Linkage Analysis and Lignin Peroxidase Gene Expression in *Phanerochaete chrysosporium*

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work and I have not previously in its entirety or in part submitted it at any university for a degree.

Signature **Date**

SUMMARY

Wood is composed of three main components: cellulose, hemicellulose and lignin. Cellulose is the main structural polymer, whereas the function of lignin in plants is to impart rigidity to the cells, to waterproof the vascular system, and to protect the plant against pathogens. A group of microorganisms, called white-rot fungi, are able to selectively degrade the lignin and hemicellulose from wood leaving the cellulose virtually untouched. The most widely studied fungus of this group is the basidiomycete *Phanerochaete chrysosporium*, which has become a model organism in studies of lignin degradation.

Lignin is a large, heterogenous and water insoluble polymer and therefore the enzymes needed to degrade it have to be extracellular and non-specific. There are a number of enzymes that are involved in the degradation of lignin, including lignin peroxidases, manganese dependent peroxidases and laccases. Laccases are blue copper oxidases that require molecular oxygen to function, whereas lignin peroxidases and manganese peroxidases are heme proteins that require hydrogen peroxide. *Phanerochaete chrysosporium* has all three of these enzymes, as well as a system for producing the hydrogen peroxide that is necessary for peroxidases to function.

For both scientific and industrial purposes, it is important to obtain linkage maps of the positions of genes in the genome of an organism. Most fungi, including *P. chrysosporium*, lack easily identifiable phenotypical markers that can be used to map the position of genes relative to each other on the genome. Previous methods of mapping genes in *P. chrysosporium* involved auxotrophic mutants, radioactivity, or the use of hazardous chemicals. Here we describe an automated DNA-sequencing based mapping technique that eliminates many of the problems associated with previous techniques. Portions of the genes to be mapped were amplified from homokaryotic single basidiospore cultures using gene specific primers using the polymerase chain reaction (PCR) technique. The PCR products were sequenced to determine the segregation of alleles. Two previously mapped lignin peroxidases, *lipA* and *lipC*, were used to develop this method, and the results obtained corresponded to the known genetic linkage. A newly characterised β-glucosidase encoding gene from *P. chrysosporium* was also mapped. Linkage was found between the β-glucosidase gene and a histone (H1) encoding gene.

In *P. chrysosporium* the lignin peroxidase isozymes are encoded by a family of at least ten genes. Previous studies with *P. chrysosporium* BKM-F-1767 in defined media, wood and soil have shown differential expression of the lignin peroxidase isozymes. In this investigation the levels of expression of lignin peroxidases in *P. chrysosporium* ME446 cultures grown in nitrogen or carbon limited defined liquid media, as well as on aspen wood chips were determined by competitive reverse transcriptase polymerase chain reaction (RT-PCR). These results were compared to those previously obtained from *P. chrysosporium* BKM-F-1767 to evaluate strain specific variation in the expression of lignin peroxidases. The results indicate that, although there were many similarities in the patterns of lignin peroxidase expression, there were also enough differences to conclude that there were strain specific variations in the temporal expression of the lignin peroxidases.

To conclude, a fast and cost effective method for mapping genes in *P. chrysosporium* was developed. Also, we showed that strain specific variation in temporal expression of lignin peroxidases occurs.

OPSOMMING

Hout bestaan uit drie hoof komponente nl. sellulose, hemisellulose en lignien. Sellulose is die hoof strukturele polimeer, terwyl die funksie van lignin in plante is om die selle te versterk, die vaskulêre sisteem waterdig te hou, en die plant teen patogene te beskerm. 'n Groep mikroörganisms, bekend as witvrotswamme, kan lignien en hemisellulose selektief uit die hout verwyder, terwyl die sellulosevesels oorbly. Vanuit hierdie groep swamme is die meeste navorsing op die basidiomiseet *Phanerochaete chrysosporium* gedoen.

Lignien is 'n groot, heterogene polimeer en is onoplosbaar in water. Die ensieme wat benodig word om lignien af te breek is daarom nie-spesifiek en kom ekstrasellulêr voor. 'n Aantal ensieme is by die afbraak van lignien betrokke, insluitend lignienperoksidase, mangaanperoksidase en lakkase. Lakkase is 'n blou koperoksidase wat suurstof benodig vir aktiwiteit. Lignienperoksidase en mangaanperoxidase is heemproteïene en benodig waterstofperoksied. *Phanerochaete chrysosporium* het al drie van hiedie ensieme, sowel as 'n sisteem wat waterstofperoksied produseer.

Vir beide wetenskaplike en nywerheidsdoeleindes is koppelingskaarte wat die posisie van gene in die genoom van 'n organisme aandui noodsaaklik. Die meeste swamme, *P. chrysosporium* ingesluit, het geen fenotipiese merkers wat maklik van mekaar onderskei kan word nie, en dit is dus moeilik om 'n kaart van die ligging van gene op die genoom te bepaal. Vorige metodes om gene in *P. chrysosporium* te karteer het auksotrofiese mutante, radioaktiwiteit of gevaarlike chemikalieë gebruik. Ons beskryf 'n metode wat van automatiese DNA-volgordebepaling gebruik maak en wat baie van die tekortkominge van die ou metodes oorkom. Dele van die gene is met geen-spesifieke PKR-amplifikasie uit kulture van homokariotiese enkel basidiospore verkry en die DNA-volgorde is bepaal om die segregasie van die allele te ondersoek. Twee gene waarvoor 'n koppelingskaart alreeds uitgewerk is, *lipA* en *lipC*, was gebruik om hierdie metode te ontwikkel. Die resultate stem ooreen met die bekende genetiese koppeling tussen hierdie gene. 'n Geen wat onlangs in *P. chrysosporium* ontdek is, nl. ß-glucosidase, is ook met hierdie metode gekarteer. Koppeling is met 'n histoon (H1) geen gevind.

Die lignienperoksidase isoensieme in *P. chrysosporium* word deur 'n familie van ten minste tien gene gekodeer. Vorige navorsing met *P. chrysosporium* BKM-F-1767 in gedefineerde media, hout en grond het getoon dat 'n variasie in die uitdrukking van

lignienperoxidase isoensieme voorkom. In hierdie ondersoek is 'n kultuur van *P. chrysosporium* ME446 in stikstof- of koolstof-beperkende vloeibare media opgegroei, as ook op aspen houtblokkies. Die vlak van uitdrukking van die lignienperoksidases is deur middel van die omgekeerde transkripsie polimerasekettingreaksie (RT-PKR) bepaal. Die resultate vir *P. chrysosporium* ME446 is vergelyk met vorige resultate van *P. chrysosporium* BKM-F-1767 om te bepaal of stamspesifieke variasies in die uitdrukking van lignienperoksidases voorkom. Daar is 'n aanduiding dat, alhoewel soortgelyke patrone in die vlakke van lignienperoksidase uitdrukking voorkom, daar ook noemenswaardige verskille is. Hieruit kan afgelui word dat stamverwante variasie van lignienperokisdase uitdrukking voorkom.

Ten slotte, ons het 'n vinnige, goedkoop metode om die gene in *P. chrysosporium* te karteer ontwikkel. Ons het ook bewys dat stam-spesifieke variasie in die uitdrukking van die lignienperoxidase gene voorkom.

BIOGRAPHICAL SKETCH

Simon Allsop is a South African citizen, born in Somerset West on the 30th of August 1968. He attended Gordons Bay Primary School, Wynberg Boys High, and Hottentots Holland High School, where he matriculated in 1986. After school, Simon attended the University of Stellenbosch where he obtained a B. Sc. Degree in Microbiology and Genetics in December 1990. Between 1991 and 1996 Simon completed his national service in the South African Defence Force and worked in the private sector before returning to the University of Stellenbosch. Simon completed his B. Sc. Hons. Degree in Microbial Ecobiotechnology in March 1997. His Masters degree was completed in the fungal biotechnology laboratory at the Department of Microbiology, University of Stellenbosch, with a portion of the practical work being done at the USDA Forest Products Laboratory in Madison, Wisconsin, USA.

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PREFACE

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately, and written according to the style of the journal to which it will be submitted.

Chapter three, "Genomic mapping in *Phanerochaete chrysosporium* BKM-F-1767 using automated sequencing", will be submitted for publication in BioTechniques.

Chapter four, "Expression of lignin peroxidase encoding genes in *Phanerochaete chrysosporium* ME446 grown on defined media and wood chips", will be submitted for publication in Applied and Environmental Microbiology.

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION AND AIMS OF STUDY

Introduction

Lignocellulose is the main building material in woody plants and is of tremendous ecological and economic importanee (2). Lignocellulose is composed of cellulose, hemicellulose and lignin. Hemicellulose and lignin polymers bind cellulose fibres, the main structural polymers in wood. This increases the strength of the plant cells, protects the plant from pathogens, and makes it waterproof (3). Cellulose is the world's largest renewable supply of polysaccharides and is therefore an important source of fuel, food and fibres. Lignin is a large heterogeneous polymer and is the world's largest renewable source of aromatic biomolecules.

Whereas many microorganisms are able to degrade cellulose and hemicellulose, a much smaller percentage are able to degrade lignin. White-rot fungi are a group of basidiomycete fungi that are almost unique in their ability to degrade all the components of wood (4). Many white-rot fungi also have the ability to degrade the lignin from wood while leaving the cellulose virtually untouched. The name "white-rot" refers to the rotted wood that has a white appearance once the brown lignin has been removed and the white cellulose remains. Apart from the very important roles of white-rot fungi in carbon cycling, food production and wood rotting, the ability to selectively degrade lignin has made white-rot fungi interesting from an industrial standpoint. Applications that have been suggested for these microorganisms and their enzymes are: the removal of lignin from pulp for the paper industry (6); the degradation of excess dyes in the textile industry (7); conversion of woody agricultural by-products to more nutritious animal feeds (5, 8) and the degradation of potentially harmful environmental pollutants (1).

White-rot fungi are able to degrade all the components of lignocellulose. This conversion of lignocellulose to carbon dioxide requires a number of different enzymes: cellulases, hemicellulases and ligninolytic enzymes. There are three main groups of lignin degrading enzymes, the lignin peroxidases (LiPs), the manganese dependent lignin peroxidases (also called manganese peroxidases or MnPs) and the laccases. Laccases are blue copper oxidases that require molecular oxygen (O_2) for activity. Lignin peroxidases and manganese peroxidases are heme proteins that require hydrogen peroxide (H_2O_2) as co-factors. Manganese peroxidase differs from lignin peroxidase in that it requires manganese for enzyme activity.

The most widely studied of the white-rot fungi is *Phanerochaete chrysosporium*, and this fungus has become a model organism in research into the mechanisms of lignin degradation. In *P. chrysosporium* the lignin degrading enzymes are encoded by families of genes, the

protein products of which are very similar. For instance, *P. chrysosporium* has at least ten isozymes of lignin peroxidase and three of manganese peroxidase, as well as a number of genes encoding enzymes for the degradation of cellulose and hemicellulose.

For any potential industrial application of white-rot fungi in general, and strains of *P. chrysosporium* in particular, more information on the function and regulation of the ligninolytic system is desirable. Firstly, the location of the lignin peroxidase genes in the genome, as well as the effect of location on the regulation of the genes, is of interest. Secondly, the question of why the fungus would carry multiple copies of what is essentially the same gene needs elucidation. Two possible explanations are: 1) that the copies are the result of random duplication events and that the superfluous copies will eventually be lost due to evolution, or 2) that each isozyme has a subtly different function and that they are therefore regulated according to the growth conditions. In view of the above the aims for this study were:

Aims

- To develop a system for quick identification of the allelic segregation of single basidiospores from *P. chrysosporium*.
- To use the system to map a previously unmapped gene (β-glucosidase) and to estimate its position in terms of other previously mapped genes.
- To develop competitive DNA templates and PCR primers that would allow the levels of lignin peroxidase mRNA to be quantified in *P. chrysosporium* ME446.
- To cultivate the white-rot fungus *P. chrysosporium* ME446 in defined liquid cultures and on wood chips and to isolate mRNA from these cultures.
- To quantify the levels of mRNA for each of the lignin peroxidase isozymes in the different media, and to compare the results to previous findings for *P. chrysosporium* BKM-F-1767.

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CHAPTER TWO

LITERATURE REVIEW

Introduction

The selective degradation of lignin from wood by white-rot fungi has brought these fungi to the attention of the pulp and paper industry where more cost-effective and environmentally friendly processes are being sought as alternatives to today's chemical and mechanical processing of wood. Paper and other fibrous materials are composed primarily of cellulose. White-rot fungi have the ability to selectively degrade the lignin from wood, leaving the cellulose virtually untouched (76). The degree that the white-rot fungi are able to degrade lignin is dependent on the type of wood. Therefore, the composition of wood in general, and the synthesis of lignin in particular, is discussed in this review.

The most widely studied of the white-rot fungi is the basidiomycete *Phanerochaete chrysosporium*. In this review the enzymes involved in lignin degradation in *P. chrysosporium* are discussed. In particular, the expression and regulation of the lignin peroxidase (LiP) enzymes, which are represented by a family of at least ten isozymes (50), are addressed. The question of why LiP is present as multiple isozymes is investigated, and in this regard the genetic organisation and the mapping of the lignin peroxidase genes are also discussed.

The Composition of Wood

Wood is mainly composed of cellulose, hemicellulose and lignin (47, 104). Cellulose is the main structural polymer of wood, and lignin and hemicellulose act as the "glue" holding the cellulose fibres together. Both cellulose and hemicellulose are composed almost exclusively of sugars and have repeating chains, whereas lignin is composed of phenolic monomers and does not have structural and stereo regularity. This difference in structure means that cellulose and hemicellulose are readily hydrolysed by enzymes, whereas lignin is relatively resistant to enzymatic attack.

The synthesis of lignin

Lignins are defined as biological macromolecules resulting from the random polymerisation of hydroxycinnamyl alcohol monomers (13, 90). Lignins are heterogeneous, three-dimensional polymers, with a high degree of cross-linking and no repetition in the pattern of the monomers (104). The synthesis of lignin can be subdivided into three stages: 1) the formation of L-phenylalanine and L-tyrosine from glucose via the shikimate pathway; 2) the formation of cinnamyl alcohols from L-phenylalanine and L-tyrosine via the cinnamate

pathway; and 3) the formation of free radicals, formed from ρ-hydroxycinnamyl alcohols during a reaction catalysed by plant laccases and peroxidases, which then polymerise in a non-enzymatic and random fashion to form a complex three-dimensional polymer. The structures of cinnamyl alcohol, and the derivatives that form the lignin precursors, are shown in Fig. 1.

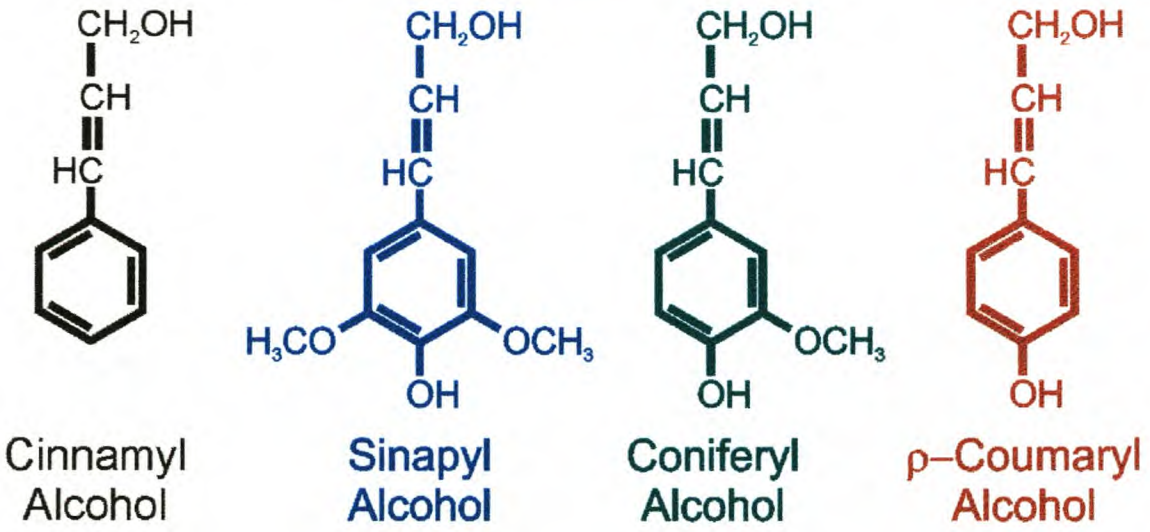

FIG. 1. The derivatives of cinnamyl alcohol: sinapyl (syringyl) alcohol, coniferyl (guaiacyl) alcohol and ρ-coumaryl alcohol. These molecules form the precursors of the lignin molecule. Adapted from Freudenberg (48).

The linking of lignin precursors is not the direct product of an enzymatic reaction. Rather, plant peroxidase enzymes form free radicals of the derivatives of cinnamyl alcohol, which then covalently link together in a random fashion to form the lignin polymer. The random polymerisation joins the lignin precursors together to form small and medium sized aggregates, which are "glued" together by further radicals to form larger entities. The sub-units are joined by carbon-carbon and ether bonds, with most of the bonds being β-aryl ether linkages, although numerous other linkages are formed (21, 48). These bonds are resistant to hydrolytic attack (14). Apart from being linked to other lignin molecules, lignin is covalently linked by ester bonds to the wood polysaccharides (cellulose and hemicellulose). Lignin impregnates the cellulose fibrils in the plant cell walls, filling spaces between the wood cells, thus forming the middle lamella (48).

The ratio between the amounts of the cinnamyl alcohol derivatives in lignin is distinctive of the wood type. In particular, softwoods (gymnosperms) typically contain larger amounts of coniferyl (guaiacyl) alcohol and less ρ-coumaryl and sinapyl (syringyl) alcohols, whereas hardwoods (angiosperms) contain approximately equal amounts of coniferyl and sinapyl alcohols, but low amounts of ρ-coumaryl alcohol (13, 48). The above is important because syringal type lignin is more susceptible to degradation by white-rot fungi (45).

A proposed model of spruce (*Picea abies*) lignin (Fig. 2), illustrates the different bonds formed (48). It must be remembered that the lignin molecule is a highly branched, three-dimensional non-repeating polymer, and no definite structure can be drawn. Therefore, the lignin structure pictured only illustrates the type of sub-units and bonds involved in the formation of lignin. The random nature of the bonds in the lignin molecule defies normal enzymatic degradation.

The function of lignin in the plant

The main functions of lignin in plants are for structural rigidity and pathogen protection (48). As a structural polymer, lignin surrounds the cellulose fibres, holding the fibres together and imparting rigidity to the cells. The heterogeneity of the lignin macromolecule also serves as a barrier against microbial attack and renders the plant immune to degradation by most plant pathogens. In fact, lignin concentration increases around the sites of wounds in plants (5, 20). After wounding there is a hypersensitive response, which includes the deposition of extracellular molecular barriers such as lignin. The lignin is very resistant to biodegradation and restricts microorganisms, that do not possess enzyme systems capable of degrading molecules that lack structural and stereo regularity, to the site of infection (21, 22, 35). A further, and very necessary, function of lignin is that it acts as a water impermeable seal for the xylem vessels of the plant vasculature (48).

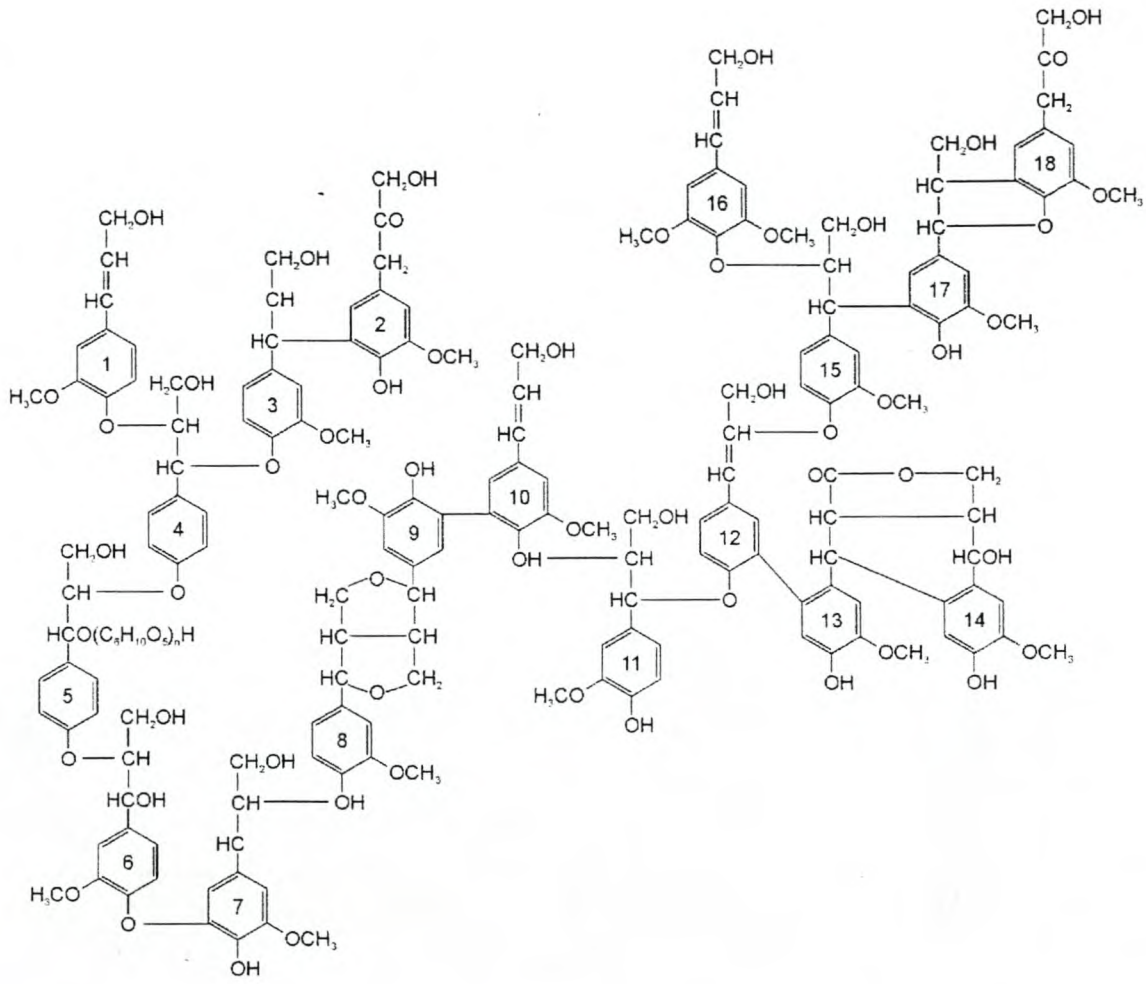

FIG. 2. A simplified schematic diagram of a lignin molecule. The cinnamyl alcohol derivatives have undergone oxidation and random coupling to form a complex molecule with no repeating units. It must be remembered that the actual lignin molecule is larger, more complex, and three-dimensional (21, 48). Adapted from Freudenberg (48).

White-rot Fungi

Collectively wood rotting fungi are able to degrade all the components of wood to carbon dioxide and they can be classified into three major categories based on the type of wood decay caused by these organisms: white-rot fungi, brown-rot fungi and soft-rot fungi (13). These terms attribute meaningful names to the groups of organisms, and describe the state of the wood after it has been rotted rather than characteristics of the fungus itself. White-rot fungi have the ability to selectively degrade the darkly coloured lignin, leaving the white cellulose virtually untouched. This gives the rotted wood a white appearance, and differentiates the white-rots from the brown rots, which degrade the cellulose and leave the

lignin and hemicellulose, thus giving the rotted wood a brown appearance. Soft rots degrade all the components of wood, which leaves the rotted wood "soft".

Phanerochaete chrysosporium

Since ligninolytic enzymes were discovered in *P. chrysosporium*, this organism has been the most widely studied of the white-rot fungi, and has been used as a model organism in studies elucidating the mechanisms involved in the degradation of lignin and lignin-like aromatic molecules. It was first isolated from wood chips in 1961 by Lyndgren and Eslyn (89). However, only the anamorphic (conidium forming) state was obtained and the fungus was identified as belonging to the *Sporotrichum* genus. Since then the anamorphic state of the fungus was variously classified as a species of either the *Sporotrichum* or the *Chrysosporium* genera. The names *Chrysosporium pruinosum*, *C. lignorum*, *Sporotrichum pruinosum* and *S. pulverulentum* have all been applied to this species in its imperfect state (24). In 1974 Burdsal and Eslyn described the telomorphic form, and named it *Phanerochaete chrysosporium* Burds (24). This name supersedes all the previous names for the fungus, although *C. pruinosum* may still be used to refer to the anamorphic state (23). *Phanerochaete chrysosporium* is classified in the division Eumycota, subdivision Basidiomycotina, class Hymenomycetes and subclass Holobasidiomycetidae. This subclass contains all the white-rot fungi, as well as most of the wood decay fungi.

The major characteristics of *P. chrysosporium* are rapid growth and metabolism of lignin, the ability to grow optimally at relatively high temperatures (39 to 40°C), the ability to grow on chemically defined media, low phenol oxidase activity and a lack of clamp connections (13, 23, 98, 120). *Phanerochaete chrysosporium* also has a high genomic G+C content of 59 %.

The life cycle of *P. chrysosporium* has not yet been studied in detail. What is known is that *P. chrysosporium* is a dikaryon and has both sexual and asexual states. *Phanerochaete chrysosporium* is one of the few white-rot fungi that produces an anamorphic state, and is able to produce conidiospores (asexual spores) as well as basidiospores (sexual spores). Only asexual conidiospores are produced during cultivation on agar with glucose, but the formation of a sexual stage including fruiting bodies and basidiospores can be induced by cultivation in a medium containing cellulose and thiamine, and with a low nitrogen content (59, 80). The basidiospores of *P. chrysosporium* are binucleate and homokaryotic (1). The ability to produce basidiospores is central to techniques used to map genes in *P. chrysosporium* that is discussed later (in the section "Genetic organisation in *Phanerochaete chrysosporium*").

It was not until 1994 that *P. chrysosporium* was isolated in South Africa (120). This specimen, isolated from indigenous forests near Knysna, was found to be identical to the description of Burdsal and Eslyn (24). Since then fifty-five strains of *P. chrysosporium* have been isolated in South Africa (36). Two were from indigenous wood samples, six from *Pinus* spp. and 47 from *Eucalyptus* spp..

Biopulping and bioremediation

The biological deterioration of wood can have major economic implications for the pulp and paper industry. Losses due to fungal growth on wood-chips in storage piles may be as high as 10 % (89). However, the biopulping process can limit losses due to deterioration of the wood chips, as well as decrease pulping costs. In biopulping selected fungi are used to pre-treat wood chips before conventional pulping occurs, removing lignin and lowering the chemical and/or energy requirements of the pulping process (13, 76). Fungal strains can be highly selective at removing lignin from wood. Removal of greater than 70 % of lignin, coupled with losses of approximately 15 % of glucose, have been reported (6, 76). Paper made of mechanical pulp from biological pretreated wood chips typically displayed lower yields than untreated wood (76, 85). However, paper strength properties increased and energy requirements decreased dramatically (up to 50 %). The disadvantages of biopulping include the long incubation times necessary to cultivate fungi on wood chips, and additional capital outlay for equipment. In a study evaluating the economic feasibility of biopulping Scott *et al.* (103) indicated that savings greater than US$10 per ton of pulp could be achieved. Although biopulping has been found to be economical, to date it has not replaced conventional pulping methods.

Other applications for fungi in the pulp and paper industry include the treatment of logs with fungi that do not stain wood to out compete staining organisms (69), as well as the pretreatment of wood chips with fungi to reduce pitch deposits in paper mills (60). Commercial applications for wood decay microorganisms are not limited to the pulp and paper industry. Increases in population and industrialisation have resulted in the release of many foreign chemicals, both natural and synthetic, into the environment (91). These foreign agents are termed xenobiotics, and are important contaminants of soil and water systems. Xenobiotic compounds released into the environment can be degraded in a number of ways: auto-oxidation, photo-oxidation and degradation by microorganisms. Compounds that are rapidly degraded are generally not an environmental problem, but compounds that are recalcitrant can accumulate in soil and sediments.

Recalcitrant xenobiotic compounds, which include pesticides, industrial wastes and dyes, are often phenolic compounds. One reason for the environmental persistence of these compounds is that they are large, insoluble in water or bound in the soil and therefore only poorly accessible to bacteria and most fungi (2, 22). However, the carbon skeletons of many xenobiotic pollutants are similar to structures within the model proposed for the lignin polymer (22), and it is logical to assume that organisms that are able to degrade lignin will also degrade these pollutants. Supporting this hypothesis are findings that white-rot fungi, including *P. chrysosporium* BKM-F-1767, are able to degrade numerous xenobiotic compounds, including: the pesticide DDT (2, 22), the phenoxyalkanoic herbicides 2,4-Dichlorophenoxyacetic acid and 2,4,5-Trichlorophenoxyacetic acid (117), the synthetic polymer nylon-66 (38), the wood preservative creosote (7) and the explosive TNT (2, 63), as well as many other compounds (2, 8, 21, 22). Similarly, white-rot fungi are able to decolourise a number of textile dyes (30, 55, 111). Although they are not poisonous, most synthetic dyes are also recalcitrant to biodegradation and cause aesthetic problems when they are discharged into bodies of water. White-rot fungi are able to degrade many of the polymeric dyes used in the textile industry, such as poly R, poly B and remazol blue, and these dyes may be used as indicators of lignin degradation (13).

Enzymology and Molecular Biology of Lignin Degradation

Although it has been known for many years that white-rot fungi can remove lignin from wood, the enzymes involved in this process have only recently been characterised. Experiments with whole cell cultures of *P. chrysosporium* showed that various growth conditions influenced the degradation of lignin (67, 75, 78). Ligninolytic activity is triggered by nutrient starvation, *viz.* limiting carbon, nitrogen and sulphur in cultures of *P. chrysosporium* increased ligninolytic activity. Nitrogen limitation is particularly effective in inducing the ligninolytic system. The source of nutrient nitrogen has little influence, but the concentration is important. The rate of lignin decomposition at a nitrogen concentration of 24 mM was found to be only 25 to 35 % of that at 2.4 mM (78). Apart from the concentration of nitrogen, Rothschild *et al.* (99) indicate that a low carbon/nitrogen ratio is also important. This ensures that the ligninolytic system is active in wood, where the nitrogen content is low (29).

While phosphorus, magnesium, calcium and other trace elements are important for fungal growth, and therefore for lignin degradation, they do not induce the ligninolytic system. The optimum pH for ligninolytic activity is between 4.0 to 4.5, with marked suppression of

activity above 5.5 and below 3.5. Oxygen tension also plays a role, with increased rates of delignification under 100 % oxygen (119).

Contrary to what is expected, lignin does not induce activity, and the appearance of ligninolytic activity occurs irrespective of the presence of lignin. Agitation of the culture, resulting in the formation of mycelial pellets, generally results in the suppression of lignin decomposition, but the addition of detergents to agitated submerged cultures permits the development of ligninolytic activity comparable to that routinely obtained in stationary cultures (65). When *P. chrysosporium* was grown in liquid cultures under conditions optimised for lignin metabolism, a reproducible sequence of events followed inoculation, as illustrated in Fig. 3 (75). In the first 24 hours germination occurred, followed by linear (primary) growth and the depletion of nutrient nitrogen. In the second 24 hours linear growth ceased and the depression of ammonium permease activity occurred (demonstration of nitrogen starvation). The appearance of ligninolytic activity occurred after 73 to 96 hours (measured as the breakdown of synthetic ^{14}C-lignin to $^{14}CO_2$). The ability to mineralise the xenobiotic chemicals mentioned in the previous section appears simultaneously with lignin degradation (2). Cultures started with fungal spores generally become ligninolytic after four days of growth. Hydrogen peroxide production, which is required by the LiP enzymes, also appears at about the same time.

The nature of the ligninolytic system is dictated by the structure of lignin. The heterogeneous nature of the lignin polymer, together with its large size (molecular weight 10^5 - 10^6 kDa) and water insolubility, dictate that lignin-degrading biocatalysts be extracellular and relatively non-specific (13). The non-specificity of the ligninolytic system is due to the enzymes acting via non-protein, diffusable mediators (14). Four classes of extracellular enzymes have been implicated in lignin degradation: lignin peroxidases (LiP), manganese peroxidases (MnP), laccases and the H_2O_2-generating enzymes (31).

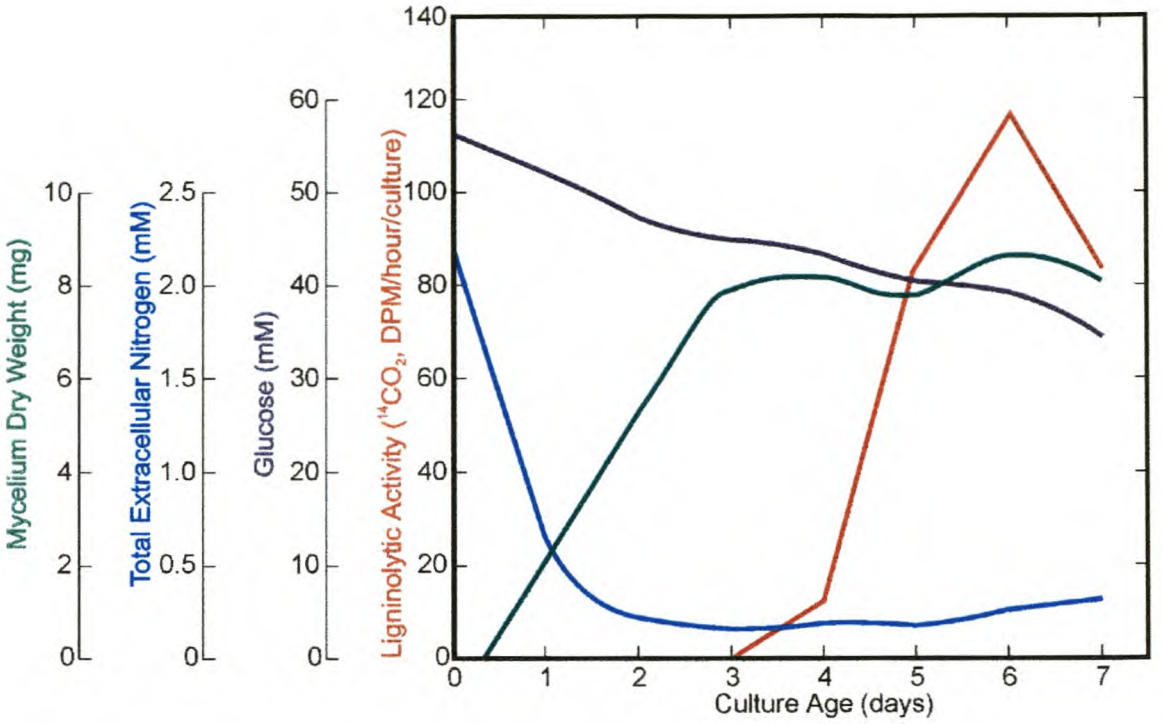

FIG. 3. ***Phanerochaete chrysosporium*** **ME446 continues to grow until nitrogen (light blue line) becomes a limiting factor. At this point the weight of the mycelium (green line) becomes stable, while the level of ligninolytic activity (red line) increases. Glucose (dark blue line) does not become limiting. Adapted from Keyser** ***et al.*** **(75).**

The first isolation of a ligninolytic enzyme was in 1983 when Glen *et al.* (57) found an H_2O_2-requiring enzyme system in the extracellular medium of ligninolytic cultures of *P. chrysosporium* ME446. The crude culture fluid oxidised a variety of lignin model compounds and the products were equivalent to the metabolic products isolated from intact ligninolytic cultures. The enzyme was not found in high nitrogen cultures or in cultures of a mutant *P. chrysosporium* strain incapable of metabolising lignin. The first isolation and purification of LiP was in 1984 by Tien and Kirk (113), and MnP was first discovered in the culture fluid of *P. chrysosporium* by Kuwahara *et al.* (83) in the same year. The heme containing extracellular ligninolytic enzymes have been classified based on their separation using high-pressure liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC). A further description of this classification is given in the section "Lignin peroxidase and manganese peroxidase isozymes".

When pre-grown on wood, the *P. chrysosporium* hyphae are encapsulated in a sheath consisting of β-1,3-1,6-D-glucan. Transmission electron microscopy and immuno-gold

staining revealed that the peroxidase enzymes was localised in the peripheral regions of the fungal cells in association with the cell membrane, fungal cell wall, and extracellular slime layer (33, 102). The localisation of the peroxidase enzymes close to the fungal hyphae was in part caused by the limited porosity of wood. Molecules as large as the peroxidase enzymes (Mr 30 000 to 50 000 Daltons) were not able to penetrate the wood structure. It has been proposed that the polysaccharide sheath provides a mode of transport of the fungal enzymes to their substrates at the surface of the wood cells (102). The sheath was also hydrolysed during the attack on lignin, indicating that it played an active role in providing the H_2O_2 necessary for the action of peroxidases. A schematic diagram of a fungal hyphal tip, together with the proposed interactions of the associated enzymes, is given in Fig. 4.

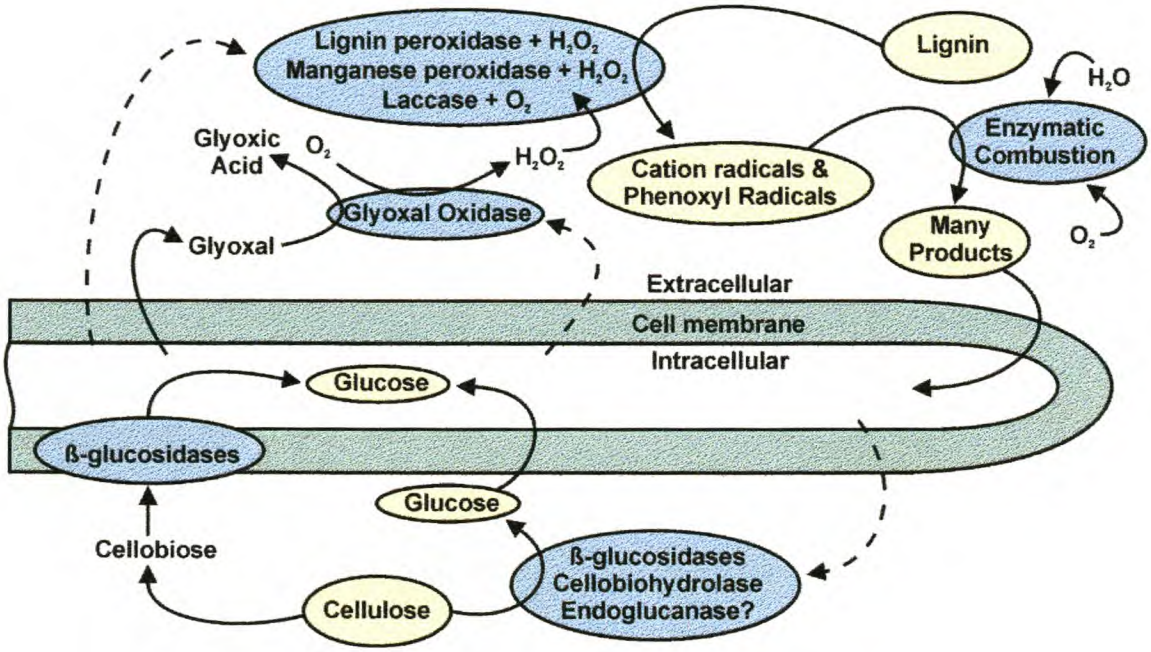

FIG. 4. A schematic representation of the ligninocellulose degradation system of *P. chrysosporium*. Adapted from de Jong *et. al.* (35) and de Koker (37).

Lignin peroxidase

Lignin peroxidase is a heme containing glycoprotein that requires H_2O_2 to function and is involved in the degradation of lignin and lignin-like compounds. Tien and Kirk (113) first described the LiP enzyme in 1984. The 42 000 dalton (SDS-PAGE) ligninase contained one protoporphyrin IX heme per molecule (90). Lignin peroxidase was found to be a monomeric

N- and probably O-glycosylated protein containing approximately 13 % by weight carbohydrate (35, 113). The glycosylation means that the original Mr of 42 000 as determined by SDS-PAGE may be an over estimation. The enzyme was able to cause the degradation of lignin and lignin model compounds, and the products were the same as with the intact fungus (61, 113). Atomic absorption spectroscopy indicated that the enzyme contained one iron atom, but not copper, zinc, manganese, molybdenum, or cobalt. The pH optimum was near pH 3.0, and the H_2O_2 optimum was at 0.15 mM H_2O_2. Although H_2O_2 is essential for activity, high concentrations (>5 mM) were found to be inhibitory. Lignin peroxidase enzymes catalyse reactions according to the multistep sequence of reactions illustrated in Fig. 5.

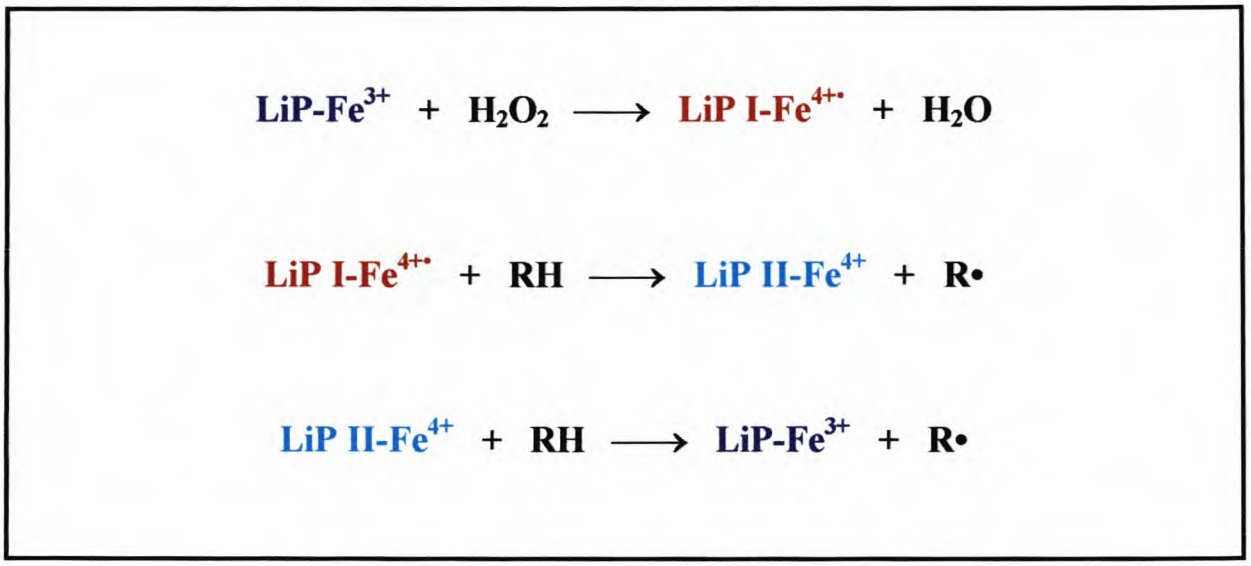

FIG. 5. Catalytic cycle of LiP indicating the various reduction states of the heme. Where LiP-Fe^{3+} (dark blue) is the native (resting) LiP enzyme, LiP I-$Fe^{4+\bullet}$ (red) and LiP II-Fe^{4+} (light blue) are the oxidised enzyme intermediates, RH is a phenolic or non-phenolic aromatic substrate or veratryl alcohol, and R• is the radical of the above compounds. Figure adapted from Boominathan and Reddy (13).

The resting LiP enzyme (LiP-Fe^{3+}) first reacts with H_2O_2 to form a two-electron oxidised intermediate (13, 32). The intermediate (LiP I-$Fe^{4+\bullet}$) causes a one-electron oxidation of substrate (RH) to form a cation radical (R•), and a one-electron oxidised intermediate is formed. The second intermediate (LiP II-Fe^{4+}) returns to its resting state by oxidising a second substrate. Both intermediates can oxidise the substrate to an aryl cation radical that

can undergo further non-enzymatic reactions to yield the final products. Many of the substrates that LiP (and MnP) is able to oxidise have redox potentials above what plant peroxidases can oxidise (13, 58, 95). This was illustrated by the ability of LiPs to oxidise milled wood lignin *in vitro*, which horse radish peroxidase could not accomplish (90). These unique oxidising potentials enable LiP to degrade a variety of aromatic compounds normally resistant to microbial degradation. Furthermore, LiPs have the ability to oxidise mediators (such as veratryl alcohol, but not Mn^{2+}) which in turn oxidise molecules that are poor substrates for LiP (32, 105). These low molecular weight mediators may be able to penetrate the wood and degrade lignin that is inaccessible to LiP.

Manganese dependent lignin peroxidase

A number of the extracellular peroxidases produced by *P. chrysosporium* require manganese as a co-factor. These enzymes are termed manganese dependent lignin peroxidases, or just manganese peroxidases (MnP). Manganese peroxidase has the ability to degrade lignins, e.g. in studies using ^{14}C-labled synthetic lignin, mutants of *P. chrysosporium* ME446 without detectable levels of LiP showed ligninolytic activity and the production of $^{14}CO_2$ (10). FPLC analysis of the concentrated extracellular fluid confirmed that the mutant produced only MnP, whilst under the same culture conditions the wild type produced both LiPs and MnPs.

The first report of a manganese dependent peroxidase purified from the extracellular medium of ligninolytic cultures of the white-rot fungus *P. chrysosporium* ME446 was in 1984 by Kuwahara *et al.* (83). The purified enzyme was capable of oxidising a wide variety of aromatic dyes, NADH and lignin model compounds in vitro, and was dependent on Mn^{2+} for activity. It had optimal activity at a pH of 4.5, at 100 μm Mn^{2+}, and at approximately 100 μm H_2O_2. Further analysis revealed that the enzyme contained one molecule of heme, as iron protoporphyrin IX, and had a molecular mass of 41 kDa determined by gel filtration and 46 kDa by SDS-PAGE (56).

Structure of lignin peroxidase and manganese peroxidase

Lignin peroxidases are heme containing proteins which are able to catalyse the oxidation of a large variety of substances through the reaction with hydrogen peroxide (3). The refined model consists of two LiP molecules in the asymmetric enzyme unit. Molecule 1 consists of all 343 residues, while molecule 2 consists of residues 1 to 341. Each monomer has one glucosamine and one heme ligand embedded in a crevice between the N- and C-terminal domains. The three-dimensional crystal structure of LiP was found to be similar to that of

cytochrome c peroxidase, despite a sequence homology of only 20 % (32, 42, 95). In Fig. 6 and Fig. 7 the structures of two common peroxidases are compared, and shared features noted. For instance LiP and cytochrome c peroxidase share catalytic site helices and scaffolding helices around the heme group. Accessible surface area views show that the peroxidases heme is buried. However, the heme is still accessible to its substrate, hydrogen peroxide, due to the small size of the hydrogen peroxide molecule.

The NMR spectrum of the MnP protein from *P. chrysosporium* is very similar to that of other peroxidases, including LiP, horse radish peroxidase and cytochrome c peroxidase (4). Some homology is also observed between LiPs and MnPs on a genetic and protein level. For instance, in *P. chrysosporium* BKM-F-1767 there is a 58 % similarity in nucleotide sequence and a 65 % similarity in amino acid sequence between MnP H4 and LiP H8 (93).

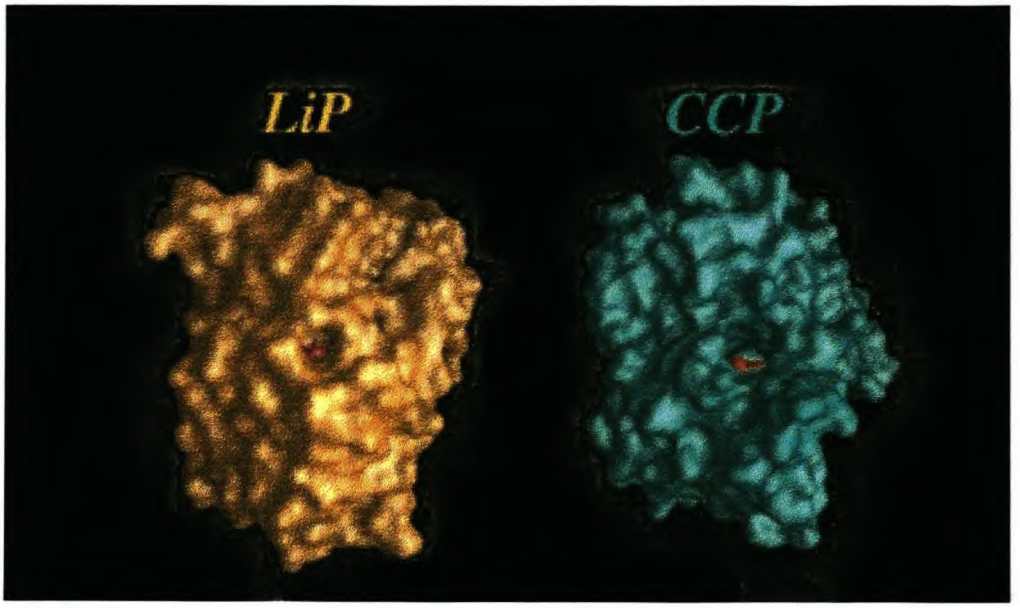

FIG. 6. Accessible surface area views of lignin peroxidase (LiP) and cytochrome c peroxidase (CCP) showing the buried heme group (red). The heme is accessible to the hydrogen peroxide molecule due to its small size. Adapted from

http://www.bch.msu.edu/labs/gravito/web/pghs_pox.html.

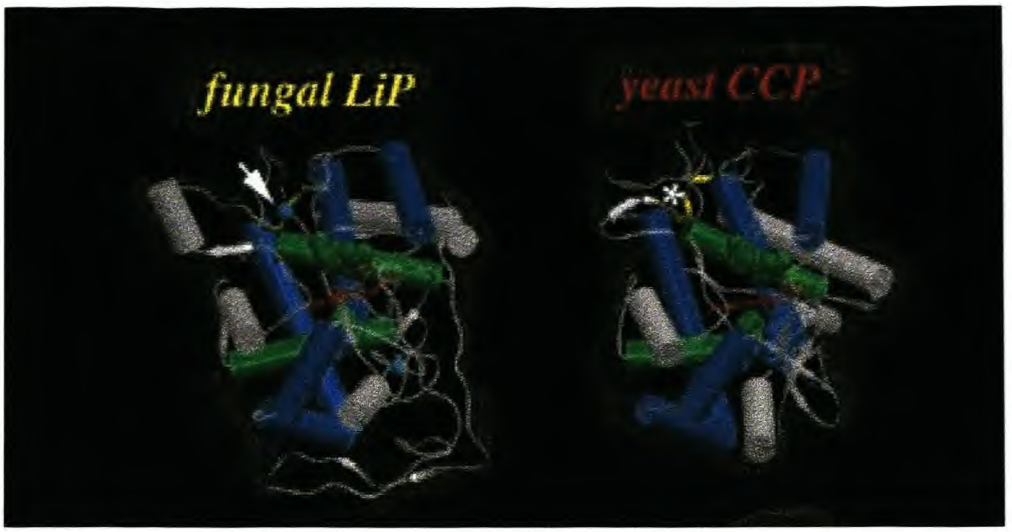

FIG. 7. The structures of lignin peroxidases (LiP) and cytochrome c peroxidase (CCP) share many of the same features, such as catalytic site helices (green), scaffolding helices (blue) and a heme core (red). Functional (arrow) and vestigial (*) calcium binding sites are also shown. Adapted from

http://www.bch.msu.edu/labs/gravito/web/pghs_pox.html

Lignin peroxidase and manganese peroxidase isozymes

Soon after the discovery of the peroxidase enzymes in *P. chrysosporium* came the discovery that the enzymes had a number of isozymes. These isozymes are very similar on both an amino acid as well as on the DNA level. The first evidence of multiple LiP and MnP enzymes was obtained when extracellular heme proteins were purified (112). Fast protein liquid chromatography (FPLC), using Mono-Q anion-exchange, revealed ten ligninase enzymes (peaks) that were labelled H1 to H10. These peaks can be seen in Fig. 8. Peaks H1, H2, H6, H7, H8 and H10 all had veratryl alcohol activity and are LiPs, while peaks H3, H4, H5 and H9 were dependent on manganese and represent MnPs (46, 112). Further purification and characterisation of the heme enzymes, especially isoelectric focussing, showed a number of differences between the isozymes. Leisola *et al.* (86) use analytical isoelectric focusing to separate the extracellular peroxidases into 21 proteins. Fifteen of these enzymes oxidised veratryl alcohol and were considered to be LiPs, while the other six were MnPs. Glumoff *et al.* (58) isolated and purified five isozymes of LiP from *P. chrysosporium*. The proteins differed from one another in terms of physical characteristics (e.g. isoelectric point and molecular mass) as well as functional characteristics (e.g. substrate specificity and stability). The N-terminal amino acid sequence was also determined for the isozymes. Differences in

the sequences between the isozymes, as well as differences in the amino acid composition of the isozymes, suggested that these proteins were the products of different genes.

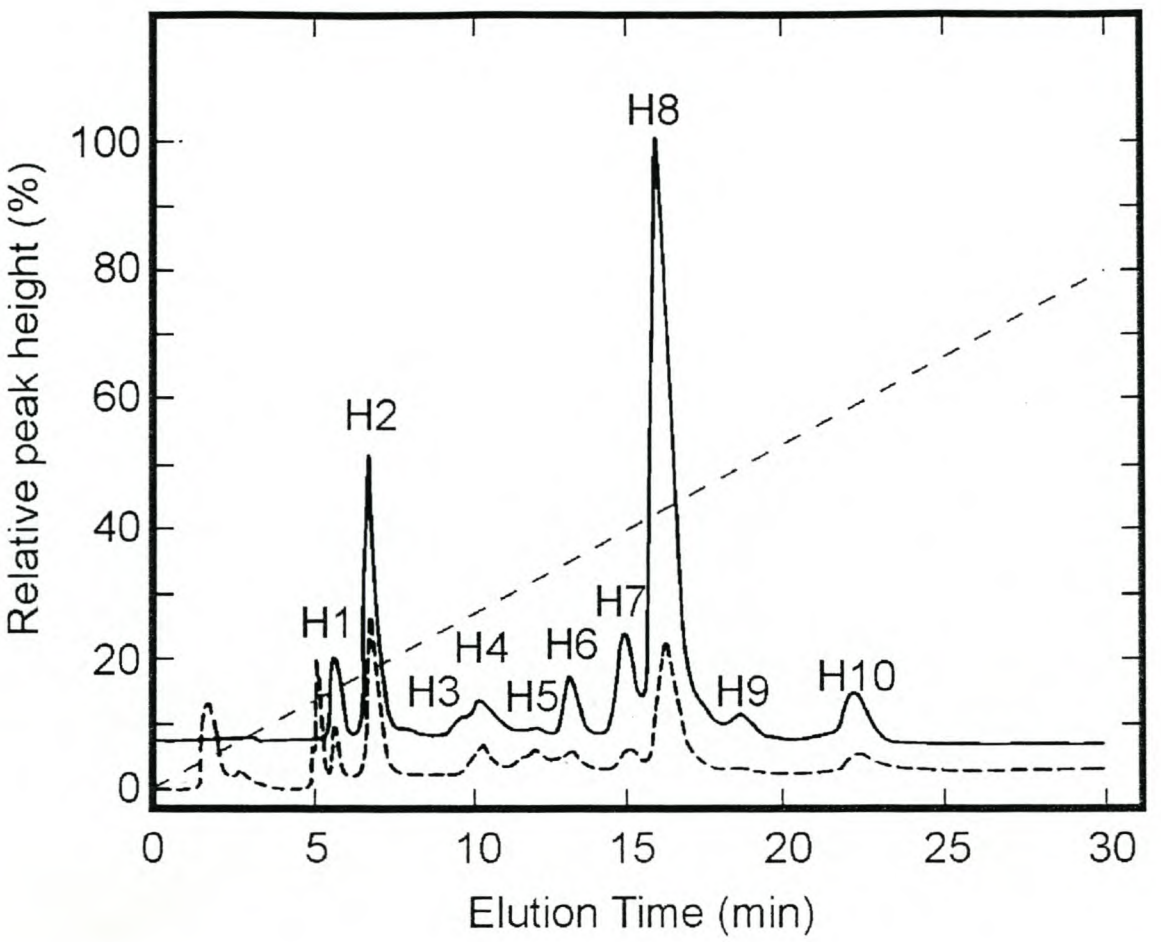

FIG. 8. FPLC profile of extracellular fluid from *P. chrysosporium* cultures. The solid and dashed lines represent absorbance at 409 and 280 nm respectively. The sloping line reflects the acetate gradient. Adapted from Tien and Kirk (112).

The number of isozymes of LiP and MnP produced by *P. chrysosporium* means that the regulation of this family of genes is still poorly understood. What is known is that the LiP and MnP genes are differentially expressed according to the nutrient conditions that the fungus finds itself in, and that this regulation takes place at the level of transcription. Post-transcriptional regulation may also be present, but at this stage the evidence suggests that regulation takes place before the formation of mRNA (discussed further in the section "Regulation of lignin peroxidase and manganese peroxidase").

The number of different LiP isozymes, and the close similarity of the enzymes, has led to confusion in the literature over the nomenclature of the isozymes. Gaskell *et al.* (50) proposed a system by which the genes are arbitrarily assigned letters A to J. Numbers 1 and 2 are assigned to the two alleles of *P. chrysosporium* BKM-F-1767, and 3 and 4 to the alleles of *P. chrysosporium* ME446. A list of the LiP encoding genes, with possible synonyms, is given in Table 1.

TABLE 1. Synonyms for the LiP enzymes and their encoding genes in *P. chrysosporium* (13, 39, 50, 79, 98, 110)

Designation	Source	Synonym
lipA1	BKM-F-1767	H8 (g); *lip*5; GLG3 (g); ML1 (c); LPOA (g)
lipA2	BKM-F-1767	LiPA (g); ML4 (c)
lipA3	ME446	LIG2 (g)
lipA4	ME446	LG1 (g)
lipB1	BKM-F-1767	none
lipB2	BKM-F-1767	LPOB (g)
lipB3	ME446	LIG3 (g)
lipC1	BKM-F-1767	H10; *lip*6; CLG5 (c); GLG5 (g)
lipC2	BKM-F-1767	H10; *lip*6; GLG2 (g); LiP6 (g)
lipD1	BKM-F-1767	H2; *lip*2; CLG4 (c); GLG4 (g); *LG2* (g); L18
lipD2	BKM-F-1767	LiP2 (g)
lipE1	BKM-F-1767	H7; *lip*4; LPO811 (c)
lipE3	OGC 101	L18 (c); LG2 (g)
lipF1	BKM-F-1767	CLG6 (c); GLG6 (g)
lipG1	BKM-F-1767	ML-5 (c)
lipH3	ME446	LIG1 (g)
lipI1	BKM-F-1767	0282 (g)
lipJ1	BKM-F-1767	V4 (g)
lipJ3	ME446	LIG4 (g)

Please note: c = cDNA clone; g = genomic clone

Laccase

Laccase is a blue-copper oxidase that is commonly found in white-rot fungi (35). It is classified as a polyphenol oxidase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2), and has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups. Laccase catalyses the four-electron reduction of oxygen to water and the simultaneous removal of one electron from the phenolic hydroxyl group of lignin model compounds to depolymerise them (in the presence of a mediator such as 2,2'-azino-di[3-ethyl-benzothiazolin-(6)-sulfonate] (ABTS)). Laccase is produced by almost all white-rot fungi,

including *Lentinula (Lentinus) edodes* and *Coriolus versicolor* (25, 118), with *P. chrysosporium* thought to be a notable exception (77). Although recent studies have detected low levels of extracellular laccase production in *P. chrysosporium* BKM-F-1767 (40, 107) these findings have been challenged by Podgornik *et al.* (94), who propose that Mn^{3+} may cause false positive reactions in laccase assays by the direct oxidation of ABTS.

Sources of hydrogen peroxide

Both LiP and MnP need hydrogen peroxide as co-factor. The necessity of H_2O_2 for lignin degradation was noted before the peroxidase enzymes were discovered. In 1983 Faison and Kirk (44) showed that addition of a H_2O_2 scavenger (catalase) to whole cultures strongly suppressed lignin degradation and noted a temporal correlation between production of extracellular H_2O_2 and lignin degradation in cultures of *P. chrysosporium*. Furthermore, H_2O_2 production was markedly enhanced by growth under 100 % O_2, mimicking the increase in ligninolytic activity characteristic of cultures grown under elevated oxygen tension. A role for H_2O_2 was established soon thereafter with the discovery, in the same organism, of H_2O_2-requiring peroxidases (ligninases) (57, 114). Since then a number of sources of H_2O_2 have been suggested, *viz.* glucose oxidase (70), glucose-2-oxidase (43) and glyoxal oxidase (GLOX) (73). Results of Kelley and Reddy (70) indicated that glucose oxidase activity was the primary source of H_2O_2 in ligninolytic cultures of *P. chrysosporium* and that the nutritional parameters which affect lignin degradation had a parallel effect on glucose oxidase activity. A second report of purification of a glucose oxidising enzyme (glucose-2-oxidase) which produces H_2O_2 during glucose starvation in *P. chrysosporium* was published in the same year (43). The following year Kersten and Kirk (73) identified a new source of hydrogen peroxide in *P. chrysosporium*, namely GLOX. They report that GLOX activity is expressed during secondary metabolism, when ligninases are also expressed, and that GLOX activity as well as its substrates are found in extracellular fluid of ligninolytic cultures.

Contrary to the results of Kelley and Reddy (70), Kersten and Kirk (73) proposed that, although glucose oxidase and glucose-2-oxidase may supplement the supply of H_2O_2, GLOX is the primary source of H_2O_2. Both glucose oxidase and glucose-2-oxidase are intracellular enzymes, but under ligninolytic conditions *P. chrysosporium* produces extracellular H_2O_2 to supply the extracellular ligninases. The close association between GLOX, LiP and enzyme substrate in cultures suggests a connection between these components, whereas the effectiveness of intracellular oxidases in supplying extracellular H_2O_2 is limited by intracellular catalase (71).

The GLOX enzyme has been purified and it's biochemical properties determined. It is a copper-containing glycoprotein with a molecular weight of 68 kDa (determined by SDS-PAGE) and two isoforms of pI 4.7 and 4.9 (72, 116). In *P. chrysosporium* GLOX is encoded by a single gene with two alleles that are located on a dimorphic chromosome unlinked to known LiP and MnP encoding genes and the cDNA and genomic DNA for both alleles have been sequenced (72, 74). The enzyme is relatively non-specific and substrates include formaldehyde, acetaldahyde, glycoaldehyde, glyoxal, glyoxylic acid, dihydroxyacetone, glyceraldehyde and methylglyoxal. These substrates are intermediary products of ligninolysis which suggests that the supply of H_2O_2 can be fuelled by the action of LiP on lignin itself (72, 113). Recent results indicate that sugars (including glucose and xylose) are also oxidised by GLOX (37).

Expression and Regulation of Lignin Peroxidase and Manganese Peroxidase

A family of at least ten genes encodes the LiP isozymes in *P. chrysosporium*. These isozymes, as well as other enzymes, are differentially expressed in response to conditions encountered during growth. However, the factors influencing the regulation of the genes and their associated proteins is still poorly understood.

cDNA as an indicator of lignin peroxidase expression

Early attempts to determine the levels of each isozyme, that are expressed under different culture conditions, relied on the extraction of the enzymes (34, 64). Fast protein liquid chromatography (FPLC) was performed on the extracellular fluid of cultures grown under different conditions. Unfortunately, analysis of gene expression at the protein level assumes total recovery of the protein. Protein isolation and purification is laborious, and yields may be variable. For instance, any membrane-bound or compartmentalised proteins may be recovered inefficiently (16).

The measurement of the mRNA encoding the protein to be studied eliminates a number of the problems associated with protein-based experiments. Northern blot analysis using gene specific probes has been used to study LiP expression over time (11). However, the reverse transcriptase enzyme allows for the rapid conversion of mRNA to DNA (called copy DNA or cDNA), which is less prone to enzymatic degradation. Subsequent amplification of the cDNA by means of the polymerase chain reaction (PCR) means that small amounts of mRNA can effectively be converted to levels of DNA that can be easily detected.

Unfortunately, the PCR process does not amplify DNA quantitatively, and further modifications to the reaction has to take place. In competitive reverse transcriptase PCR (RT-PCR) reactions a second DNA template of known concentration (called the competitive template) is added to the PCR mixture, along with the cDNA. After the PCR process the relative amounts of the two types of DNA are determined. It is then possible to determine if the cDNA in question has a higher or lower concentration compared to the competitive DNA template. By doing a number of PCR reactions using varying concentrations of competitive template it is possible to determine the concentration of the cDNA. Gilliland *et al.* (54) report that as little as 1 pg of cDNA can be quantified from 1 ng of total mRNA. They state that the technique is accurate enough to distinguish a two-fold difference in mRNA concentration. Souazé *et al.* (106) determined that when the ratio of mRNA to competitive template was between 0.66 and 1.5 the error of the final result was approximately 10 %. They also found that the technique was sensitive to increases or decreases in RNA concentration as low as 20 %.

Ideally the competitive template should be identical to the cDNA so that neither should be preferentially amplified (92). In practice the DNA molecules used are virtually identical, but with a significant difference that will allow them to be differentiated. One approach is to introduce a unique restriction site into a copy of the cDNA to form the template DNA. The template DNA can then be distinguished from the cDNA by restriction enzyme digestion following PCR. Alternatively, genomic plasmid DNA can be used as a competitive template provided the PCR primers are in separate exons that flank a small intron (100 to 200 bp). The amplified competitive template can be distinguished from the target cDNA by size. The disadvantage in using genomic DNA is the possibility that it may not be amplified as efficiently as target DNA, either because of its increased size or increased duplex melting temperature (54). Gilliland *et al.* (53) used competitive templates with either introns or restriction enzyme sites to quantify cytokine mRNA. They found that the ratio of products remains constant during the amplifications, and that both methods can provide accurate quantification of mRNA levels.

Chandler *et al.* (27), who observed reverse transcription inhibition of the PCR reactions at low concentrations, voiced reservations on the accuracy of the quantitative RT-PCR technique. They found that quantitative RT-PCR systems underestimated the actual RNA concentrations, and that the inhibitory effect of reverse transcriptase on the PCR was removed with increasing template concentrations beyond 10^5 to 10^6 copies. They concluded that RT-PCR quantification of mRNA at concentrations below 10^5 to 10^6 copies would not be an absolute

measure of RNA abundance. This issue was addressed by Janse *et al.* (66), who found no evidence of reverse transcriptase inhibition when quantifying LiP encoding mRNA in *P. chrysosporium* BKM-F-1767. Subsequently quantitative RT-PCR has successfully been used to quantify the levels of proteins in *P. chrysosporium* involved in lignin degradation. Stewart *et al.* (110) used a modification of the protocol of Gilliland *et al.* (54) to quantitate LiP transcripts in *P. chrysosporium* BKM-F-1767. They found the technique to be particularly suited for gene families such as LiPs.

Regulation of lignin peroxidase and manganese peroxidase

The ligninolytic system of *P. chrysosporium* is activated in response to nutrient limitation, sometimes called secondary metabolism. The organism will grow and not produce LiP and MnP, as well as GLOX, until either carbon, nitrogen or sulphur become limiting. Lignin peroxidase and MnP gene expression appears to be differentially regulated depending on the intracellular concentration of cAMP. Production of LiPs and MnPs are preceded by a sharp rise in intracellular cAMP concentration, and compounds that lower the intracellular cAMP concentrations affect the production of LiP and MnP. Lignin peroxidase production was generally more sensitive to the inhibitors than MnP production (12). Extracellular protease activity also decreases during the early stages of secondary metabolism when LiP activity is at its peak (26).

Contrary to expectations, lignin and veratryl alcohol, the substrates of LiP, are not inducers of the ligninolytic system. Veratryl alcohol is, however, a stabiliser of LiP activity and has been shown to increase the activity of LiP. Haemmerli *et al.* (62) and Tonon and Odier (115) concluded that veratryl alcohol protects LiP from excess H_2O_2 produced by the culture. Veratryl alcohol had no effect on MnP activity and inhibited GLOX activity (26, 62, 73).

Although little is known of the regulation of the LiP genes, analysis of the DNA sequences upstream of the LiP genes has revealed putative regulation sites. TATA boxes have been identified upstream of LG2 (*lipE*) (98) and H8 (*lipA*) (64). Putative activator protein-2 (AP-2) sites are also present in the upstream regions of 11 of 15 published *P. chrysosporium* LiP genes. In other organisms these elements have been shown to be responsible for gene activation in response to elevation of the intracellular level of cAMP, which in turn is associated with the onset of secondary metabolism in *P. chrysosporium* (39, 98).

There is also evidence to suggest that post-transcriptional modification may take place, and that LiPs are modified extracellularly. Rothschild *et al.* (100) showed that the predominant isozymes at peak LiP activity are modified extracellularly in older cultures. In Fig. 9 it can be

seen that the predominant isozymes in a four-day old culture of *P chrysosporium* were H1, H2, H6 and H8. After incubation of the extracellular fluid there was a decrease in the levels of these isozymes, and a corresponding increase in the in H1, Ha and Hb. Using ^{32}P-labeled LiP enzymes in conjunction with partially purified extracellular fluid they were able to show that this process took place extracellularly, and that the LiP isozymes H2, H6 and H8 were phosphorylated, while H1, Ha and Hb were not. One molecule of phosphate was incorporated per molecule of enzyme in the form of mannose-6-phosphate contained on an asparagine-linked oligosaccharide (82). These changes were the result of the dephosphorylation of the LiP isozymes by the extracellular enzyme mannose-6-phosphatase (101).

Johnston and Aust (68) found that LiP protein expression was not regulated by nutrient nitrogen but was rather inactive in nitrogen sufficient conditions due to lack of heme. However, their work was contradicted by Li *et al.* (88) whose results indicated that LiP expression was regulated at the level of gene transcription by nutrient nitrogen. They used Western blot (immunoblot) and Northern (RNA) blot analysis to show that protein and mRNA from LiP isozyme H8 (LiPA) were present in nitrogen-limited but not nitrogen-sufficient cultures.

Fungal cultures grown in the absence of Mn^{2+} develop normally but show no MnP activity. The MnP protein cannot be detected in the extracellular fluid or intracellularly, and no MnP mRNA is produced (Immunoblot and Northern blot analysis). These results indicate that Mn^{2+}, the substrate for the enzyme, not only affects the activity of MnP but is also involved in the transcriptional regulation of the MnP encoding genes. The amount of MnP mRNA is a direct function of Mn^{2+} concentration in the culture up to a maximum of 180 μm (17, 18). This phenomenon is not limited to *P. chrysosporium*. Similar results were found for *L. edodes*, where the appearance of MnP was affected by the concentration of Mn^{2+} in the culture medium (under low nitrogen conditions) (25). Northern blot analysis of mRNA from ligninolytic *P. chrysosporium* BKM-F-1767 cultures indicates that the level of MnP mRNA correlates with expression of the enzyme and its activity. This is consistent with the regulation of the enzyme being at the level of transcription (93). Apart from manganese, heat shock has also been shown to induce the production of MnP mRNA (but not LiP mRNA) (19, 52, 87).

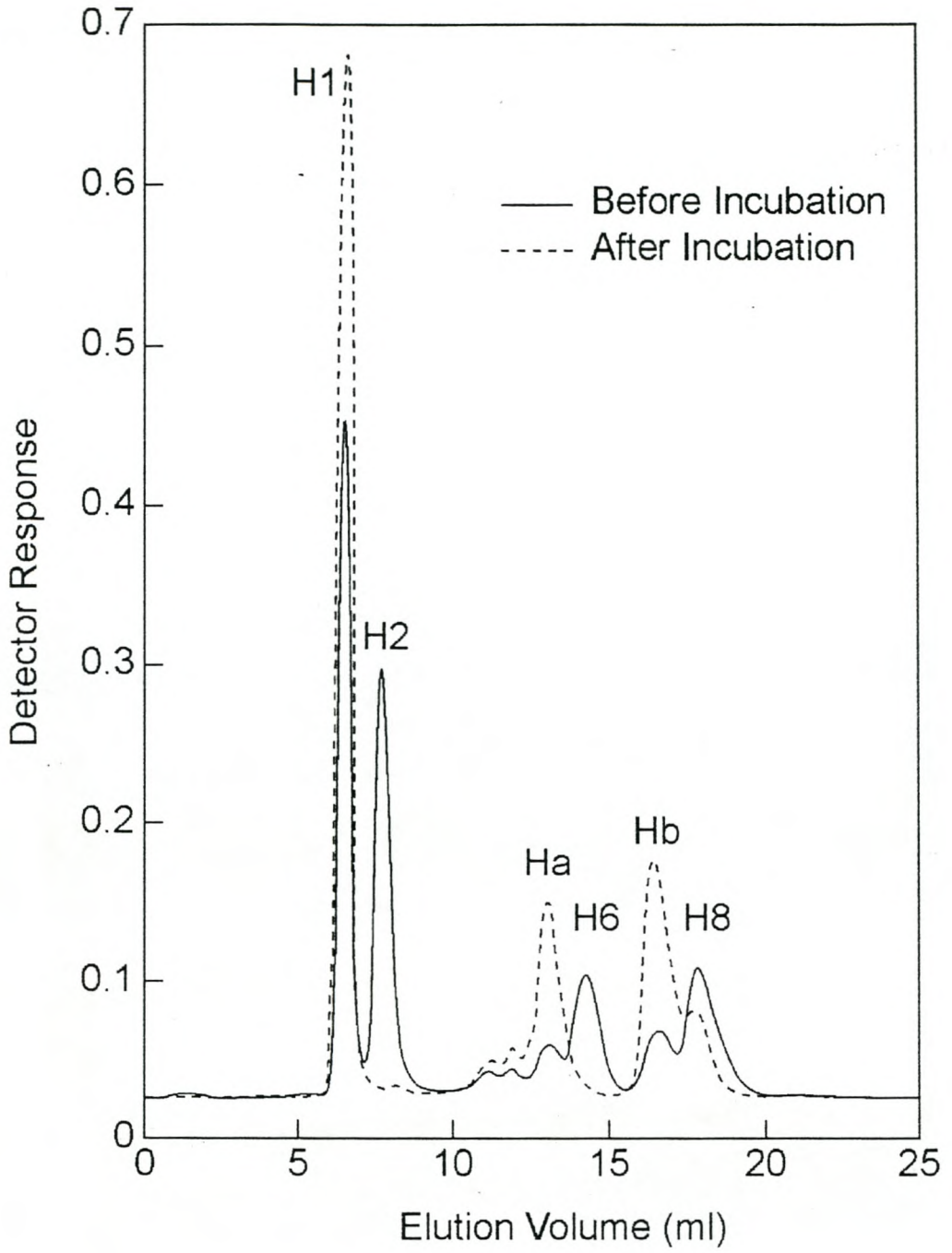

FIG. 9 The HPLC profile of the concentrated and dialysed extracellular fluid of a four-day old *P. chrysosporium* BKM-F-1767 culture. The heme protein levels were determined using strong anion-exchange HPLC (with monitoring at 409 nm) before and after incubation of the extracellular fluid for eight hours at 37°C. Adapted from Rothschild *et al.* (100).

The estimation of protein concentration by the quantification of mRNA levels (described in the previous section) assumes that no post transcriptional regulation of the protein occurs, and that the ration of mRNA to protein remains constant. Although knowledge of the regulation of the LiP genes is still incomplete, the quantitative RT-PCR protocol proposed by Gilliland *et al.* (54) and modified by Stewart *et al.* (110) has successfully been used to quantify the levels of proteins involved in lignin degradation in *P. chrysosporium* (66, 110).

Lignin peroxidase expression in liquid cultures

Holzbaur and Tien (64) compared the expression of extracellular peroxidase enzymes in *P. chrysosporium* BKM-F-1767 grown in carbon limiting and nitrogen limiting conditions. FPLC indicated that under nitrogen limitation the fungus produces a range of extracellular peroxidases, with major peaks for LiP isozymes H2 (*lipD*) and H8 (*lipA*). However, the carbon-limited cultures show a single major peak for isozyme H2 (*lipD*). Northern blot analysis using gene specific probes was used to detect the presence of mRNA encoding these enzymes. Messenger RNA for *lipD* and *lipA* was detected in nitrogen-limited cultures, while only *lipD* mRNA was detected in carbon deficient cultures.

In 1992 Stewart *et al.* (110) evaluated the expression of a larger number of LiPs in *P. chrysosporium* BKM-F-1767 grown in either nitrogen deficient or carbon deficient liquid media. Competitive RT-PCR reactions were used to determine the levels of LiPA (*lipA*), LiPB (*lipB*), 0282 (*lipI*), GLG5 (*lipC*), V4 (*lipJ*) and GLG4 (*lipD*). They found the level of *lipD* to be 1000-fold higher in carbon deficient cultures than in nitrogen deficient cultures. Conversely, *lipC* and *lipJ* were present in nitrogen deficient cultures, but were undetectable or present in very low levels in carbon limited cultures. The levels of *lipA*, *lipB* and *lipI* did not vary significantly between the two media, although *lipA* was at least ten fold more abundant than *lipB* and *lipI*. These results for *lipA* differ from the results of Holzbaur and Tien (64), which may be due to differences in experimental protocols, but may also be due to the high concentrations of *lipD* in cultures grown on carbon deficient media overshadowing the detection of *lipA*.

In a more comprehensive study Stewart *et al* (108) evaluated the expression of all ten known LiPs in *P. chrysosporium* BKM-F-1767 grown in either nitrogen deficient or carbon deficient liquid media. Their results confirmed the earlier work in that *lipD* was the dominant transcript in carbon limited cultures, and that *lipC* and *lipJ* were present in nitrogen limited cultures but present in low levels in carbon limited cultures (*lipC* was the dominant transcript). Furthermore, *lipE* and *lipI* were expressed at higher levels in nitrogen limited

cultures, while *lipH* and *lipF* were higher in carbon limited cultures. The three remaining *lips*, *lipA*, *lipB* and *lipG* were expressed in similar levels in both carbon and nitrogen limiting cultures. Stewart *et al* (108) also compared expression and genomic organisation of the LiPs in *P. chrysosporium* BKM-F-1767 (discussed further in the section "Genetic organisation in *P. chrysosporium*").

The high levels of *lipD* in *P. chrysosporium* BKM-F-1767 grown on carbon deficient media corresponds to the findings of Broda *et al.* (15) in *P. chrysosporium* ME446. They used gene-specific PCR amplification of cDNA to investigate the expression of enzymes involved with lignocellulose degradation, including LiPs, MnPs and cellobiohydrolases. They could not detect cDNA corresponding to these enzymes in cultures grown on media sufficient in carbon and nitrogen. However, in glucose deficient media *P. chrysosporium* ME446 produced abundant levels of LIG5 (*lipD*).

Lignin peroxidase expression in complex substrates

The use of quantitative RT-PCR is particularly advantageous when attempting to determine the levels of gene expression in cultures grown on complex substrates. *Phanerochaete chrysosporium* occurs naturally in soil and wood. The levels of enzyme produced in these conditions can be very low, and contaminating substances may hamper purification (84). Lamar *et al.* (84) reported a method for the quantification of fungal mRNAs in complex substrates using RT-PCR. Specifically the technique was used to detect transcripts of *lip* (*lipA*, *lipC*, *lipD* and *lipE*) and other genes in *P. chrysosporium* BKM-F-1767 grown in soil amended with the organo-pollutant pentachlorophenol (PCP). Transcript levels for *lipC* were at least 10-fold greater than *lipA* transcript levels in 4-day-old soil cultures. No *lipD* transcripts (common in carbon deficient liquid media) were detected when most PCP degradation occurs (days 2 to 4). In a similar evaluation using soil contaminated with the polycyclic aromatic hydrocarbon anthracene the levels of nine LiP encoding mRNAs from *P. chrysosporium* BKM-F-1767 were observed over a period of 25 days (9). Initially *lipA* and *lipD* had the highest levels. However, the highest levels recorded were for *lipJ* on day 20. Further investigation revealed that the levels of the mRNA transcript (*lipA*) and the LiP protein (H8) were well correlated, although they were separated by a 2-day lag period.

Similar results were found for *P. chrysosporium* BKM-F-1767 grown on Aspen wood (66). The expression of ten LiP genes, as well as other genes involved with lignocellulose degradation, was determined for two- and eight-week old cultures. At two weeks *lipA, lipD* and *lipJ* were the dominant cultures. After eight weeks *lipD* and *lipJ* were still high, but the

levels of *lipA* had decreased dramatically and *lipF* had increased and was the dominant transcript.

The above results for LiP expression in *P. chrysosporium* BKM-F-1767 grown on complex substrates, as well as the previous results on defined media, indicate that the LiPs are differentially expressed in response to nutrient conditions. As yet the results for *P. chrysosporium* BKM-F-1767 have not been compared to other strains of *P. chrysosporium*.

Genetic Organisation in Phanerochaete chrysosporium

Chromosome mapping is a term used to describe the process of assigning genes to chromosomes and, more specifically, mapping the genes to positions on the chromosomes. This process is important for a number of reasons, including the breeding of certain desirable characteristics. When genes are on separate chromosomes they will be inherited independently of one another. If these genes are close together on the same chromosome the possibility will be greater that they will be inherited together.

There are two types of genetic mapps: physical and genetic. Genetic mapping is the oldest technique and involves tracing characteristics from parents to offspring. By investigating the frequency that characteristics occur together in offspring it is possible to calculate how closely linked the genes encoding the characteristics are. In genetic mapping the position of genes are found relative to one another and the distance between them given as the relative frequency of recombination. In theory the closer two genes are to one another the less often there will be any recombination of the alleles. While genetic mapping can give an indication of the physical distance between genes on the chromosomes, there are many factors that play a part in making this relationship between physical distance and genetic mapps imprecise. In physical mapping the position of the genes on the chromosomes are accurately determined and the distances between these genes given in the number of base pairs. The disadvantage of physical mapping is that it does not take into account other factors that will influence the frequency of recombination between the genes. Raeder *et al.* (97) determined an approximate relationship between physical map length (expressed as kilo bases (kb)), and genetic map distance (expressed as centi Morgan (cM)), for *P. chrysosporium*. They estimated that for *P. chrysosporium* ME446 one cM corresponds to less than 59 kb.

The ability to separate intact fungal chromosomes using electrophoretic methods has led to the discovery that most species exhibit chromosome length polymorphisms (CLP). Polymorphisms have been observed in both asexual and sexual fungi and most likely result

from both mitotic and meiotic processes. *Phanerochaete chrysos*porium is a dikaryon and has chromosome length dimorphism (28, 121). In 1991 Gaskell *et al.* (49) were able to resolve seven chromosome bands in *P. chrysosporium* BKM-F-1767 using clamped homogeneous electric field (CHEF) electrophorsis. In addition, they speculated that two of the bands might be doublets (based on the intensity of the ethidium bromide staining). Later the genomic DNA of *P. chrysosporium* BKM-F-1767 was resolved into ten chromosomes, and the genomic DNA of *P. chrysosporium* ME446 was resolved into 11 chromosomes, using transverse alternating field electrophoresis (TAFE) (41). The isolation of a homokaryotic derivative of *P. chrysosporium* BKM-F-1767 by Stewart *et al.* (109) promises to allow higher resolution of the chromosomes.

Mapping in *Phanerochaete chrysosporium*

Early attempts at mapping *P. chrysosporium* produced linkage maps that could not be coupled to specific LiP encoding genes. Krejcí and Homolka (81) mapped *P. chrysosporium* 284B using auxotrophic isolates. Raeder *et al.* (96, 97) used sequences specifically expressed during the ligninolytic phase of the fungus to probe DNA from meiotically derived haploid recombinants. The results were as expected from meiosis of a diploid or dikaryotic organism where chromosomes undergo recombination and independent segregation. The data suggested that the genes were not completely randomly distributed and that the genetic map length of *P. chrysosporium* ME446 was fairly small. However, the above results could not be coupled to specific genes. A method developed by Gaskell *et al.* (51) determined linkage between certain genes. This method involved the mapping genes from single basidiospore isolates of *P. chrysosporium* using PCR amplification of the genes.

As previously described (see Table 1) *P. chrysosporium* contains at least ten LiP encoding genes, designated *lipA* to *lipJ* by Gaskell *et al.* (50). Gaskell *et al.* (49) mapped a 30kb cosmid insert containing *lipA*, *lipB* and *lipC*: *lipA* and *lipB* were found to be separated by 1.3 kb from each other, and *lipC* was approximately 15 kb downstream of *lipB*. Furthermore, they located six *lip* genes, including GLG5 (*lipC*) and the two transcriptionally convergent genes (*lipA* and *lipB*), on a single dimorphic chromosome. These three genes hybridised to two bands (3.7 and 3.5 mb) on a CHEF gel. Further investigation using sexual fruiting determined that *lipE* was closely linked to *lipA* and *lipB*, and that *lipG* and *lipH* were closely linked to *lipI* and *lipJ* (50). The remaining two LiP genes, *lipD* and *lipF*, were found to be inherited separately to the above clusters (Table 2), and were located on dimorphic chromosome with sizes of 4.4 & 4.8 mb and 1.8 & 2.0 mb, respectively (28, 41).

TABLE 2. The percent co-segregation of *P. chrysosporium* LiP encoding genes. Adapted from Gaskell *et al.* (50)

	lipC	*lipD*	*lipE*	*lipF*	*lipG*	*lipH*	*lipI*	*lipJ*
lipA/B	98	**53**	100	**58**	97	97	97	97
lipC		**52**	98	**56**	98	98	98	98
lipD			**51**	**64**	**50**	**50**	**52**	**52**
lipE				**59**	97	97	97	97
lipF					**58**	**58**	**58**	**58**
lipG						100	100	100
lipH							100	100
lipI								100

Numbers in bold indicate statistically significant (P = 0.01) by χ^2 analysis.

Stewart *et al* (108) further refined the map of the eight clustered LiP encoding genes (Fig. 10). The LiP encoding genes are arranged in two groups of four genes with a recombination frequency of 3 % between lipE and lipJ. Six of the genes are trascriptionally convergent (*lipA* & *lipB*, *lipI* & *lipG*, and *lipH* & *lipJ*). Comparison of the organisation of the LiP encoding genes with the expression of these genes in different growth conditions (discussed in the section "Lignin peroxidase expression in liquid cultures") revealed no correlation.

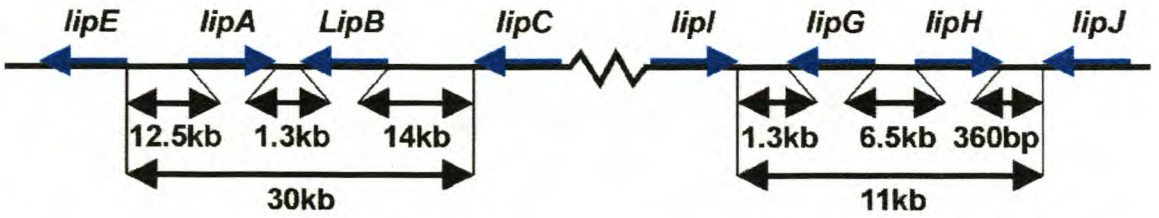

FIG. 10. Genetic linkage in the LiP encoding gene cluster (not to scale). Blue arrows indicate the trascriptional orientation of the genes. Adapted from Gaskell *et al.* (49) and Stewart *et al.* (108, 109).

Discussion and Conclusions

The complex structure of wood, and in particular the heterogeneous nature of lignin, make it resistant to degradation by most microorganisms. However, certain fungi have evolved a unique enzymatic system that allows for the total degradation of wood to carbon dioxide,

thereby playing an important role in carbon cycling in nature. Some fungi (termed white-rot fungi) are able to selectively degrade lignin from wood, leaving the cellulose virtually untouched. This characteristic has been successfully utilised in biopulping (13, 76), where fungal pretreatment of woodchips has led to a lowering of the energy and chemical requirements of the pulping process.

The same characteristics that allow for the mineralisation of wood also allow the fungi to degrade a wide range of chemicals, including a number that pose a threat to the environment. White-rot fungi have been associated with the degradation of textile dyes, pesticides, herbicides and many other compounds (2, 8, 21, 22, 30, 55, 111, 117). Therefore white-rot fungi, and the enzymes they produce, are of great importance industrially and environmentally, as well as being of scientific interest.

The most widely studied of the white-rot fungi is the basidiomycete *P. chrysosporium*. However, this literature review indicates that analysis of the genetics of *P. chrysosporium* is hampered by the lack of suitable techniques. In particular, previous attempts at mapping the *P. chrysosporium* genome have relied on the use of auxotrophic strains, chemical or physical mutagens, and radioactive labelling. The advent of powerful techniques such as PCR and automated sequencing have allowed us to develop an automated sequencing method for identifying alleles and mapping genes that is rapid, reliable, and cost effective. This technique is described in the manuscript in chapter three.

Although the knowledge of the genetics of *P. chrysosporium* is important, the regulation of the ligninolytic enzymes is directly applicable from an industrial standpoint. This fungus has a number of enzymes that are closely associated with the degradation of lignin, including LiP, MnP, and laccase. In *P. chrysosporium* the LiPs and MnPs are encoded by families of closely related genes, and these isozymes are expressed differentially according to growth conditions (31). Whether the multiple copies of the ligninolytic genes are the results of random genetic duplication, or whether the different enzymes have varying functions, is an important consideration when developing industrial bio-processes. In an attempt to clarify this matter competitive RT-PCR has been used to quantify the levels of the different ligninolytic enzymes from *P. chrysosporium* grown on various substrates, including defined media (110), wood chips (66) and soil (9). Results indicate that all genes encoding ligninolytic enzymes are transcribed according to a complex system of regulation in response to changing nutrient conditions. Although different strains of *P. chrysosporium* have been used in studies of lignin degradation, as yet no comparison has been made for *lip* expression between different strains

of *P. chrysosporium*. The results presented in chapter four compare the expression of LiP encoding genes in strains ME446 and BKM-F-1767 of *P. chrysosporium* using RT-PCR.

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CHAPTER THREE

GENOMIC MAPPING IN *PHANEROCHAETE CHRYSOSPORIUM* BKM-F-1767 USING AUTOMATED SEQUENCING

MANUSCRIPT IN PREPARATION FOR BIOTECHNIQUES

GENOMIC MAPPING IN *PHANEROCHAETE CHRYSOSPORIUM* BKM-F-1767 USING AUTOMATED SEQUENCING

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A technique was developed to map genes from the basidiomycete fungus *Phanerochaete chrysosporium* using amplification of selected portions of genes by polymerase chain reaction (PCR) and subsequent automated sequencing to determine the allelic segregation. The technique was developed using two lignin peroxidase (LiP) encoding genes with known genetic and physical linkage, *viz. lipA* and *lipC*. The results from our technique were consistent with previously published results. A recently identified and sequenced extracellular cellulose-binding β-glucosidase (CBGL) encoding gene was subsequently mapped, and linkage was found to a histone encoding gene (H1).

Introduction

The white-rot basidiomycete *Phanerochaete chrysosporium* is commonly used as a model organism in studies concerned with lignin biodegradation. In contrast to the enzymology of ligninolysis, the genetics are characterised to a lesser degree. Like many other fungi, *P. chrysosporium* lacks easily identifiable phenotypical markers. The absence of these markers means that they cannot be used to find genetic linkage, and other methods must be employed. Restriction mapping or direct sequencing of cosmid libraries can be used for mapping closely linked genes. These techniques were used to show genetic linkage of closely linked lignin peroxidase (*lip*) (6) and cellobiohydrolase (4) gene families in *P. chrysosporium*.

Although *P. chrysosporium* has a primary homothalic mating system, crosses between different strains are possible (1). Therefore, the analysis of nutritional requirements of the

progeny of crosses between auxotrophic strains can be used as a method of determining genetic linkage (2). This method requires the mutagenesis of *P. chrysosporium* conidia and the isolation of auxotrophic marker strains (9, 11), and was used by Krejcí and Homolka (12) to map mutations to auxotrophy in *P. chrysosporium* 284B. Homothalic mating was used to produce homokaryotic single basidiospore cultures in *P. chrysosporium* BKM-F-1767. These basidiospore cultures were used to map LiP encoding genes by amplifying portions of the genes using the polymerase chain reaction (PCR) and probing with allele-specific radioactively labelled oligonucleotide probes (7, 8). Similarly, *P. chrysosporium* ME446 single basidiospores were used by Raeder *et al.* (14) for a RFLP-based map of the LiP encoding genes. Gaskell *et al.* (7) published a map of the physical and genetic linkage of genes involved with lignocellulose degradation. Southern blots of CHEF gels localised *lipA* and *lipC* to the same dimorphic chromosome, and subsequent partial restriction mapping indicated that the genes are convergent and approximately 14 kb apart (16) with a genetic distance of 2% (7).

Phanerochaete chrysosporium has a number of different forms of extracellular cellulose-binding β-glucosidases (CBGL), which form part of a complex cellulose-degrading system (10). Deshpande *et al.* (5) reported five extracellular and cell wall bound CBGL's in *Sporotrichium pulverulentum* (the anamorphic state of *P. chrysosporium* (3)), and Smith and Gold (15) reported both intracellular and extracellular CBGL's in *P. chrysosporium* ME446. Li and Renganithan (13) sequenced both the genomic and the cDNA alleles of an extracellular cellulose-binding CBGL from *P. chrysosporium* OGC101. They report a 98% similarity between the two alleles, with a total of 50 base mismatches (in the exon regions) that cause four variations in amino acid sequences.

In this study we report on a time and cost effective method based on automated sequencing that can be used to monitor the allelic segregation of single basidiospore cultures. This method was authenticated using two previously mapped *lip* genes, as well as an unmapped CBGL gene.

Experimental Protocols

Preparation of DNA

The genomic DNA used in this study was kindly provided by Jill Gaskell (Forest Products Laboratory, Madison, WI) and was previously isolated from single homokaryotic basidiospore cultures of *P. chrysosporium* strain BKM-F-1767 (ATCC 24725) (7, 8). The

linkage map of the LiP encoding gene alleles, as well as linkage to other genes, is known for these cultures (7). A total of 58 single basidiospore isolates were selected as to include representatives of both allelic variants of *lipA* and *lipC*, as well as a rare isolate (SB56) that contained the recombination event (based the on previous mapping data). Heterokaryotic DNA isolated from the parental mycelia was also used. All the DNA samples were diluted to 100 ng/µl prior to use.

PCR reactions

Primers (Table 1) were designed so that selected regions of *lipA* and *lipC* genes could be amplified using the polymerase chain reaction (PCR). The primers were selected in such a way that they were: (a) gene specific, (b) that they spanned an area where there were allelic differences (based on previously published sequences or database depositions (7)) and (c) that they included the sequence previously used as probes by Gaskell *et al.* (7). Primers (Table 1) designed for the sequencing reactions were complementary to an area within the PCR fragment, within 100 bp of the 5' end. The primers for the *cbgl* gene were similarly designed based on the sequences for the *cbgl* gene from *P. chrysosporium* OGC101 (GeneBank accession numbers AF036873 and AF036872). All PCR reactions were done in 100 µl reaction volumes containing: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 100 ng of each primer, 2.5 units Taq DNA polymerase (Promega), and 400 ng DNA. The PCR cycling parameters used were the same as was previously employed by Gaskell *et al.* (8). All PCR reactions were done on an Applied Biosystems 391 thermocycler (Perkin Elmer). A 5 µl sample of each PCR reaction was run on a 2 % agarose gel, stained with ethidium bromide, and visualised under ultraviolet light.

Prior to sequencing, several methods of removing unincorporated PCR primers from the PCR products were evaluated. Firstly, the Wizard DNA clean-up system (Promega) was used, following the manufacturers instructions, and the DNA was eluted with 50 µl sterile deionised water (65°C). Secondly, a series of PCR reactions were done using varying concentrations of primers. The highest concentration was 100 ng per 100 µl reaction and then a two fold dilution series was done until the lowest concentration of 3.2 ng per reaction was reached. The PCR products (5 µl) were run on a 2 % agarose gel (Biorad), stained with ethidium bromide, and visualised using ultraviolet light. The reaction that had the lowest primer concentration and still gave efficient amplification was used for further sequencing reactions without any further removal of the primers. Thirdly, excess PCR primers were also removed by precipitating the DNA using 2 volumes 95% ethanol and 1/10 volume 3 M NaOAc (pH

5.4). The DNA was resuspended in 50 µl sterile deionised water. The concentration of the DNA was determined by measuring the absorbance at 260 nm on a spectrophotometer.

TABLE 1. PCR and sequencing primers

Gene	*lipA*	*lipC*	CBGL
5' PCR Primer	GCT TTG GCG GAT GGT ACG AAC TAA C	AAC GGT TGC CTT TGG ACA GAC	CAT CTT GCT CTA GGG TTG CTG ACT
3' PCR Primer	GAA ATC TAG TAA AGC CGA AGT TCC	GAA GTT GGT CTC GAT CTC GT	GTT GCC GAC ACA TGG ACC TTT CT
PCR Product Length (bp)[1]	970	499	383
Sequencing Primer	CGG AGG CGG AGG GCT GGA G	GCC AGT GCA GAA GCC GAG GGA TAC	CCA CCA CGA GTG TTT CGT CTA
Sequencing Length (bp)[2]	853	398	331
Probes[3]	TTT CAG GAA ATG (C/T)A ATC	TCC G(C/T)G CTG C(G/T)C AGG GT	

[1]Approximate PCR product lengths for both alleles.

[2]Maximum sequencing length.

[3]A pair of oligonucleotide probes was synthesised to differentiate between the two alleles of each gene.

Sequencing

For sequencing reactions 20 µl reaction volumes were used, containing 240 ng of DNA, 5 % DMSO, 3.3 pmol gene specific sequencing primer (Table 1), and an Applied Biosystems prism dye terminator cycle sequencing kit (Perkin Elmer) at half the recommended concentration. The sequencing parameters were as follows: denaturation (30 s, 96°C) followed by 25 cycles of denaturation (10 s, 96°C), annealing (5 s, 50°C), and extension (4 min, 60°C). Excess fluorescent dye was removed from the sequencing reactions using sephadex G50 fine gel filtration matrix (Sigma) in micro-centrifuge spin columns (Pharmacia). The DNA was then dried in a vacuum drier and resuspended in 4 µl gel loading buffer (Sigma). Sequencing reaction products (1.5 µl) were loaded onto a 29:1 acrylamide gel on an Applied Biosystems 377-18 DNA sequencer. A series of sequencing reactions were performed using varying concentrations of big dye terminator. DNA that had previously displayed good sequencing results was used for this test. Volumes of 1, 2 and 4 µl of big dye terminator were tried, and 7, 6 and 4 µl of big dye buffer were added to each of the reactions

respectively, to bring the total volume to 8 µl. The sequencing reactions and clean up were done in the same manner as before.

The results were analysed using DNASTAR DNA analysis software (DNASTAR). Two methods were used to identify the different alleles, based on previously published data for the *lip* and *cbgl* alleles. Firstly the bases present at five points on the sequence that were known to give alleleic variations were checked. Secondly, the sequences were aligned against the consensus of each alleleic sequence for *lipA* and *lipC* respectively, and the similarity tabulated. Furthermore, consensus sequences were obtained for the CBGL alleles, including the intron regions not published by Li and Renganithan (13), and these sequences used for allele identification as above.

Southern bloting

The PCR products of both *lipA* and *lipC* from four selected isolates (SB25, SB17, SB56 and parental) were run on two agarose gels simultaneously. The gels were stained with ethidium bromide and photographed before the DNA was transferred to a nitro-cellulose membrane using the Southern blot protocol of Gaskell *et al.* (8). The membranes were then probed with allele specific oligonucleotides end labelled with ^{32}P (8).

Results and Discussion

Method Development

Automated sequencing can be successfully used to determine genetic linkage in *P. chrysosporium*. However, a number of important factors have to be taken into account. Firstly, care must be taken to ensure that the primers designed are specific for the gene to be mapped and that both alleles are amplified. When there is a closely related family of genes, as is often the case in filamentous fungi, it may be difficult to differentiate between the genes, and it is essential to ensure that the PCR primers used are gene specific.

Secondly, sequence template purity is another factor to be taken into account. Although Wizard DNA purification columns (Promega) were routinely used in this study, both the low primer concentration and the ethanol precipitation methods of DNA purification yielded DNA of sufficient purity for the sequencing reactions. The Wizard-column cleaned samples gave better sequencing results, but the sequence results from the other two methods gave sequence of sufficient quality to differentiate between the two alleles (data not shown). The disadvantage of the low primer concentration method was that multiple PCR reactions had to

be done to ensure that a low carry over of PCR primers to the sequencing reactions occurred. Our experience showed that, as with automated sequencing in general, template DNA uncontaminated with PCR primers was essential, but that the method of purification did not influence the result.

The length of the PCR products also influenced the quality of the results and choosing PCR primers that amplified a fragment of approximately 500 to 600 base pairs in length was found to be optimal. This allowed for quick and efficient PCR amplification that results in a fragment that was easily visualised on an agarose gel. The sequencing primer should be chosen within the first 100 base pairs of the fragment to give a sequencing length of 400 to 500 base pairs. This allows a sufficient amount of sequence to align the fragments effectively, as well as limiting the length of the sequencing run to save time. Short PCR and sequencing products reduced the cycle times for the PCR reactions and the sequencing reactions, as well as the running times for the agarose and polyacrylamide gels. The area where there were differences in base sequence between the alleles should not be within the first 100 bases of sequence as this region in often subjected to unusually high peaks that interfere with the readability of the sequence. A half-strength sequencing reaction (50 % big dye terminator and 50 % buffer) was found to give as good a signal as the full strength sequencing reactions. Further dilution, however, resulted in weaker signals and a corresponding drop in the quality of the sequence obtained.

Both methods of allele identification worked well. Where good quality sequence was obtained each single basidiospore DNA would align well to one of the allelic sequences, and to a far lower degree to the other. The later sequences typically showed all the point differences corresponding to one allele or the other. As the quality of the sequence obtained decreased, the number of point differences that could be used for differentiation also decreased, lowering the accuracy of the results. As the computer based alignment method scored a higher number of allelic differences this method was found to be less susceptible to decreased sequence quality. However, neither method worked when the sequence obtained was of very bad quality.

Lignin peroxidase A and C

The results for the Southern blot for *lipA* and *lipC* are shown in Fig. 1, and a selected portion of the corresponding electropherograms showing allelic variations is shown in Fig. 2. In Fig. 1 the heterokaryotic parental strain can clearly be seen to have a band for each of the allelic probes, whereas the homokaryotic single basidiospores display a band in only one of

the blots. The strain containing the recombination (SB 56) event displays a band on the blot for *lipA1* (as does the single basidiospore 25), but not one on the blot for *lipC1*. Rather, a band is visible on the blot for *lipC2* (as for the single basidiospore 17), showing that a recombination event had taken place between the two genes.

The same results were obtained for our sequencing method of allele identification. In Fig. 2 the heterokaryotic parental strain displayed double peaks in the electropherogram, whereas the homokaryotic single basidiospores had a single peak at the respective points on the electropherogram. The sequencing method of allele identification was also able to distinguish the recombination event (data not shown). The above data indicated that the use of an automated sequencing method was effective for allele identification.

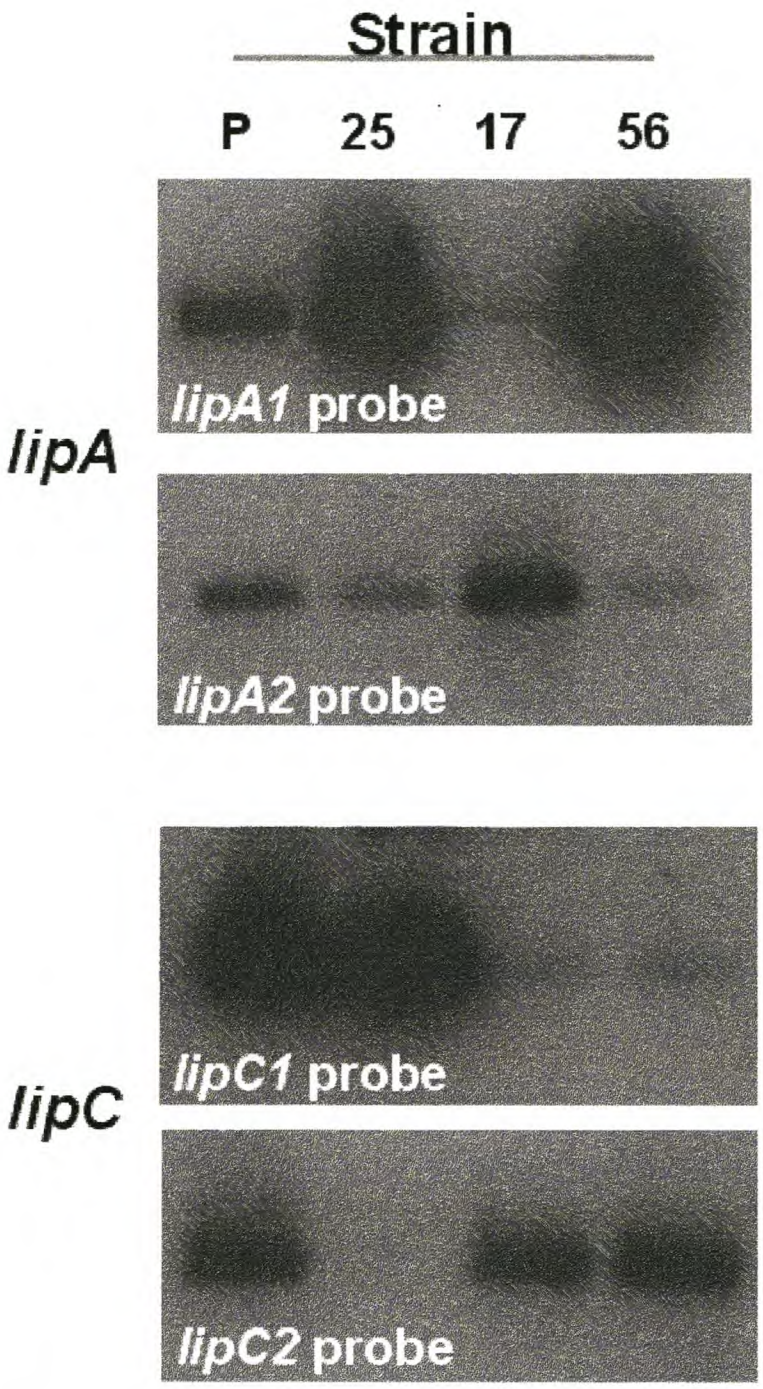

FIG. 1. Southern hybridisation of gene-specific PCR products probed with allele specific probes for *lipA* and *lipC*. Lanes contain the heterokaryotic parental DNA (P), and three selected homokaryotic single basidiospores, SB 25, SB 17 and SB 56. Both allele specific probes hybridise to the parental DNA, whereas only one of the probes hybridise to each of the single basidiospore DNA.

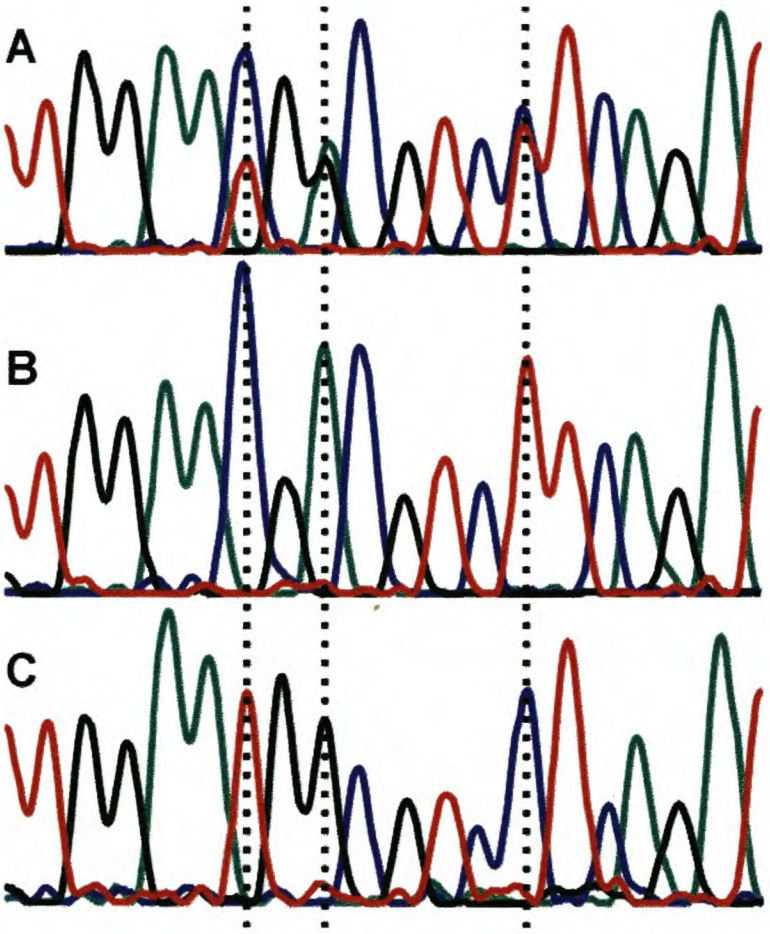

FIG. 2. The first electropherogram (A) is of the sequence of the parental (heterokaryotic) DNA. Three points can be seen where double peaks occur in the parental strain (indicated with dotted lines). Below the parental sequence are the sequences of the two homokaryotic single basidiospores, SB17 (B) and SB25 (C). Electropherograms B and C show only one peak at each of the marked points, and the bases encoded differ between the two alleles.

β-glucosidase

In the small area we sequenced the DNA sequence from *P. chrysosporium* BKM-F-1767 matched that of the exon sequence of *P. chrysosporium* OGC101 published by Li and Rengenithan (13), including the allelic mismatches. They reported a 98% similarity between the two alleles, with a total of 50 base mismatches (in the exon regions) that cause four variations in amino acid sequences. We found far more variation between the alleles within

the intron regions sequenced, so that the similarity between the alleles for the short regions sequenced was 90%.

Of the 58 single basidiospores sequenced, 22 of the cultures were of one allele (corresponding to the sequence for *cbgl*-2 determined by Lee and Rengenithan (13)) and 29 were the other allele (corresponding to the cDNA sequence *cbgl*-1), while seven gave results similar to the heterokariotic strain. The frequency of segregation of the two *cbgl* alleles was thus 56.8 %, indicating that the segregation was Mendelian in nature. Comparison of the *cbgl* results with previous mapping data revealed linkage between *cbgl* and a histone (H1) encoding gene. The percentage co-segregation of alleles was calculated to be 92.3% (χ^2 analysis indicates $P < 0.01$).

Apart from the considerations listed, the technique was found to be simple and sufficient for the differentiation of alleles and the determination of genetic linkage in *P. chrysosporium*. The use of fully automated reaction and sequencing machinery would allow large numbers of samples to be processed simultaneously.

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CHAPTER FOUR

EXPRESSION OF LIGNIN PEROXIDASE ENCODING GENES FROM *PHANEROCHAETE CHRYSOSPORIUM* ME446 GROWN ON DEFINED MEDIA AND ON ASPEN WOOD CHIPS

MANUSCRIPT IN PREPARATION FOR APPLIED AND ENVIRONMENTAL MICROBIOLOGY

EXPRESSION OF LIGNIN PEROXIDASE ENCODING GENES FROM *PHANEROCHAETE CHRYSOSPORIUM* ME446 GROWN ON DEFINED MEDIA AND ON ASPEN WOOD CHIPS

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The levels of expression of lignin peroxidase encoding genes from *Phanerochaete chrysosporium* ME446 grown on defined liquid media and Aspen wood chips was estimated by determining the levels of messenger RNA (mRNA) produced by the fungus. Magnetic capture and competitive reverse transcriptase PCR reactions were used to quantify the amounts of mRNA in the cultures. The levels of mRNA varied both over time, as well as between growth substrates used. The results in this study also differed from previous results for *P. chrysosporium* BKM-F-1767 for both defined media as well as wood chips.

Introduction

Certain basidiomycete fungi have the ability to selectively degrade lignin from wood, leaving the cellulose virtually intact, a characteristic that has earned them the name of white-rot fungi. Apart from their importance in carbon cycling in nature these fungi are also being studied for potential applications in the pulp and paper industry and in bio-remediation. *Phanerochaete chrysosporium*, one of the best-studied white-rot fungi, has a complex and poorly understood system for the degradation of lignin that includes lignin peroxidases (LiP), manganese peroxidases (MnP) and glyoxal oxidase (8, 23). This system allows *P. chrysosporium* to degrade not only lignin but also a variety of phenolic compounds, including organic pollutants (6, 17).

In *P. chrysosporium*, LiP is represented by a family of at least ten closely related isozymes (22), designated *lipA* to *lipJ* (11). Although these isozymes have different K_m, K_{cat} and substrate affinities (10, 16), they are structurally very similar and isolation and quantification of the separate isozymes are difficult. These problems are compounded in natural substrates such as wood and soil where the enzymes are present in low concentrations and contaminants may effect their extraction. However, a reverse transcriptase polymerase chain reaction (RT-PCR) method has been developed that allows for the quantification of relative amounts of mRNA in fungal cultures (15). This method uses magnetic particles to purify poly adenylated mRNA. It has been successfully used to quantify the mRNA levels of enzymes involved in lignin degradation from cultures grown in defined culture media (5, 14, 26, 28), in soil (2, 3, 24), and on wood chips (20). There have been reservations as to the accuracy of the RT-PCR method of mRNA quantification (18) and fears of reverse transcriptase inhibition of the PCR reactions (7). However, these matters have been adequately addressed (18, 20). In particular, Janse *et al.* (20) found no evidence of inhibition when varying amounts of LiP encoding mRNA was used in RT-PCR reactions.

Due to differences in experimental procedures, previously published results of LiP expression in *P. chrysosporium* ME446 (4) are not directly comparable to findings in *P. chrysosporium* BKM-F-1767 (20, 26). We report here the quantification of mRNA levels for the LiP encoding genes from *P. chrysosporium* ME446 grown on both defined media and on Aspen wood chips and compare the results obtained with the results of previous studies using *P. chrysosporium* BKM-F-1767 (20, 26).

Materials and Methods

Bioreactors

Approximately 1,5 kg dry weight of fresh aspen wood chips were sterilized and inoculated with $1{,}0 \times 10^5$ *P. chrysosporium* ME446 spores per gram of wood-chips according to standard biopulping methods (1). The bioreactors were incubated at 37°C with constant humidity. At two week intervals samples of approximately 20 g of wood chips were harvested from the bioreactors. The samples were flash frozen in liquid nitrogen and stored at –80 °C. Portions of the samples were ground in a clean coffee grinder (washed with DEPC treated water and dried) in the presence of liquid nitrogen.

Liquid medium

Basal medium (B III) described by Kirk *et al.* (22) was used for the liquid cultures and carbon or nitrogen were limited to promote the production of ligninolytic enzymes (21). The carbon limited media had a total carbon content of 0.2 % (w/v), and the nitrogen limited media had a total nitrogen content of 1.2 mM. The media (100 ml) was filter sterilised into pre-sterilised 125 ml flasks and inoculated with 5 x 10^6 *P. chrysosporium* ME446 spores. The flasks were incubated at 37°C for 24 hours and then the headspace was flushed for 10 minutes with sterile oxygen. The flasks were immediately sealed with rubber stoppers and incubated stationary at 37°C. Each day one flask was removed and veratryl alcohol assays were done according to the method of Tien and Kirk (29) to determine the LiP activity of the culture. The cultures were harvested on day 5 and day 6 for the carbon and nitrogen limited cultures, respectively. The cultures were filtered through miracloth (Calbiochem) to remove the media, frozen in liquid nitrogen, and ground fine in a mortar and pestle.

DNA and mRNA isolation

Genomic DNA (competitive template) was isolated from *P. chrysosporium* ME446 (obtained from the Centre for Forest Mycology Research, Forest Products Laboratory, Madison, Wisconsin) using the method of Raeder and Broda (25). Polymerase chain reaction (PCR) primers designed to amplify the complete *lip* gene from *P. chrysosporium* BKM-F-1767 were used to amplify each of the corresponding LiP encoding genes from *P. chrysosporium* ME446. The PCR products were ligated into the pGEM-T Easy vector (Promega), as per manufactures instructions, and amplified using a suitable *E. coli* host. After plasmid isolation the concentrations of the plasmid DNA was determined by using a spectrophotometer, and then diluted to 100 ng/µl. A ten fold dilution series was then made for each plasmid, starting at 100 ng/µl and ending at 10 fg/µl.

Poly adenylated mRNA was extracted using poly T-tailed paramagnetic beads (Dynel). The protocol for the extraction from wood was as described by Vallim *et al.* (30). The protocol for the extraction from liquid cultures was as described by Stewart *et al.* (26). One-tenth volume of sodium hydroxide and two volumes of 95 % ethanol were added to the extracted mRNA, and the mRNA was stored at –20 °C. The reverse transcriptase reactions were done in multiple 100 µl reaction volumes using 250 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (GIBCO BRL) according to the method of Gilliand *et al.* (15) as modified by Janse *et al.* (20).

Competitive RT-PCR

Competitive RT-PCR primers were designed according to sequence data previously published for *P. chrysosporium* ME446. Many of the primers used were identical to those used for *P. chrysosporium* strain BKM-F-1767 (11, 20), but where these primers were not suitable new primer pairs were designed (Table 1). The primers were designed to ensure a gene specific PCR product of approximately 600 base pairs from genomic DNA, and to be homologous to both of the alleles of the gene in question as well as to include an intron region so that the shorter cDNA PCR product could be differentiated from the template DNA on an agarose gel.

TABLE 1. Gene specific competitive PCR primers for *P. chrysosporium* ME446

Gene	5' Primer	3' Primer
lipA[a]	5'-TTCATCGCCTTCGCTGGTGCTGTC-3'	5'-AAGATTCCGGGGGTCGAGTCAAAG-3'
lipB[a]	5'-ACACGAGCGATGATCTGG-3'	5'-GCTATTGCCATCTCTCCT-3'
lipC[a]	5'-ACACGGTCGATGATTTGG-3'	5'-GCCATCGCTATCTCTCCC-3'
lipD[b]	5'-TCCATCGCTATCTCGCCC-3'	5'-ATGCGAGCGAGAACCTGA-3'
lipE[b]	5'-TCCATCGCCATCTCGCCC-3'	5'-ACGCGGGCGATGATCTGG-3'
lipF[a]	5'-GCGCTGCTGTGAAGGAGAAG-3'	5'-GCGGAGCACCAGGGCAGTTG-3'
lipG[a]	5'-GTGCCGTAGTTAAGGAGAAAC G-3'	5'-GGCGCGACCGGTGAAGAA G-3'
lipH[b]	5'-GCAATTGCCATCTCGCCC-3'	5'-ACACGGTTAATGAGCTGG-3'
lipI[a]	5'-AGGCCCAGGGCAAGTTCG-3'	5'-TCGAGCTCATCGAACTGTC-3'
lipJ[a]	5'-CACGGTGTTACTCCTGGCGACTTC-3'	5'-GTGACATCCCTTCCCCTTGGTTGC-3'

[a] Primers designed from previously published sequences for *P. chrysosporium* ME446.

[b] Primers identical to previously published primers for *P. chrysosporium* BKM-F-1767 (20).

Competitive PCR reactions were done for each of the genomic template dilutions using Taq enzyme (Promega) and the reactions run on an Applied Biosystems 9600 thermal cycler (Perkin Elmer) using the cycling parameters described by Gaskell *et al.* (13). The products from the PCR reactions were run on a 1% agarose gel stained with ethidium bromide and photographed.

Each of the gel photographs were scanned and cropped using Photoshop computer software (Adobe). The intensity of all the bands was determined using NIH image analysis software 1.58 (National Institute of Health). Regression analysis and tabulation of the results was done using the Excel spreadsheet program (Microsoft). The concentration of mRNA was determined by solving the regression formula of Gilliland *et al.* (15) with an x value of 1,5 to compensate for the difference in size between the cDNA and the competitive template (as determined by Stewart *et al.* (28)).

Results

Lignin peroxidase activity and mRNA levels

The results of the veratryl alcohol assays are presented in Fig. 1. Lignin peroxidase activity in the carbon-limited liquid media peaked on day 4, and in the nitrogen-limited liquid media the highest activity was recorded on day 7.

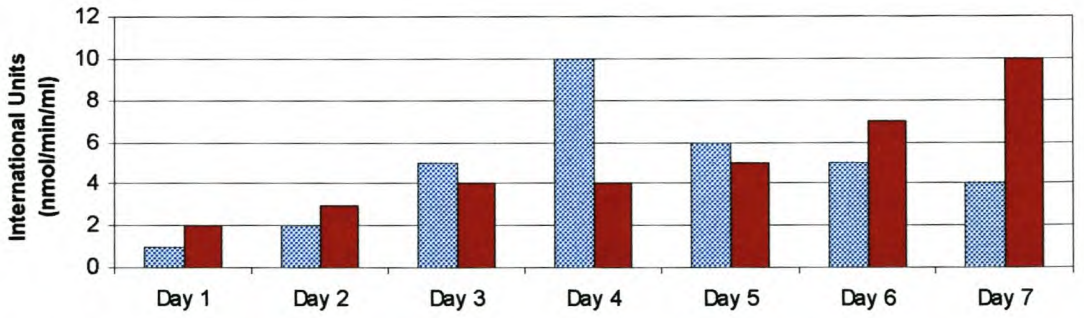

FIG. 1. Comparisons of lignin peroxidase activities for *P. chrysosporium* ME 446 grown in liquid medium determined by veratryl alcohol assays (29). ■ = carbon limited liquid medium; ■ = nitrogen limited liquid medium. Values are given as international units (nmol/min/ml).

Examples of the agarose gels, used to separate the genomic DNA from the cDNA, are shown in Fig. 2. The differentiation between the larger genomic DNA bands (480 bp) and the smaller cDNA bands (326 bp) can easily be seen. The approximate titration point of equivalence is marked with an arrow. A complete list of the levels of mRNA extracted from the different *P. chrysosporium* ME446 cultures is given in Table 2. No suitable internal control for the RT-PCR reactions was available. Therefore the values between different mRNA extractions cannot be compared directly. Consequently, a maximum of 100 % for the highest value of each sample was assumed and the rest of the results were transformed to a percentage thereof to normalise the results.

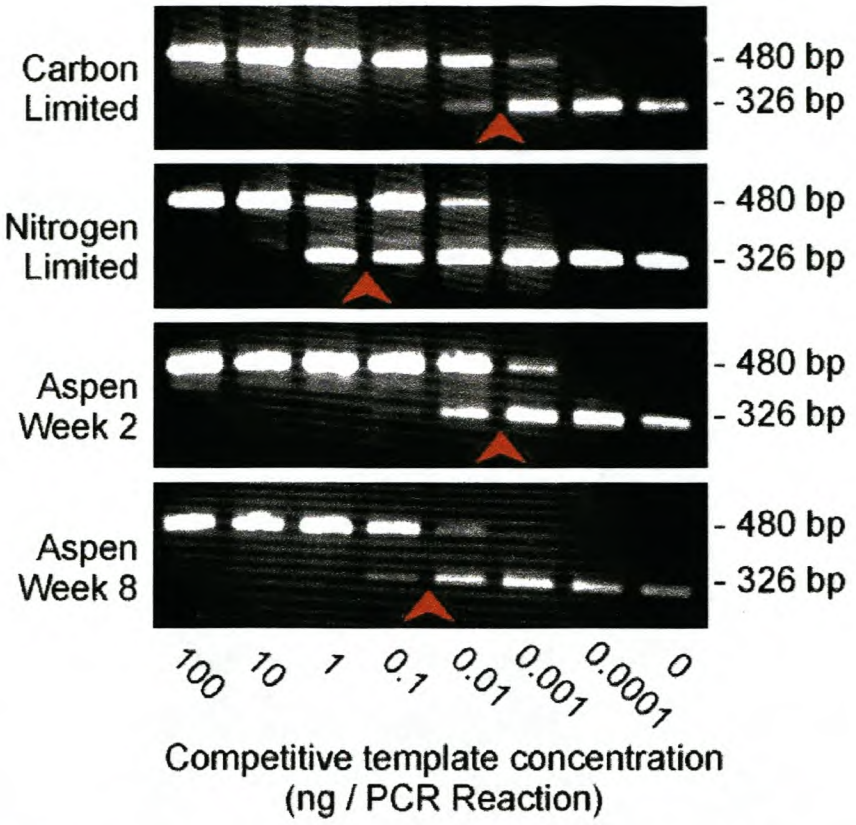

FIG. 2. Competitive PCR products for *lipC* from *P. chrysosporium* ME446 grown on defined liquid media and aspen wood chips. Arrows indicate the approximate equivalence points.

TABLE 2. Lignin peroxidase mRNA level from *P. chrysosporium* ME446 grown in liquid media and on wood chips (expressed as a percentage of the maximum value)

	lipA	*lipB*	*lipC*	*lipD*	*lipE*	*lipF*	*lipG*	*lipH*	*lipI*	*lipJ*
Week 2[a]	**100**	**5.43**	**34.0**	**0.439**	**ND**	**25.0**	**1.11**	**ND**	**ND**	**ND**
Week 4[a]	**2.61**	**0.184**	**100**	**0.035**	**0.010**	**0.826**	**0.161**	**1.82**	**0.666**	**0.001**
Week 6[a]	**4.71**	**9.84**	**100**	**0.078**	**ND**	**55.2**	**7.72**	**39.6**	**0.531**	**0.802**
Week 8[a]	**1.15**	**0.107**	**2.04**	**0.332**	**ND**	**0.411**	**100**	**6.82**	**1.00**	**ND**
Carbon Limited[b]	**9.89**	**1.49**	**0.090**	**100**	**13.1**	**1.373**	**28.4**	**6.23**	**0.324**	**0.291**
Nitrogen Limited[b]	**100**	**7.07**	**82.9**	**5.56**	**26.2**	**0.094**	**9.81**	**2.10**	**0.195**	**3.06**

[a]Woodchips.

[b] Liquid media.

ND = Not Detected.

Comparison to results for *P. chrysosporium* BKM-F-1767

The results obtained for *P. chrysosporium* ME446 were compared to those previously obtained for *P. chrysosporium* BKM-F-1767 by Janse *et al.* (20) and Stewart *et al.* (26). A comparison of the relative levels of LiP mRNA for each of the *P. chrysosporium* strains grown on carbon limited and nitrogen limited liquid media is given in Fig. 3, and on aspen wood chips is given in Fig. 4.

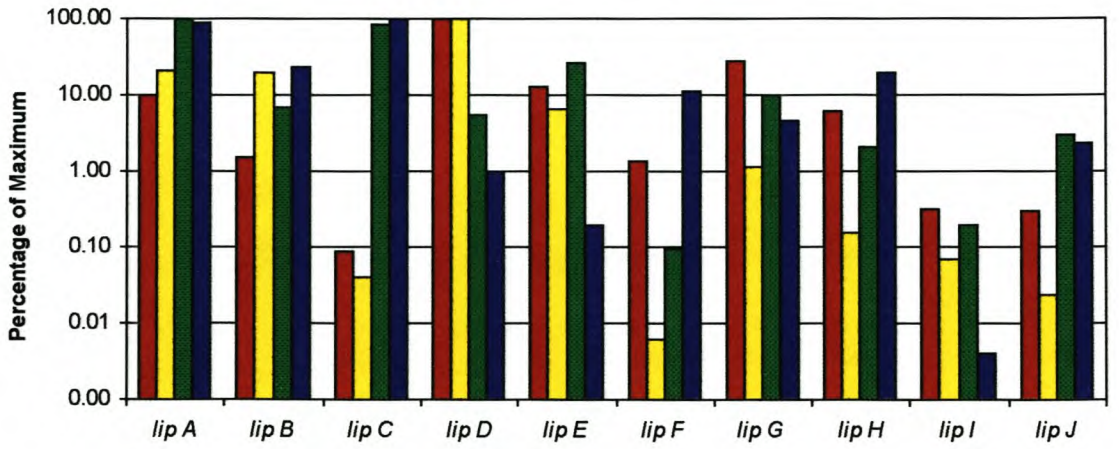

FIG. 3. Comparisons of transcript levels of the LiPs from *P. chrysosporium* BKM-F-1767 and *P. chrysosporium* ME446 grown on carbon limited and nitrogen limited liquid media. ■ = *P. chrysosporium* ME446 grown on carbon limited medium; ■ = *P. chrysosporium* BKM-F-1767 grown on carbon limited medium; ■ = *P. chrysosporium* ME446 grown on nitrogen limited medium; ■ = *P. chrysosporium* BKM-F-1767 grown on nitrogen limited medium. The values are given as a percentage of the highest value for each series. The values for *P. chrysosporium* BKM-F-1767 are those previously published by Stewart *et al.* (26).

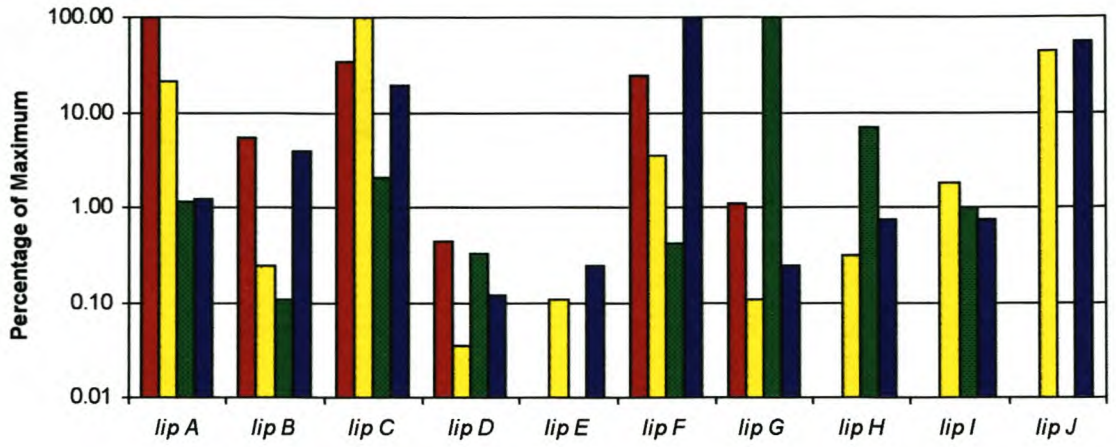

FIG. 4. Comparisons of transcript levels of the LiPs from *P. chrysosporium* BKM-F-1767 and *P. chrysosporium* ME446 grown on aspen wood chips. ■ = *P. chrysosporium* ME446 harvested on week 2; ■ = *P. chrysosporium* BKM-F-1767 harvested on week 2; ■ = *P. chrysosporium* ME446 harvested on week 8; ■ = *P. chrysosporium* BKM-F-1767 harvested on week 8. The values are given as a percentage of the highest value for each series. The values for *P. chrysosporium* BKM-F-1767 are those previously published by Janse *et al.* (20).

Discussion

Lignin peroxidase activity and mRNA levels

The peak LiP activities observed in the liquid cultures of *P. chrysosporium* ME446 (Fig. 1) were later than those observed for *P. chrysosporium* BKM-F-1767 by Stewart *et al.* (26). Also, in Table 2 it can be noted note that transcripts for *lipE*, *lipH*, *lipI* and *lipJ* were detected at week 4 for *P. chrysosporium* ME446 grown on Aspen wood chips, all of which were not detected at week 2 and were therefore not represented in Fig. 4. The contrast in these results for *P. chrysosporium* ME446 to those for *P. chrysosporium* BKM-F-1767 grown on liquid media (26) and Aspen wood chips (20), and are most likely the result of slower growth by *P. chrysosporium* ME446. However, the results of Bogan *et al.* (2) for *P. chrysosporium* BKM-F-1767 grown in soil show that mRNA levels can vary on a daily basis, which may also account for some of the variation.

No correlation was found between the pattern of transcription of the LiP genes of *P. chrysosporium* ME446 and the structural organisation of the genes, or the five intron/exon subfamilies of the LiPs (26). Similar results were obtained by Stewart *et al.* (26) for *P. chrysosporium* BKM-F-1767. This indicates that, although the lignin peroxidase encoding genes are arranged in clusters in *P. chrysosporium*, no relationship exists between the structural organisation of the genes and the levels of transcription of these genes.

Comparison to results for *P. chrysosporium* BKM-F-1767

The results for the LiP levels of cultures grown in liquid media were more reliable for comparing the two strains of *P. chrysosporium* strains than those for the cultures grown on wood chips, as the culture parameters were more closely controlled. In liquid media some of the LiP genes were similarly expressed in both strains: *lipA*, *lipB*, *lipC*, *lipD* and *lipI* all showed similar patterns of expression for carbon and nitrogen limited liquid media. However, the rest of the LiP genes differed in their expression. For instance, *lipF* from *P. chrysosporium* ME446 had a relatively high level of mRNA in carbon limited conditions and over a 10 fold decrease in nitrogen limited compared to carbon limited conditions. The pattern of mRNA levels for *lipF* from *P. chrysosporium* BKM-F-1767 was inverted with over a 1000 fold increase in nitrogen limited media as compared to carbon limited media. Contrary to earlier results for *P. chrysosporium* ME446 (5, 19), transcripts of *lipH* (LIG1), *lipA* (LIG2), *lipB* (LIG3) and *lipJ* (LIG4) were detected in both nitrogen limited and nitrogen sufficient liquid cultures, as well as in all but the earliest wood sample, and *lipD* (LIG5) did not dominate in the nitrogen deficient liquid media. These discrepancies may possibly be attributed to variations in culture parameters as well as the age of the cultures at the time of harvesting.

Similarities and discrepancies in gene expression were also observed when comparing the strains grown on wood chips. Datta *et al.* (9) suggested that the pattern of ligninolytic enzymes expressed on solid substrates differ from those expressed in liquid culture. This held true for the mRNA levels we detected. Of the LiP mRNA's that showed similarities in the patterns between the strains in liquid cultures, only *lipA* and *lipC* showed consistent patterns for both strains of *P. chrysosporium* grown on wood chips, decreasing from week 2 to week 8. The remaining mRNA levels varied considerably, which may be due to the heterogeneous nature of the growth substrate and differences in growth rates as well differences between the regulation of the LiP genes in the *P. chrysosporium* strains.

Of special interest were the results for *lipI*. Gaskell *et al.* (12) found an insertional element (Pce1) in one of the alleles of the *lipI* gene in *P. chrysosporium* BKM-F-1767. Further investigation revealed that the *lipI* allele containing the insertional element was not transcribed (27). This element is not present in *P. chrysosporium* ME446 and it was initially thought that there could be marked differences between the different strains due to *P. chrysosporium* ME446 having two active alleles of the *lipI* gene. However, the variation in expression between the strains was not pronounced, and we concluded that the insertional element had no effect on the expression of *lipI*.

The above results confirmed that in *P. chrysosporium* ME446 the lignin peroxidase encoding genes were regulated in response to nutrient conditions. Furthermore, the large differences in expression between media indicated that the regulation of the LiP gene family was both complex and subtle. The similarities in the expression of the lignin peroxidase encoding genes between *P. chrysosporium* ME446 and *P. chrysosporium* BKM-F-1767, especially in defined liquid media, suggested that both strains were regulated in a similar manner, although strain specific variations do exist.

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CHAPTER FIVE

GENERAL DISCUSSIONS AND CONCLUSIONS

In the literature review (chapter two) the structure of wood, as well as fungi that are able to efficiently degrade wood to carbon dioxide, were discussed. These microorganisms, called white-rot fungi, are of special interest to the pulp and paper industry, where biopulping promises to lower chemical and energy costs involved in the pulping of wood (4, 17). Furthermore, white-rot fungi have also been advanced as potential organisms to be used in the bioremediation of toxins and pollutants contaminating the environment (1, 2, 6-8, 13, 25, 26).

The best studied of the white-rot fungi is the basidiomycete *Phanerochaete chrysosporium*. In *P. chrysosporium* a number of enzymes are involved with the degradation of wood, including cellulases, hemicellulases and ligninases. The ligninases can be further subdivided into lignin peroxidases (LiPs), manganese peroxidases (MnPs) and lacasses. In *P. chrysosporium* the LiPs are encoded by a family of at least 10 genes, the products of which have been found to be very similar (53-98%) on a protein level (18). The large number of LiP isozymes leads us to ask why an organism would have many copies of what is essentially the same gene. Possible explanations for this phenomenon are that the extra genes are products of a random copying of an original gene (and will eventually be lost to evolution), or that isozymes have subtle differences in their action that help the fungus degrade the complex lignin molecules.

The question remains unanswered, in part due to lack of suitable techniques for genomic mapping in *P. chrysosporium*. In particular, a fast and efficient method of differentiating gene and their alleles has previously been unavailable. In chapter three the use of automated DNA sequencing to map genes in *P. chrysosporium* was described. The technique we described, and possible variations thereof, will be a useful tool to increase our knowledge of genetic organisation in *P. chrysosporium* and other organisms. Previous mapping techniques involved either the laborious analysis of crosses between auxotrophic strains (19), or the use of hazardous radioactive chemicals (11, 12, 21). In our technique genes were PCR amplified from DNA extracted from single basidiospore cultures using gene specific primers, followed by automated DNA sequencing to determine the allelic segregation. The technique is simple and quick to perform, and is above all very accurate. Although it is possible for false results to occur due to DNA contamination, non-specific PCR products or inefficient sequencing, these errors are easily distinguished from the correct results. Further development of this technique may lead to fast, fully automated methods of genetic mapping that will allow the segregation of a large number of genes to be determined simultaneously.

The expression of lignin peroxidases on defined media and complex substrates was also discussed in the literature review. Several researchers have found significant differences in

the levels of LiP isozyme expression when *P. chrysosporium* BKM-F-1767 was grown under varying conditions. In particular, isozymes *lipA* and *lipC* were predominant in nitrogen deficient liquid media, whereas *lipD* was more dominant in carbon deficient liquid media (15, 22, 24). Furthermore, the expression of the LiP encoding genes in *P. chrysosporium* grown on complex natural substrates like wood (16) and soil (3) differed from the above mentioned defined media. This suggested that, although the LiP proteins were similar, there were subtle differences in enzyme action and the LiP encoding genes were differentially expressed in response to external influences. How the differential expression of the LiP encoding genes corresponds to the differences in enzyme action (e.g. K_m, K_{cat} and substrate specificity (9, 14)) needs further elucidation.

The above results on different media also did not take into account the differences in isozyme expression between strains. Although Broda *et al.* (5) published results of LiP expression in *P. chrysosporium* ME446 their results were not directly comparable to findings in *P. chrysosporium* BKM-F-1767 due to differences in experimental procedures. In chapter four we described the evaluation of strain specific differences in the expression of LiPs in both defined media as well as on wood. Levels of expression of the ten known *lip* genes from *P. chrysosporium* ME446 were determined and compared to similar results from *P. chrysosporium* BKM-F-1767. In defined liquid media the results for most of the genes corresponded to the results of Stewart *et al.* (22), *i.e.* genes *lipA* and *lipC* were dominant in nitrogen deficient media, and *lipD* was dominant in carbon limited media. However, significant differences were observed for *lipF* and *lipH*. In *P. chrysosporium* BKM-F-1767 the levels both isozymes were higher in nitrogen limited media than in carbon limited media. In *P. chrysosporium* ME446 the results were inverted and the levels of *lipF* and *lipH* were higher in carbon limited media.

More variations were found between the strains when grown on Aspen wood chips. In two-week old cultures the differences of *P. chrysosporium* ME664 and BKM-F-1767 are marked. In *P. chrysosporium* ME446 the dominant transcript was *lipA*, and no mRNA was detected for *lipE*, *lipH*, *lipI* and *lipJ*. This is in contrast to the results of Janse *et al.* (16), which showed that all these genes were expressed in *P. chrysosporium* BKM-F-1767, with *lipC* being dominant. It is interesting to note that the levels of expression of the *lip* genes in four-week old cultures of *P. chrysosporium* ME446 correspond more closely to the levels in two-week-old cultures of *P. chrysosporium* BKM-F-1767, with transcripts of *lipE*, *lipH* and *lipI* being detected, as well as *lipC* being the dominant transcript. This could be due to the fact that *P. chrysosporium* ME446 produces the LiP's later than *P. chrysosporium* BKM-F-1767,

possibly as the result of slower growth, although no studies have compared biomass and enzyme production between the two strains. Furthermore, *lipJ* was only detected in very low levels in *P. chrysosporium* ME446, while high levels of *lipJ* were detected in both two-week and eight-week old cultures of *P. chrysosporium* BKM-F-1767. These results indicated that there were definite strain variations in the expression of the LiPs on natural media.

Analysis of the results in chapter four, as well as previously reported results (3, 5, 15, 16, 20, 22, 24), revealed no link between LiP expression and genetic organisation. Most of the LiP genes (*lipA*, *lipB*, *lipC*, *lipE*, *lipG*, *lipH*, *lipI*, and *lipJ*) were found on a single dimorphic chromosome, with *lipD* and *lipF* being found on separate chromosomes (10, 23), but no similarities in expression were detected between the closely linked genes. Although no link was detected between *lip* expression and genetic organisation or gene orientation, the LiP encoding genes are differentially regulated in response to growth conditions. The method of this regulation is still poorly understood, partially due to the fact that *P. chrysosporium* is dikaryotic (23), and inadequate techniques are available to study the genetics of dikaryotic fungi.

In summary, the main aims of this study were to investigate the influence of strain variations on *lip* expression in *P. chrysosporium*, as well as to develop a simple and efficient method of determining allele segregation. In chapter three we describe an efficient and cost effective method of identifying alleles using automated DNA sequencing. In chapter four we showed that although there are many similarities in the LiP expression between the two strains on *P. chrysosporium*, there are also sufficient variations to conclude that there is strain variations in the regulation of the LiP genes.

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