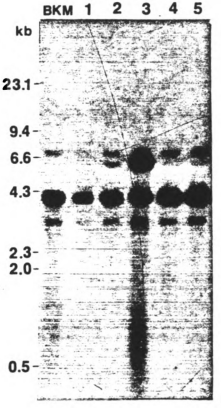


Figure 5. Analysis of individual basidiospore progeny of a pUGLG1:kan transformant of P. chrysosporium. Basidiospores were isolated as described(Alic et al., 1989). Lane BKM contained total DNA from untransformed P. chrysosporium BKM-F. Lanes 2 and 3 contained DNA from G418-resistant basidiospore progeny of a pUGLG1:kan transformant, whereas lanes 1, 4, and 5 contained G418-sensitive basidiospore progeny of the same transformant. All DNA samples were digested to completion with BamH1 and the Southern blot was probed with the labelled GLG1:kan insert shown in Fig. 1.

examined (Fig. 5, lanes 1,4, and 5) which were G418 sensitive did not show the 5.8 kb band of hybridization. Note that the BamH1 digests of all the basidiospore progeny as well as that of undigested P. chrysosporium BKM-F DNA gave one major band of hybridization (4.1 kb) representing the GLG1 locus in the chromosome and two minor bands of hybridization which are most probably one or two other LIP genes of P. chrysosporium related to the LIP2 gene. Upon further Southern analysis of a larger number of basidiospore progeny pUGLG1:kan was found to be transmitted to about 12% of the basidiospore progeny of the pUGLG1:kan transformants tested. These data indicated that the vector is transferred to the progeny at a relatively low rate as would be expected for an extrachromosomally maintained vector (van Heeswijck, 1986; Tsukuda, et al., 1988).

A mitotic stability assay using conidia isolated from G418-resistant and G418-sensitive basidiospore progeny (lanes 3 and 5 of Fig. 5, respectively), was then performed to determine how well pUGLG1:kan is passed to conidial progeny. Only 3% of the conidia derived from a G418-resistant strain grew on G418 media, whereas none of the conidia derived from a G418-sensitive strain grew on the G418 plates. These results indicate that pUGLG1:kan could be transferred to conidial progeny, although at a low frequency.

(g) Recovery of pUGLG1:kan in *E. coli*

Further evidence for the presence of pUGLG1:kan in G418-resistant *P. chrysosporium* transformants as well as in the G418-resistant basidiospore progeny was obtained by using total DNA of these strains to transform *E. coli* to either Km or Ap resistance, and by recovering intact pUGLG1:kan from the *E. coli* transformants. In contrast, total DNA from wild type *P. chrysosporium* and G418-sensitive basidiospore progeny did not yield either Km^R or Ap^R transformants of *E. coli*.

(h) Conclusions

1) The results of this study show that vector pUGLG1:kan containing the *P. chrysosporium* *LIP2* gene, disrupted by the Km^R gene and flanked on either side by approximately 1 kb of genomic sequences functions as a relatively high copy number vector for transforming *P. chrysosporium* BKM-F to G418 resistance.

2) Vector pUGLG1:kan is maintained intact as an autonomous, extrachromosomal element in a relatively high copy number compared to the previously described pG12-1 vector plasmid(Randall et al., 1990) which was found to be maintained in too low a copy number to be detected by standard Southern hybridization procedure and could be demonstrated in the total DNA of fungal transformants only after amplification by polymerase chain reaction (PCR).

Thus, pUGLG1:kan should be a particularly useful improvement as the basis for a shuttle vector over our previously described vector, pG12-1.

3) Vector pUGLG1:kan could be passed through basidiospore and conidial progeny of the transformants at a relatively low rate. This is quite similar to the maintenance of high copy, unstable, extrachromosomal vectors in both Ustilago maydis (Tsukuda, 1988) and Mucor circinelloides (van Heeswijck, 1986)

4) Intact pUGLG1:kan could be recovered by transforming E. coli using total DNA from either the initial G418 resistant fungal transformants or from G418 resistant basidiospore progeny of these transformants. The general behavior of pUGLG1:kan in P. chrysosporium transformants is quite similar to ars vectors of S. cerevisiae (Williamson, 1985) and of filamentous fungi (Tsukuda, et al., 1988; van Heeswijck, 1986; Roncero, et al., 1990). Specific ars elements have been identified for both U. maydis (Tsukuda, 1988) and M. circinelloides (Roncero, et al., 1990) and these are apparently analagous to those of S. cerevisiae (Williamson, 1985).

Identification of the DNA sequences responsible for the autonomous replication of pUGLG1:kan in P. chrysosporium is an important future goal in our laboratory as it should be very useful in further developing a shuttle vector which can be used to isolate P. chrysosporium genes directly by complementation of appropriate mutants.

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APPENDIX A

USE OF A SHUTTLE VECTOR FOR THE TRANSFORMATION OF THE THE WHITE ROT BASIDIOMYCETE PHANEROCHAETE CHRYSOSPORIUM

By

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USE OF A SHUTTLE VECTOR FOR THE TRANSFORMATION OF THE WHITE ROT BASIDIOMYCETE, Phanerochaete chrysosporium+

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A novel shuttle vector based spheroplast transformation system for the lignin degrading filamentous fungus P. chrysosporium is described. The transformation vector, designated pRR12, consists of the yeast integration plasmid YIp5, a putative autonomous replication sequence (ars) of P. chrysosporium, and a 2.2 kb PvuII fragment carrying kan^r determinant from plasmid pNG35, which confers resistance against both kanamycin and the related antibiotic G418. Two different strains of P. chrysosporium (ME446 and BKM-F) were transformed to G418 resistance using vector pRR12. Approximately 20 transformants per µg of vector DNA were obtained. The transforming vector pRR12 could be recovered from the total DNA of transformants by E. coli transformation, albeit at a low frequency. © 1989 Academic Press, Inc.

Lignin is a highly complex, amorphous, aromatic polymer, which is a major component of woody plants, and is the second most abundant renewable organic resource on the earth (1). Research has intensified worldwide on ligninolytic microorganisms and their enzymes because of their potential industrial applications in biopulping and in the conversion of lignin and lignocellulosic materials to useful chemicals (1,2). Phanerochaete chrysosporium, a white-rot basidiomycete, is a rapid lignin degrader and is the most widely studied organism from both the basic and applied standpoints. Genetic analysis of this organism has been limited, but recently auxotrophic mutants and mutants lacking various secondary metabolic activities have been isolated (3-6) and a protoplast fusion system has been described (7,8).

One of the long range objectives of research in our laboratory is to develop a molecular genetic approach to the analysis of lignin degradation by P. chrysosporium and to study the regulation of secondary metabolism in this organism. To this end, cDNAs coding for lignin peroxidases, H_2O_2 dependent extracellular heme proteins expressed during secondary metabolism, and genomic clones encoding these proteins have been isolated and sequenced (9-13; Zhang, Y. Z. and Reddy, C. A. 1988. Abstr. Annu. Mtg. Am. Soc. Microbiol. H51).

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An integrative transformation system for P. chrysosporium has also recently been described (14). We describe here the development of a novel transformation system for P. chrysosporium in which the transforming vector can be isolated intact from the genomic DNA of G418 resistant fungal transformants by E. coli transformation.

MATERIALS AND METHODS

Strains and Media. P. chrysosporium strains ME446 (ATCC 34541) and strain BKM-F (ATCC 24725) were maintained on malt extract slants as previously described (2). E. coli DH5α [F-,endA1,hsdR17(rk-,mk+),supE44,thi-1,recA1, gyrA96,relA1, Δ(argF-lacZYA)U169, P80dlacZ ΔM15, λ-], used for the maintenance of plasmid vectors, was maintained as described (15). Gold's medium B (16) supplemented with 10% sorbose and 3% agar (Difco) was used for regeneration of P. chrysosporium spheroplasts. A sterile aqueous solution of G418 (Giboo Laboratories, Chagrin Falls, OH) was added to the autoclaved, cooled medium to a final concentration of 100 μg/ml. E. coli was grown in L broth (15) supplemented with 50 μg/ml of kanamycin.

DNA Isolations and Manipulations. Plasmid DNA was isolated from E. coli by the method of Holmes and Quigley (17) and was purified through a two step CsCl-Ethidium bromide gradient (18) for use in transformation. P. chrysosporium DNA was isolated by the method of Rao and Reddy (19). All molecular biological techniques were performed according to Maniatis et al. (15).

Construction of pRR12. A 5.8 kb derivative of plasmid pRR2, which contains a putative ars of P. chrysosporium inserted into YIp5, has previously been described (19). This plasmid was further modified by the addition of the 2.2 kb kan^r determinant from pNG35 (20) and was designated pRR12 (Fig. 1).

Transformation of P. chrysosporium. Conidial suspensions (2×10^8 ml^{-1}) were prepared from malt extract plate cultures of P. chrysosporium as described (2) and used to inoculate 100 ml of malt extract broth in 250 ml foam plugged Erlenmeyer flasks, which were then incubated with shaking (150

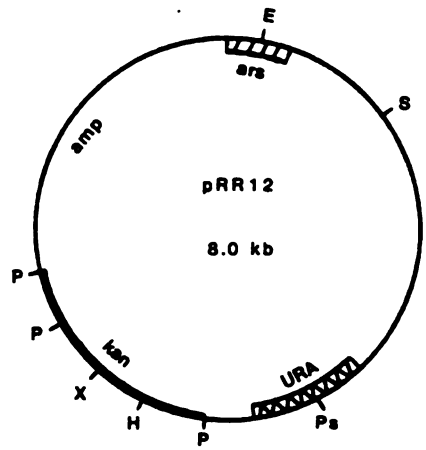

Figure 1. Construction of vector pRR12. A 5.8 kb derivative of pRR2 (16) which contains a putative autonomous replication sequence (ars) of P. chrysosporium was cut with PvuII and was ligated to a 2.2 kb PvuII fragment of pNG35 (20) which contains the kan^r determinant of Tn903. Legend: Thick line within the PvuII sites is the kan^r determinant, with the arrows indicating inverted repeat regions. E: EcoRI; H: HindIII; P: PvuII; Ps: PstI; S: SalI; X: XhoI.

rpm) at 30°C until small germ tubes were visible microscopically on a small fraction of the conidia (approximately 8 hrs). The conidia were then spun down in a clinical centrifuge, rinsed in sterile H_2O, resuspended in 10 ml of 1 M sorbitol containing 5 mg/ml novozyme 234 (Novo Industries, Copenhagen, Denmark) and incubated with shaking (150 rpm) for 80 min at 30°C. Spheroplast formation was monitored by the addition of 20 µl of 10% sodium dodecylsulfate to 20 µl of the above spheroplast suspension and checking for lysis under a phase contrast microscope. The spheroplasts were then spun down at low speed in a clinical centrifuge and were washed twice in 1 M sorbitol, once in SMC [1 M sorbitol, 10 mM MOPS(3-(N-Morpholino) propanesulfonic acid), 50 mM $CaCl_2$, pH 6.3] and resuspended in 0.4 ml SMC plus 0.1 ml PMC [40% PEG (Polyethylene glycol, BDH Chemicals Ltd., Poole, England), 10 mM MOPS, 50 mM $CaCl_2$] and 10 µl dimethyl sulfoxide. An equal volume of spheroplasts were added to each DNA sample, preincubated in 80 µl of 5 mg/ml heparin in 10 mM MOPS for 30 min, and to a negative control containing no DNA. The tubes were incubated on ice for a further 30 min, vortexed after adding 2.5 ml PMC and incubated at room temperature for 20 min. Each sample was then split into two aliquots, 10 ml top agar (Golds medium B; 16) supplemented with 10% sorbose and 100 µg/ml G418 was added to both aliquots, and each reaction mixture was poured onto a plate of the spheroplast regeneration medium described above. For the determination of regeneration frequency 50 µl of the negative control (no DNA) is added to the same regeneration medium but without G418. After 5-7 days incubation at 37°C, individual colonies become visible on this medium.

Recovery of Vector DNA from Transformants. Colonies of G418 resistant transformants from the previous step were inoculated onto malt extract plates supplemented with 100 µg/ml G418. After 4 days of growth, the mycelia were scraped from these plates and DNA was isolated as described (19). Total DNA from G418 resistant P. chrysosporium transformants was used to transform E. coli using the method of Hanahan (21). High frequency transformation of E. coli is critical since no transformants were recovered unless the transformation frequency was at or above 1×10^8/µg DNA standard (CsCl-EtBr gradient purified pUC18). Transformed E. coli were plated on LM medium (21) containing 50 µg/ml of kanamycin.

RESULTS AND DISCUSSION

The kan^r determinant of Tn903 (20), which encodes an aminoglycoside phosphotransferase is known to confer resistance to both kanamycin and G418, and has been shown to be expressed in E. coli as well as in several eukaryotic systems (22,23). Our initial results showed that growth of P. chrysosporium was completely inhibited on regeneration medium containing 50 µg/ml of G418. A concentration of 100 µg/ml G418 was thus chosen for selection in all transformation experiments.

Transformation of P. chrysosporium.

Vector pRR12 transformed both strains of P. chrysosporium (ME 446 and BKM-F) yielding about 20 G418 resistant transformants per µg DNA (Table 1). Negative controls with no DNA added or with salmon sperm DNA substituted for pRR12 DNA did not yield any transformants. The purity of the vector DNA appears to influence the transformation efficiency as the plasmids purified in a CsCl-ethidium bromide gradient gave approximately three-fold higher transformation efficiency as compared to that observed with plasmids isolated by the method of Holmes and Quigley (17). Similar variation in transformation

Table 1. Transformation of P. chrysosporium with vector pRR12[a]

Strain	Plasmid	Transformation Frequency (no./µg DNA)	Transformation Efficiency (no./regenerated spheroplast)
ME446	no DNA	0	0
	pRR12	6.6	3.3×10^{-3}
	pRR12[b]	22	1.1×10^{-2}
BKM-F	no DNA	0	0
	pRR12[b]	19.9	9.9×10^{-3}

[a]Each strain was transformed with 10-20 µg vector DNA. A sample with no DNA added was used as a negative control. Transformants were counted as colonies growing on regeneration medium containing 100 µg/ml G418.

[b]Purified through a CsCl-ethidium bromide gradient (18).

efficiency based on the purity of the vector DNA has been reported in other organisms also (24). The transformation efficiency, as determined by the number of transformants per regenerated spheroplast was comparable to that obtained for high frequency transformation of Neurospora crassa (25), and Aspergillus nidulans (26).

Recovery of Transforming Vector pRR12.

We used two approaches to recover the transforming vector pRR12 from P. chrysosporium transformants. The first was to attempt to recover plasmids from individual transformants. The second was to make DNA preparations from a pool of individual transformant colonies on a plate. Both approaches were successful in recovering intact pRR12 by E. coli transformation using genomic DNA of P. chrysosporium transformants. The plasmids recovered in E. coli were identical to pRR12 (Fig. 2, panel A) in that all had the same restriction pattern as the original vector. Furthermore, identical Southern hybridization patterns were seen with both pRR12 and the plasmids recovered from individual G48-resistant transformants when the kan^r determinant of pRR12 was used as the probe (Fig. 2, lanes c and d). However, this plasmid appears to be present in too low a copy number in the total genomic DNA of fungal transformants to be detectable by Southern hybridization using the kan^r determinant as a probe. Maintenance of the transforming vector in low copy number is also suggested by the low frequency of recovery of pRR12 (~ one transformant/10-30 µg genomic DNA) from fungal transformants. It is important to emphasize, however, that pRR12 was consistently recovered, although at a low frequency, from the transformants by E. coli transformation. The fungal transformants have been maintained on selective medium (malt extract slants containing 200 µg/ml G418) for

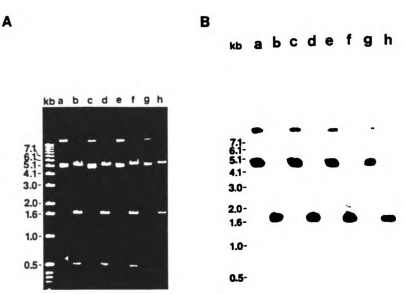

Figure 2. Recovery of pRR12 from P. chrysosporium transformants. One µg of plasmid DNA was electrophoresed on a 0.7% agarose gel, blotted onto nitrocellulose and hybridized with the kan^r determinant (1.7 kb PvuII fragment of pRR12). Panel A is the ethidium bromide stained photograph of the agarose gel and Panel B is the Southern blot of the same gel. Lanes a, c, e, and g contain uncut plasmid DNA and lanes b, d, f, and h contain PvuII digested plasmid DNA. Lane a and b contained pRR12; lanes c and d, and e and f contain separate plasmids recovered from the genomic DNA of fungal transformants via E. coli transformation; plasmid shown in lane e was used to retransform wild type P. chrysosporium to G418 resistance, and from the total DNA of one of those transformants the plasmid shown in lanes g and h was recovered.

over a year and the vector recovered from such transformants was identical to pRR12.

Plasmids recovered from the genomic DNA of transformants (Fig. 2, lanes e and f) were used to retransform P. chrysosporium to G418 resistance successfully at frequencies similar to that observed with the original pRR12. Plasmids recovered from these second generation transformants (Fig. 2, lanes g and h) were also identical to pRR12. These results indicate that pRR12 can be passed intact through P. chrysosporium repeatedly and recovered from G418 resistant fungal transformants by E. coli transformation. Further studies are in progress to understand the nature of this novel transformation system mediated by vector pRR12.

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