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Molecular Aspects of Cellobiose Dehydrogenase Produced by *Trametes versicolor*

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in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

"The worst thing that has happened to science education is that the fun has gone out of it. A great many good students look at it as slogging work to be got through on the way to medical school. Others are turned off by the pre-medical students themselves, embattled and bleeding for grades and class standing. . . Very few recognize science as the high adventure it really is, the wildest of all explorations ever taken by human beings, the chance to glimpse things never seen before, the shrewdest manouevre for discovering how the world works. . . Part of the intellectual equipment of the educated person . . . ought to be a feel for the queerness of nature, the inexplicable thing, the side of life for which informed bewilderment will be the best way of getting through the day."

Lewis Thomas

ACKNOWLEDGMENTS

The work described in this thesis was carried out in three laboratories located in two continents. Accordingly, there is a large number of people to thank. I thank my supervisor, Dr. Fred Archibald, who was always patient and affable, and shared with me and with all of his students a little of his own sense of "informed bewilderment". Dr. Trevor Charles, my supervisor at Macdonald Campus, was likewise patient and encouraging, and an endless source of good ideas. In addition, I thank Dr. Kirk Bartholomew, an "unofficial" advisor for many of these experiments, who generously shared his extensive expertise in fungal molecular genetics.

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The generosity of Dr. V. Renganathan in providing the cDNA clone of *Phanerochaete chrysosporium* CDH, along with purified protein and anti-CDH antibodies is gratefully acknowledged. Also, Dr. Edgar Ong is thanked for providing the laccase cDNA clones, along with PCR primers specific for *T. versicolor* laccase.

Finally, I thank my wife, Ronda Appell, whose unconditional support and constant encouragement made the completion of these studies possible.

Generous scholarship support for these studies was provided by the Pulp and Paper Research Institute of Canada and by the J.W. McConnell Memorial Foundation. A stipend was also provided by the *Centre Technique du Papier* (France).

ABSTRACT

Under cellulolytic conditions, the white-rot fungus *Trametes versicolor* produces cellobiose dehydrogenase (CDH), an enzyme with a number of biochemical properties that are potentially relevant to the degradation of lignin and cellulose. To clarify its biochemical properties, CDH was purified from cultures of *T. versicolor*. Two isoforms of CDH were found: a 97 kDa isoform with both heme and flavin cofactors, and an 81 kDa isoform with a flavin cofactor. Both isoforms of CDH were found to be quite non-specific in their reductive half reactions. The flavin enzyme catalyzed many of the same reactions as the heme/flavin enzyme, but less efficiently. The flavin isoform reduced Fe(III) and Cu(II) only at concentrations well above those found physiologically. Thus the heme/flavin enzyme, but not the flavin enzyme, could be involved in promoting and sustaining the generation of hydroxyl radicals (•OH) by Fenton's chemistry.

To characterize further the structural features of CDH, a genomic clone was isolated and sequenced. CDH was found to consist of 748 amino acids, without its predicted 19 amino acid signal peptide. Consistent with the domain structure of other CDHs, *T. versicolor* CDH appeared to be divided into an amino terminal heme domain and a carboxy terminal flavin domain, connected by a hydroxyamino acid-rich linker. Within the flavin domain, a putative cellulose-binding domain (CBD) was found by alignment to the hypothesized CBD of *P. chrysosporium* CDH. The CBD of CDH appeared to be structurally unrelated to other CBDs which have been reported.

A cDNA clone encoding *T. versicolor* CDH was isolated by RT-PCR. Using this clone, three vectors for the heterologous expression in *Aspergillus oryzae* of CDH were prepared. These vectors were built by performing in-frame fusions of the cDNA to control sequences from the highly expressed *A. oryzae* amylase gene. These vectors were transformed into *A. oryzae* and one strain was isolated which contained the expression construct DNA.

A rapid method for cloning *cdh*-like genes was developed. Using short stretches of amino acids completely conserved within *T. versicolor* and *P. chrysosporium* CDH,

PCR primers were designed to amplify a homologous gene from other fungi. The primers were tested using genomic DNA of *Pycnoporus cinnabarinus*. A 1.8-kb fragment of *P. cinnabarinus cdh* was thereby amplified and cloned, and its sequence was determined. The three CDHs displayed very high homology at the amino acid level.

Finally, to probe the role of CDH in lignocellulose degradation by *T. versicolor*, a "knockout" vector was constructed consisting of a phleomycin-resistance cassette inserted into the protein coding sequence of cloned *T. versicolor cdh*. *T. versicolor* was transformed with the knockout vector and the transformants were analyzed for their CDH-producing phenotype. Three isolates were found that produced no detectable CDH. Biobleaching and delignification by the CDH(-) strains appeared to be unaffected, suggesting that CDH does not play an important role in these processes.

RÉSUMÉ

La moissisure blanche *Trametes versicolor* produit de la cellobiose déshydrogénase (CDH) dans des conditions cellulolytiques. Cet enzyme a plusieurs propriétés biochimiques qui pourraient être utiles dans la dégradation de la lignine et de la cellulose. Pour clarifier ses propriétés biochimiques, la CDH fut purifiée à partir de cultures de *T. versicolor* et deux isoformes furent trouvées. La première a 97 kDa et possède un cofacteur hème et un cofacteur flavine. La deuxième a 81 kDa et contient seulement la flavine. Les deux isoformes ne sont pas très spécifiques par rapport aux substrats réduits. L'enzyme possèdant uniquement la flavine catalyse, de façon moins efficace, beaucoup de réactions semblables à celles de l'enzyme ayant les deux cofacteurs. La protéine ayant seulement la flavine réduit le Fe(III) et le Cu(II) à des concentrations supérieures aux concentrations physiologiques. Donc l'enzyme hème/flavine, contrairement à l'enzyme flavine, pourrait promouvoir et soutenir la génération de radicaux hydroxyles (•OH) par la chimie de Fenton.

Pour mieux déterminer les caractéristiques structurelles de la CDH, un clone génomique fut isolé et séquencé. La CDH est constituée de 768 acides aminés, incluant un peptide signal de 19 acides aminés. La CDH de *T. versicolor* semble avoir un domaine hème amino terminal et un domaine flavine carboxy terminal reliés par une connexion riche en acides hydroxy-amine. Ceci est semblable aux domaines structurels d'autres CDH. Un domaine probable d'attache à la cellulose (CBD) a été déterminé avec le domaine hypothétique (CBD) de CDH de *Phanerochaete chrysosporium*. Le CBD de la CDH ne semble pas de s'apparenter à d'autres CBD rapportés dans la littérature.

Un clone d'ADNc codant pour la CDH de *T. versicolor* fut isolé par RT-PCR. Utilisant ce clone, trois vecteurs, pour une expression hétérologue de CDH par *Aspergillus oryzae*, furent préparés. Ces vecteurs furent créés en faisant des fusions de l'ADN pour contrôler les sequences du gène amylase. *A. oryzae* fut transformé avec ces vecteurs et une souche contenant l'ADN d'expression fut isolée.

Une méthode rapide pour cloner des gènes semblables au gènes de CDH fut dévelopée. Des amorces de PCR furent mises au point en utilisant de courtes séquences d'acides aminés retrouvés dans la CDH de *T. vesicolor* et de *P. chrysosporium*. Les amorces furent testées en utilisant l'ADN génomique de *Pycnoporus cinnabarinus*. Un fragment de 1,8 kb de la *cdh* de *P. cinnabarinus* fut alors amplifié et cloné, et sa séquence fut également déterminée. Les trois CDH étaient très similaires au niveau des acides aminés.

La séquence codant pour la proteine clonée de *cdh* fut interompue par une cassette contenant une résistance à la phléomycine. Le vecteur ainsi construit servira à déterminer le rôle de la CDH dans la dégradation de la lignine par *T. versicolor*. *T. versicolor* fut transformé avec ce vecteur et les transformants analysés pour la production de CDH. Trois souches ne produisant pas de CDH détectable furent isolées. Le bioblanchiment par les souches CDH(-) ne semble pas affecté, suggêrant que la CDH ne joue pas un rôle important dans ce processus.

CLAIMS OF CONTRIBUTIONS TO KNOWLEDGE

1. Showed that the true molecular weight of *T. versicolor* CDH, previously thought to be approximately 50 kDa, is nearer to 97 kDa. Expanded the known substrate range of CDH, and showed that it does not reduce oxygen or oxidize glucose as previously thought.

2. Showed that the heme/flavin isoform of CDH reduces Fe(III) and Cu(II) under physiologically relevant conditions, while the flavin isoform reduces these metals only at higher (non-physiological) concentrations. This implied that the heme/flavin isoform, but not the flavin isoform, can promote and sustain a Fenton's reaction which generates hydroxyl radicals. In addition, this finding suggested that the proteolysis of CDH is one means by which *T. versicolor* can control CDH-mediated hydroxyl radical production.

3. Demonstrated that the nature of the reduced substrate influences the observed temperature and pH ranges of CDH. Since its *in vivo* substrate is unknown, the effective temperature and pH ranges of CDH are unknown.

4. Showed that antibodies raised against CDH of *Phanerochaete chrysosporium* sensitively detect the two isoforms of *T. versicolor* CDH, in both non-denatured and SDS-denatured forms. This implies that the two isoforms of CDH share common sequence elements and that *T. versicolor* and *P. chrysosporium* CDH are highly similar to one another.

5. Isolated a genomic clone encoding *T. versicolor* CDH and determined its complete sequence. This is the first *cdh* sequence outside of *P. chrysosporium* that has been reported. Also determined that *T. versicolor* CDH consists of 769 amino acids with a 19 amino acid signal peptide, and confirmed its high homology to *P. chrysosporium* CDH.

6. Determined that *T. versicolor* CDH is encoded by a single gene. Taken together with the observation that the two CDH isoforms share common sequence

elements, this strongly suggests that the flavin isoform is produced from the heme/flavin isoform by proteolysis. Also showed that the expression of *T. versicolor cdh* is controlled at the transcriptional level.

7. Isolated a cDNA clone encoding *T. versicolor* CDH and thereby confirmed the tentative intron assignments of the genomic clone.

8. Using the gene splicing technique of splicing by overlap extension (SOE-PCR), constructed several vectors for the heterologous expression of *T. vesicolor* CDH and laccase in *Aspergillus oryzae*. Found that the success of SOE-PCR is strongly dependent upon a 1:1 molar ratio of the products to be spliced.

9. Developed PCR primers for amplification of *cdh*-like sequences from any organism containing similar genes. Using these primers, a near full-length CDH-encoding clone from *Pycnoporus cinnabarinus* was amplified. This is only the third organism whose *cdh* sequence has been determined. *P. cinnabarinus* CDH was found to be very similar to *P. chrysosporium* and *T. versicolor* CDHs, but, consistent with the taxonomic relationships of the three fungi, was more similar to *T. versicolor* CDH than to *P. chrysosporium* CDH.

10. Constructed a vector for the targeted disruption of *T. versicolor cdh* by inserting a phleomycin resistance cassette into the *cdh* genomic clone. Showed that two of three CDH(-) strains isolated using this vector have an ostensibly normal *cdh* locus, while the third has a disrupted *cdh* locus and a normal laccase locus. This is the first report of a *cdh* mutant.

11. Produced a cDNA library of *T. versicolor* 52J designed to include clones of importance to the biobleaching effect. This library was used by other workers to isolate two full-length and one truncated laccase-encoding clone. Also produced a representative genomic library from *T. versicolor* 52J DNA which was used to isolate the genomic clone of *cdh*.

CONTRIBUTIONS OF AUTHORS

This thesis is presented as a compilation of six manuscripts, all or parts of which have been or will be published in the scientific literature. Various co-authors contributed to these manuscripts, as detailed below.

In Chapter 2, the procedure for purification of cellobiose dehydrogenase was developed by Brian Roy, who appears as a co-author on the manuscript which was published in *Applied and Environmental Microbiology* (B. Roy et al., Appl. Environ. Microbiol. 62 (12): 4417-4427, 1996). The manuscript is reprinted with permission from the publishers. Most of the data appearing in this chapter are from the purification I undertook, with some data from B. Roy included for comparison. Specifically, B. Roy contributed the most of the data for the carbohydrate oxidations (except as indicated in the footnote of Table 2.1); the pH and temperature optima using TBBQ as electron acceptor; and the cofactors of CDH 4.2 and CDH 6.4. Moreover, the original text was written by B. Roy, and was modified according to the data I contributed.

In Chapter 3/Appendix 2, the size fractionated DNA for the genomic DNA library was prepared by K. Bartholomew. The experiments were performed under the guidance of K. Bartholomew, S. Moukha, T. Charles, and F. Archibald. A minor portion of the sequence of the *cdh* genomic clone was done using a commercial service, and the entire *cdh* cDNA sequence was done commercially. This chapter is reprinted with permission from Gene 210(2): 211-219, 1998.

All of the experiments described in Chapter 4 were undertaken by the current author, under the guidance of S. Moukha for the SOE reactions, and under the guidance of K. Bartholomew, T. Charles, and F. Archibald for the heterologous expression trials. The sequencing reactions confirming the structure of the spliced constructs were done using a commercial sequencing service. The data presented in this chapter has not yet been published in the scientific literature.

In Chapter 5, I was responsible for designing the PCR primers, performing the PCRs, and cloning the interesting PCR products. The sequence of the longest PCR product, 2U/4D, was determined by a commercial sequencing service. Since this data was obtained, S. Moukha has isolated a full-length clone of *P. cinnabarinus cdh* and analyzed its expression. This data will be included with that reported in Chapter 5 before submission to a scientific journal for publication.

Chapter 6 was a collaborative effort involving myself, K. Bartholomew, L. Valeanu, T. Charles, and F. Archibald. I designed and constructed the knockout vector for *T. versicolor cdh*, designed the primers for the genetic screen of the CDH(-) mutants, and performed the genetic screen of the mutants. K. Bartholomew performed all of the transformations; L. Valeanu screened the phleomycin resistant transformants for CDH activity and performed the biobleaching and delignification assays; and all of the experiments were done under the guidance of T. Charles and F. Archibald. This work has not yet been submitted to a scientific journal.

Appendix 3 describes several experiments I undertook using an MnP-deficient mutant originally isolated by K. Addleman. The work represents an expansion on the characterization of this mutant, and was added to the data contributed by K. Addleman before publication in *Applied and Environmental Microbiology* (Appl. Environ. Microbiol. 61(10): 3687-3694). This excerpt is reprinted with permission from the publishers.

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aa	amino acid	Da	dalton
Ab	antibody	DCIP	dichlorophenolindophenol
ABTS	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid	DEAE	diethylaminoethyl
AOX	adsorbable organic halogens	DNA	deoxyribonucleic acid
ATCC	American type culture collection	dNTP	deoxyribonucleoside triphosphate
bp	base pair(s)	DTT	dithiothreitol
C-terminus	carboxy terminus	EDTA	ethylene diamine tetraacetic acid
CBD	cellulose binding domain	FAD	flavin adenine dinucleotide
CBQ	cellobiose:quinone oxidoreductase	gal	galactose
cdh	gene encoding cellobiose dehydrogenase	glc	glucose
CDH	cellobiose dehydrogenase	h	hour(s)
cDNA	DNA which is copied from RNA	ha	hectare
CPPA	Canadian Pulp and Paper Association	HWKP	hardwood kraft pulp

IEF	isoelectric focusing	mRNA	messenger RNA
IPTG	isopropyl β-D-thiogalactoside	MW	molecular weight
kb	kilobase pairs	N-terminus	amino terminus
KMB	α-keto-γ-methiolbutyric acid	ng	nanogram(s)
L	litre	nt	nucleotide(s)
LB	Luria-Bertani medium	PAGE	polyacrylamide gel electrophoresis
lcc	gene encoding laccase	PCR	polymerase chain reaction
LiP	lignin peroxidase	PEG	polyethylene glycol
MES	2(N-morpholino) ethane sulfonic acid	pI	isoelectric point
mg	milligram(s)	poly(A)	polyadenosine
min	minute(s)	polyAb	polyclonal antibody
mL	millilitre(s)	PVDF	polyvinyldiene fluoride
MnP	manganese peroxidase	RNA	ribonucleic acid
MOPS	4-morpholinopropane sulfonic acid	RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction	µg	micrograms

s	second(s)	µL	microlitres
SDS	sodium dodecyl sulfate	UTR	untranslated region(s)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis	XGal	5-bromo-4-chloro-3-indolyl β-D-galactoside
SOE-PCR	splicing by overlap extension	YM	3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose
SSC	0.15 M NaCl/0.015 M Na_3 citrate pH 7.6		
SSPE	0.15 M NaCl/0.010 M NaH_2PO_4/0.001 M EDTA		
STC	1.2 M sorbitol/10 mM $CaCl_2$/10 mM Tris pH 7.5		
t	tonne		
TAE	0.04 M Tris-acetate/0.001 M EDTA		
TBBQ	3,5-di-*tert*-butylbenzoquinone		
TBE	0.09 M Tris-borate/0.002 M EDTA		
Tris	tris (hydroxymethyl) amino methane		
u.v.	ultraviolet light		

CHAPTER 1 LITERATURE REVIEW

1.1. General Introduction

Life has existed on the Earth for at least 3.5 billion years. Throughout this time, carbon has cycled endlessly through ponderous geotectonic cycles acting in concert with relatively rapid biospheric cycles in a process that has come to be known as the biogeochemical carbon cycle (291). Essentially, the carbon cycle is a balance between atmospheric CO_2 and various carbon sinks which exist on land and in the ocean. The existence of such a balance has been critical over geologic time in maintaining the habitability of the planet; this is due to the fact that atmospheric CO_2 absorbs and re-radiates much of the heat (12-17 μm infared radiation) radiated from the Earth's surface, thereby insulating the Earth and acting as a "greenhouse gas". This effect is important in maintaining the global average temperature above the freezing point of water. CO_2 is removed from the atmosphere in a number of ways. Among the most important of these is photosynthesis, in which CO_2 is fixed into phytomass by organisms on land and in the ocean. In addition, the hydration of CO_2 in rain water as carbonic acid (H_2CO_3) removes the gas from the atmosphere and promotes the slow weathering of limestone rock which is abundant in the Earth's crust. If these carbonates were not returned to the atmosphere, life would eventually stop as CO_2 reserves became depleted and the planet cooled. Of course, this has not happened, since CO_2 is returned to the atmosphere, principally via metabolic respiration by plants, animals, and microorganisms, volcanic eruptions, and degassing along spreading oceanic ridges (291).

A large proportion of the biomass present in terrestrial biota is phytomass (42). Moreover, approximately 85% of the land biota is contained in forests of various types (42). Each year some 100×10^9 tons of new phytomass are produced through the fixation of atmospheric CO_2 (51). The major proportion of this biomass is composed of just two molecules: lignin and cellulose. These two molecules thus form a major sink of atmospheric CO_2, and the recycling of the carbon contained in these molecules is of

central importance to the biogeochemical carbon cycle. However, cellulose and especially lignin are big, highly polymerized, insoluble molecules that are very recalcitrant to degradation. In fact, the extensive decomposition of lignin can be accomplished by very few organisms, mainly a single class of the fungi. These unique degraders thus have an ecological role that is far from insignificant, and the study of the enzyme systems used by these fungi to degrade lignin can only lead to an improved understanding and appreciation of an essential component of the carbon cycle.

The subject of this thesis is the mechanism by which lignin is degraded by a certain "white-rot" fungus, *Trametes versicolor* (Figure 1.1). This basidiomycete is one of the most effective lignin degraders described to date, and its ligninolytic enzyme system has been the subject of considerable scientific scrutiny. Like many related fungi, *T. versicolor* has been found to use a series of oxidoreductase enzymes to bring about the degradation of lignocellulose. Included among these are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, all of which appear to mediate the degradation of lignin primarily through oxidative processes involving one-electron abstractions. In addition, *T. versicolor* produces cellobiose dehydrogenase (CDH), an enzyme which possesses many biochemical capabilities potentially relevant to lignocellulose degradation, but its role in the process has remained largely speculative. The majority of the work described herein focuses on various features of the latter enzyme, in an effort to characterize its features and elucidate its role. The literature review will therefore aim to summarize current knowledge of the ecology of wood rotting fungi, lignin biochemistry, and the mechanism of lignin biodegradation. Specifically, the biochemical and molecular features of the various enzymes thought to be involved in ligninolysis will be described. In addition, potential and actual industrial applications of ligninolytic fungi and their enzyme systems will be reviewed, specifically in the realm of bioremediation and in the replacement of chlorine-based bleaching chemicals in the pulp and paper industry.

1.2. Wood Rotting Fungi

1.2.1. Taxonomy

The term "fungus" is a rather generic description of an incredible array of eukaryotic organisms which have several features in common but are spread across an enormous range of taxonomic groups. Fungi are represented in two Kingdoms, Protoctista and Eumycota; the latter Kingdom contains the vast majority of known fungal species, over 70,000 (172). Two Phyla of eumycotan fungi are recognized: Zygomycotina and Dikaryomycotina. The Zygomycetes are coenocytic, fast-growing primary colonizers; the common bread mould *Rhizopus stolonifer* is one example. The Dikaryomycetes are distinguished from the Zygomycetes by the presence of septae at regular intervals within the fungal mycelium. Furthermore, Dikaryomycetes tend to grow on much less accessible substrates such as cellulose and lignin; unsurprisingly, the wood-rotting fungi are contained within this group. Their most distinguishing characteristic, however, is the presence at some phase of the life cycle (except for the anamorphs, which reproduce asexually) of a dikaryon, a state in which two haploid nuclei co-exist within a single cell without fusing and undergoing meiosis. This stage is very brief in some fungi and very stable in others, and may depend upon growth conditions (172). The Dikaryomycotina sub-phyla, Ascomycotina and Basidiomycotina, are distinguished by their meiosporangia. Ascomycetous meiosporangia are called asci and contain (usually) 8 haploid meiospores (ascospores) which are the product of meiosis and a subsequent mitosis within the meiosporangium of the dikaryon. Basidiomycetes possess basidia, which contain four sterigmata, each bearing a single haploid meiospore (basidiospore). The Basidiomycetes are further sub-divided into three Classes (172), including the Holobasidiomycetes, the common mushroom-bearing fungi. Included within this Class are several Orders; many lignin degraders are classified among the Aphyllophorales, of which there are about 400 genera and 1200 species (172). These fungi are almost all wood degraders, and include eight Families. The major Families of lignin degraders are the Polyporaceae (the bracket or shelf fungi, of which *T. versicolor* is a member) and the Schizophyllaceae (which includes another important white-rot fungus, *Pycnoporus cinnabarinus*). Another Order

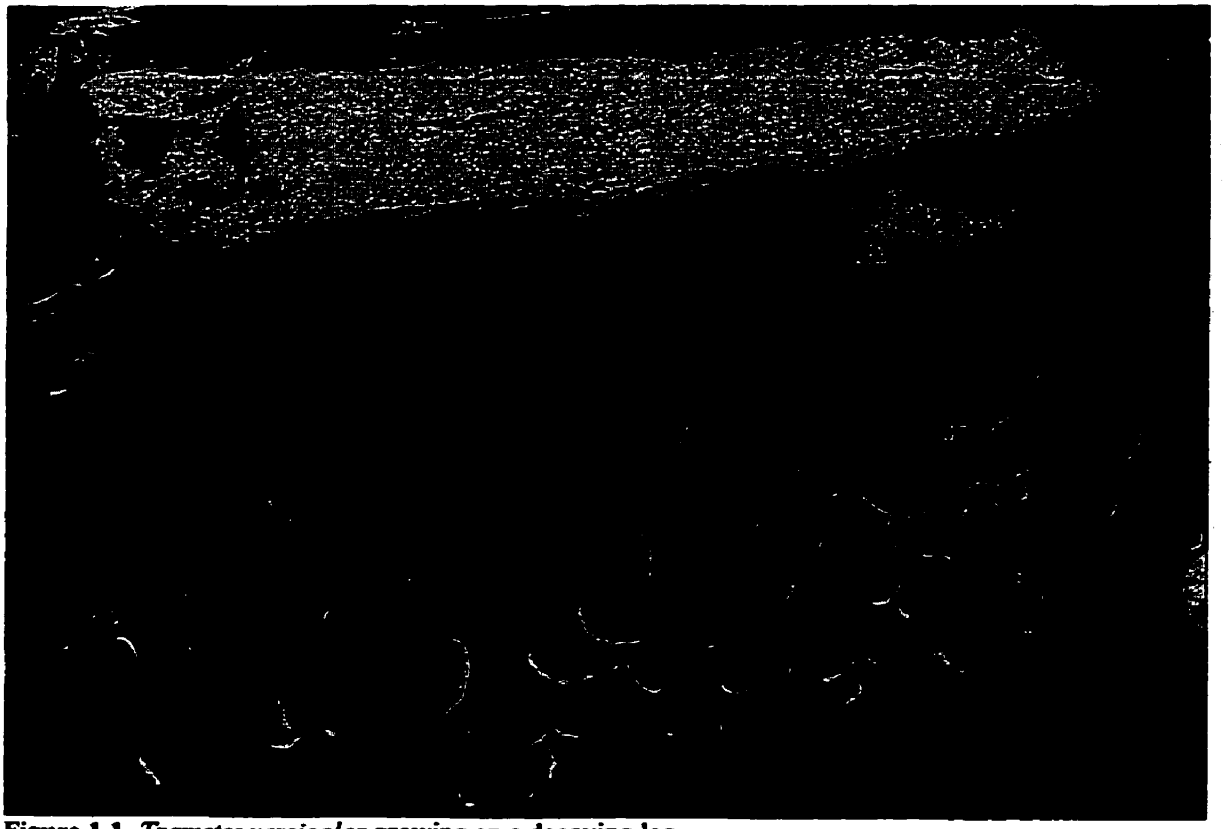

Figure 1.1. ***Trametes versicolor*** **growing on a decaying log.**

of lignin-degrading fungi are the Stereales, of which the best-studied lignin-degrading basidiomycete, *Phanerochaete chrysosporium*, is a member.

1.2.2. Genetics

The fungi are in general very good model organisms for the study of eukaryotic genetics. This is because, among other reasons, of the fact that fungi possess a manageably small genome, the large majority are haploid throughout most of their life cycle, and most produce very large numbers of uninucleate, haploid spores (172). The majority of genetic studies which have been carried out on fungi have used ascomycetes such as the yeast *Saccharomyces cerevisiae*, or filamentous ascomycetes such as *Neurospora crassa* or *Aspergillus nidulans*. Only more recently have genetic studies been extended to basidiomycetes (65, 125). Specifically, the mating-type genes have been well-studied in the plant pathogen *Ustilago maydis* and the wood-degrading fungus

Schizophyllum commune (65, 226). These studies have provided a wealth of valuable information on the cellular processes of transcription regulation and signal transduction. In addition, lignin degradation by *P. chrysosporium* has been studied at the molecular level, with a number of clones encoding lignin degrading enzymes having been isolated (124, Section 1.4.4). For *T. versicolor*, very few genetic studies have been carried out, probably due to the fact that this fungus does not readily produce haploid basidiospores in laboratory cultures. In fact, despite numerous attempts in this laboratory, the production of fruit bodies by *T. versicolor* has not been observed (K. Addleman, G. Dos Santos, unpublished observations). Thus the study of *T. versicolor* genetics has so far been limited to gene cloning (Section 1.4.4).

1.2.3. Ecology

Most wood rotting fungi exist within the forest ecosystem. Swift (296) has described the functional components of a forest ecosystem in terms of three subsystems: decomposition, herbivore, and primary producers (Figure 1.2). In forest ecosystems, the herbivore subsystem is a relatively minor component, and most of the carbon and energy coming from the green plant subsystem enters the decomposition subsystem as plant litter. The decomposition of plant litter is due to the combined actions of several hundred species, among which the ligninolytic basidiomycetes are prominent. This is because the large majority of the litter, some 0.7-14 t/ha (296) or 70% by weight of the total, is in the form of lignin and cellulose, and basidiomycetes are the best adapted of all of the decomposer organisms to degrade these polymers. Basidiomycetes therefore play an important and central role in the decomposition subsystem of forest ecosystems, and are responsible for recycling a large proportion of the carbon fixed into the phytomass of the primary producers. The basidiomycete biomass, including fungal mycelia and fruit bodies, is then a source of carbon, energy, and nutrients for other organisms. These nutrients are recycled into the other subsystems by various means, including mycotrophy (other organisms eating basidiomycete mycelia or fruit bodies), and mineralization — the production of inorganic molecules during the process of degradation, including CO_2

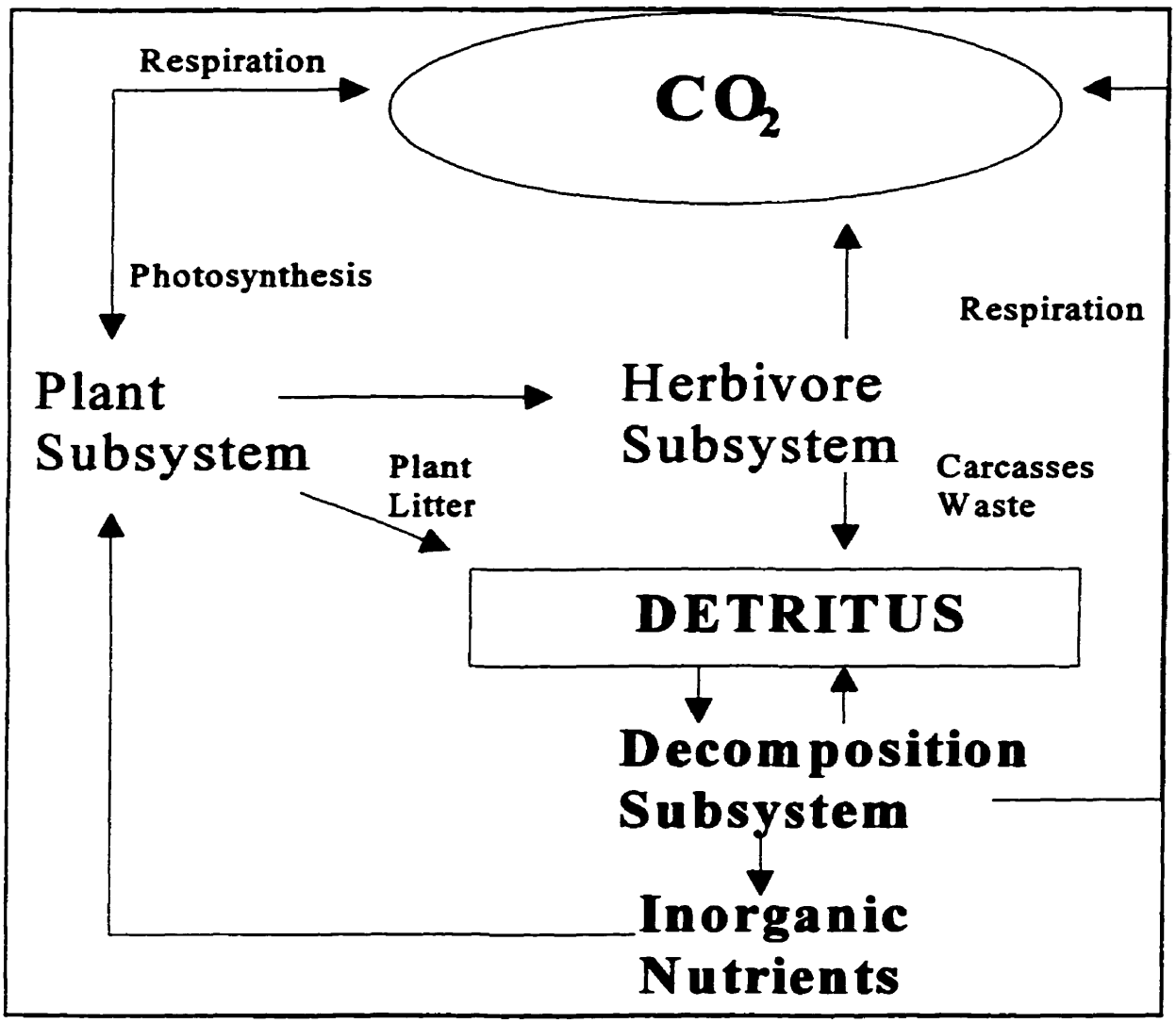

Figure 1.2. The major subsystems of the forest ecosystem. The main flows of carbon, energy, and nutrients are indicated by arrows. Adapted from reference 296.

which is incorporated into new phytomass by photosynthesis (296). In addition, the action of decomposer fungi leads to the formation of humic and fulvic acids which ultimately becomes humus, a mixture of amorphous chemical compounds that form the basis of the forest soil.

The process of decomposition of woody tissues is normally slow, especially in temperate forests (291), but follows a defined pattern. Levy (199) has identified a number of factors which influence the process of wood decay, including the structural and chemical characteristics of the wood, the rate of growth of the wood when formed, its

moisture content, pH, temperature, *etc*. The particular combination of these factors present gives rise to a series of ecological niches which are filled by a range of microorganisms which together account for the complete degradation of the log. These organisms, representing a wide range of taxonomic groups, attack woody substrates in a defined order and give rise to a nearly invariant succession of organisms that mediate the decay (199). These organisms can be classified into groups according to their effect on the wood, a more important consideration than their taxonomic status.

Under most circumstances, the first group of organisms to colonize wood which is in contact with the ground are bacteria. These are mostly gram positive bacilli, some of which can fix atmospheric nitrogen, although actinomycetes are also commonly represented. Their effects include the breakdown of pit membranes (pectin and pectin-like carbohydrates), which results in the opening up of the wood structure and facilitates the diffusion of gases. This, in combination with the fixation of nitrogen (often a limiting nutrient in a rotting log), can allow other organisms to colonize the wood. Thus the bacteria, which flourish early, are normally quickly succeeded by a second group of organisms, the "primary moulds". These are the first fungal colonists, but these organisms lack the ability to degrade cellulose and lignin, hallmarks of the fungi which flourish later in the succession. The food source of these fungi are simple carbohydrates present in the wood; they can only penetrate into natural openings or those which have been produced by other organisms. The primary moulds are succeeded by the "stainers", most of which are ascomycetes. These fungi possess pigmentation in their hyphal walls and cause a discoloration of infected wood. There is some temporal overlap between the stainers and the following group, the "soft-rots". These cellulolytic organisms are grouped together on the basis of their ability to form cavities in lignin-poor areas of the log, and become well established only when there is little competition from other fungi. The climax mycoflora of a rotting log, and the group responsible for the majority of the decomposition, are normally the basidiomycetes. These are classified into two groups: the "white-rot" fungi, which destroy both cellulose and lignin and cause a characteristic bleaching of infected wood, and the "brown-rot" fungi, which degrade primarily cellulose and hemicellulose,

leaving the lignin as a brown-colored, powdery residue. The final group of fungi to invade a rotting log, which are closely associated with the appearance and dominance of the basidiomycetes, are known as the secondary moulds. These are fungi which do not attack the wood itself, but which possess an active cellulolytic system. These organisms likely grow on cellulose which is exposed during lignin breakdown by the white rot basidiomycetes, and their appearance may represent a competition between true wood rotters and opportunistic secondary moulds. The ligninolytic enzyme system of the basidiomycetes has probably evolved to minimize the exposure of cellulose in areas far from the hyphae in order to decrease the growth of the secondary moulds (199, 216).

Lignin-degrading basidiomycetes are therefore an important component of the decay subsystem of forest ecosystems, primarily due to their ability to completely degrade lignin and cellulose. Before describing the molecular mechanisms employed by these organisms to bring about this decomposition, it is prudent to consider the nature of the substrate that is acted upon by the wood-rotting basidiomycetes.

1.3. Characteristics of the Wood Substrate

1.3.1. Wood Structure and Composition

A cross section of a woody stem reveals a series of tissue types (292). Just inside the outer bark is the phloem tissue (inner bark), which is a narrow layer through which carbohydrate-rich sap flows. Next is the cambium, a thin layer of cells which causes the yearly increase in girth of the stem by forming the sapwood. The familiar annual ring structure of the sapwood derives from the increased density of the wood formed at the end of the growth year (latewood) as compared to that formed at the beginning of the next growth year (earlywood). The sapwood, which contains some physiologically active cells, provides structural support for the tree but also serves as a food storage reservoir and in water conduction. Finally, at the center of the woody stem is the heartwood, a region of dead cells which provides structural support only. The heartwood is often discolored compared to the sapwood due to the deposition of resinous organic compounds in this region, which can affect the growth of microorganisms.

The vertical structure of the sapwood consists primarily of long cells called tracheids or fibres. The inter-fibre material is the middle lamella (ML), a lignin-rich "glue" which serves to stick the fibres together. The wall of a tracheid is rich in cellulose fibrils and consists of several layers (Figure 1.3). The primary wall is a very thin (0.05 μm), impermeable layer. The secondary wall comprises the bulk of the cell wall, and consists of three layers: an outer layer (S_1) of 0.1-0.2 μm thickness, a middle layer (S_2) which is 2-10 μm thick, and an inner layer (S_3), similar in thickness to S_1. Of the various layers of the cell wall, only S_2 contains a substantial amount of lignin. Inside S_3 is the lumen, a void area where the cytoplasm of the cell which formed the tracheid existed when the cell was part of the cambium tissue.

For all its structural complexity, wood is composed primarily of only four types of molecules. The most abundant of these, comprising approximately half of the total carbon found in plants (334), is cellulose. Cellulose is a homopolymer of glucose linked in a β1−4 linkage; the repeating unit of cellulose, cellobiose (glcβ1−4glc) thus forms a linear chain which has many free hydroxyl groups. These hydroxyl groups form powerful interchain hydrogen bonds, which cause the individual cellulose chains to aggregate together into microfibrils. Their interactions with one another are more powerful than with water, rendering the cellulose chains insoluble. Within a given microfibril of cellulose, two distinct regions can be discerned: crystalline cellulose, where the cellulose chains fit snugly together, becoming physically more rigid and chemically more resistant; and amorphous regions, which are generally more penetrable and susceptible to hydrolysis (292). Second in abundance after cellulose is lignin. This molecule is responsible for a great deal of the structural strength of the tree, and is found mainly in the middle lamella and in the S_2 layer of the cell wall of the tracheids. The details of its structure and biosynthesis will be considered in the next section. Another carbohydrate present in wood is known as hemicellulose. This diverse group of sugars, primarily hexoses (glucose, mannose, and galactose), and pentoses (xylose and arabinose), as well as the sugar acid 4-

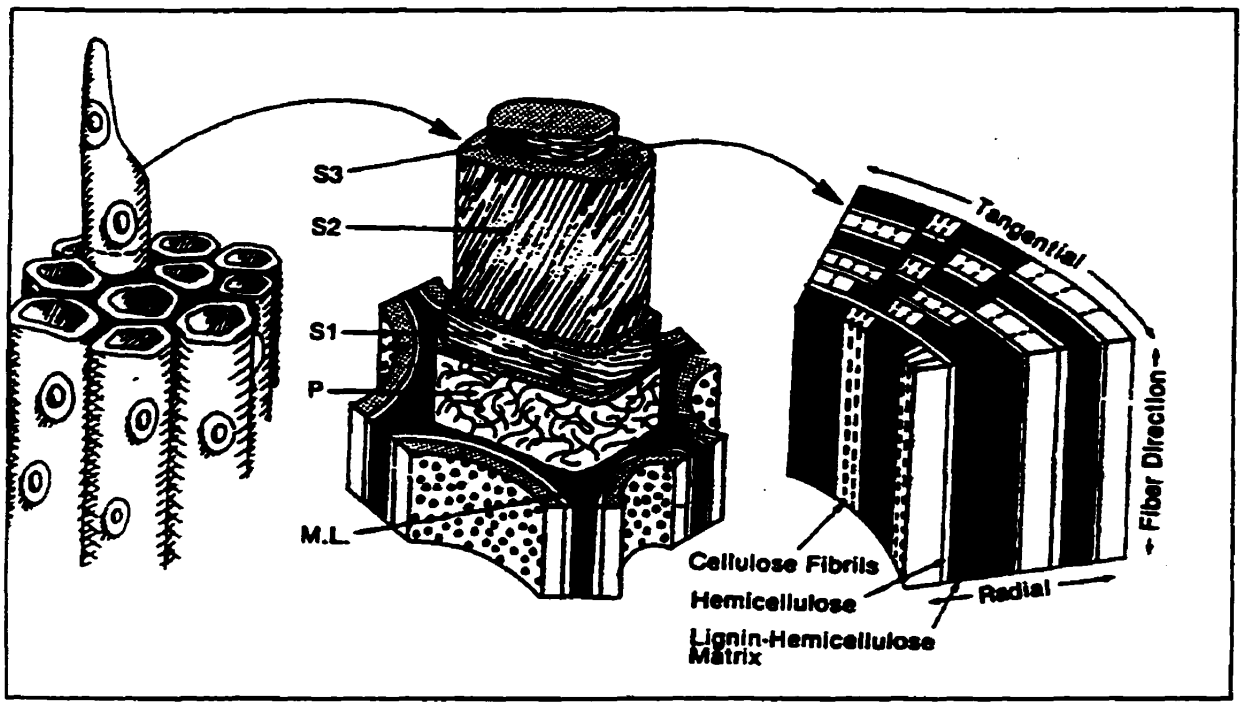

Figure 1.3. Structural detail of wood. Depicted at the left is a bundle of wood cells, in the centre an exploded view of a single cell, and at the right a section of the secondary wall (S_2) showing the relationship between lignin, cellulose, and hemicellulose in this matrix. The cell wall layers are primary (P), and S_1, S_2, and S_3. M.L. = middle lamella. Figure taken from reference 57.

O-methyl glucaronic acid, combine in a variety of ways to form a number of polymeric structures (292). Some of the hemicellulose component is closely associated with (hydrogen bonded to) the cellulose, and some is covalently bonded to the lignin component of wood. Compared with lignin and cellulose, these polymers do not pack well, and therefore are noncrystalline, open, and more easily degraded by organisms in nature. The term "extractives" is used to refer to a heterogeneous group of molecules which are extractable from wood in water or neutral organic solvents. This family of molecules, which normally comprise only a minute fraction by weight of the wood (except in certain very resinous gymnosperms), includes resin acids, fatty acids, terpenoids, and alcohols (292). Some of these molecules can inhibit microbial growth.

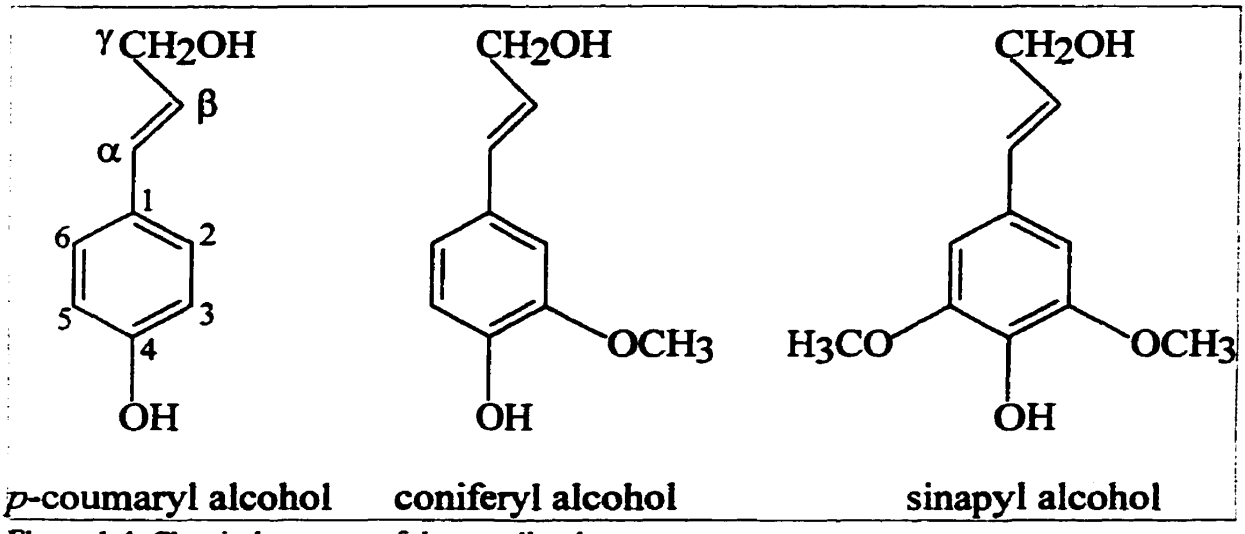

Figure 1.4. Chemical structure of the monolignols.

1.3.2. Lignin Structure and Biosynthesis

Lignin is a highly polymerized, branched network of three aromatic compounds (146). The three lignin monomers, known collectively as *p*-hydroxycinnamyl alcohols (or more simply as monolignols), are *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 1.4). The structure of the lignin depends upon the relative abundance of the three monolignols, and differs among woody plant species. Lignin from gymnosperms (also called softwoods or evergreens) is called guaiacyl lignin and is composed primarily of polymerized coniferyl alcohol (Figure 1.6), while lignin from angiosperms (hardwoods or deciduous trees) contains a roughly equal proporation of polymerized coniferyl and sinapyl alcohols, and is known as guaiacyl-syringyl lignin. Only in lignin derived from grasses is there a substantial amount of polymerized *p*-coumaryl alcohol, although the other monolignols are also present.

Lignin biosynthesis begins with the synthesis of the monolignols. The biosynthetic pathway of monolignols has been worked out by feeding radiolabelled intermediates to cut plant stems and measuring their incorporation into new lignin (reviewed in reference (146)). As with most aromatic cell constituents (334), the monolignols are synthesized via the shikimate-chorismate pathway which results in the production of the amino acids phenylalanine and tyrosine (Figure 1.5). In gymnosperms

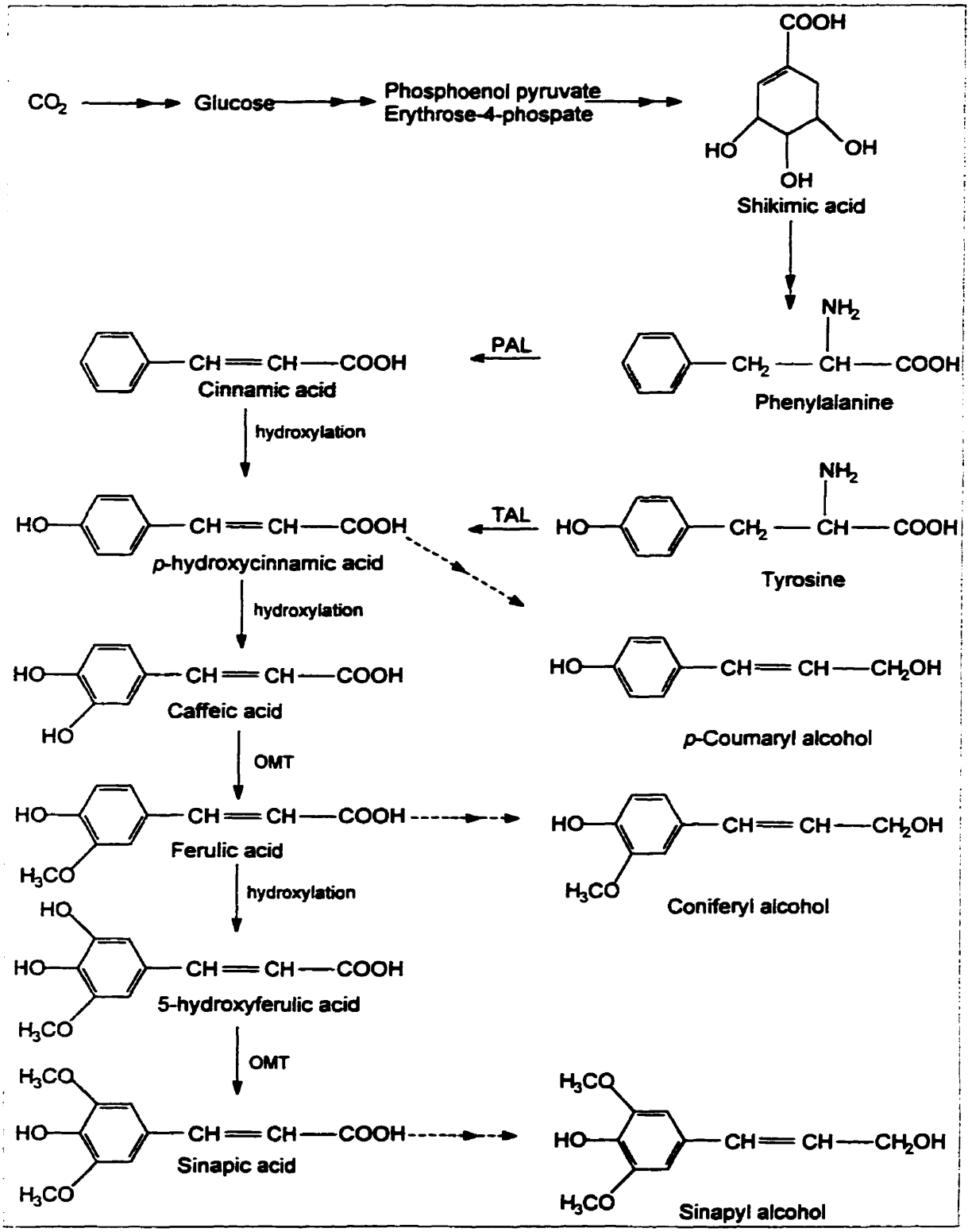

Figure 1.5. Biosynthetic pathway of monolignols. PAL = phenylalanine ammonia lyase; TAL = tyrosine ammonia lyase; OMT = *O*-methyl transferase. Dotted arrows indicate the reduction of the *p*-hydroxycinnamic acids to the respective alcohols via an intermediate aldehyde. Adapted from references 121, 146.

and angiosperms, phenylalanine is acted upon by a lyase (phenylalanine ammonia lyase, PAL), resulting in the formation of cinnamic acid, the precursor of all three monolignols. In grasses, an additional enzyme, tyrosine ammonia lyase (TAL) converts that amino acid to *p*-hydroxycinnamic acid. Thus grasses possess an additional pathway for monolignol synthesis which allows monolignols to be produced from tyrosine (146).

The biosynthesis of the monolignols starts from the common precursor, cinnamic acid, which is produced from phenylalanine by PAL. This molecule is then hydroxylated in two steps to form caffeic acid; the intermediate step forms *p*-hydroxycinnamic acid, which can be reduced to *p*-coumaryl alcohol. The *m*-phenolic hydroxyl group of caffeic acid is then methylated by an *O*-methyl transferase (OMT), yielding ferulic acid which can be reduced to coniferyl alcohol. Another hydroxylation step forms 5-hydroxyferulic acid, which is again acted upon by an OMT to form sinapic acid, the direct precursor of sinapyl alcohol.

The different composition of lignins from different species can be traced at least in part to differences in the activity and specificity of the enzymes synthesizing the monolignols (146). In angiosperms, these enzymes tend to be multifunctional, so that the same enzyme catalyzes more than one reaction. Thus, in angiosperms, the OMT catalyzes the addition of the methoxyl group to both caffeic acid and 5-hydroxyferulic acid, while in gymnosperms the analogous enzyme only works on caffeic acid. Similarly, the *p*-hydroxycinnamyl alcohol oxidoreductase, which catalyzes the final step in monolignol formation from the aldehyde intermediate, works on both coniferyl aldehyde and sinapaldehyde in angiosperms but on coniferyl aldehyde only in gymnosperms (146). In grasses, the unique presence of TAL may help explain why these lignins tend to be enriched in *p*-coumaryl alcohol, although it is unclear whether this is due to a relative abundance of *p*-hydroxycinnamic acid or to differences in the activity of the enzymes which reduce this compound to the corresponding alcohol (146).

The monolignols, once formed, are quickly stabilized by the addition of a glucose residue at the phenolic hydroxyl group (121). This improves the solubility of

these compounds and reduces their reactivity to oxidants, enabling the plant cell to store these reactive molecules and to transport them safely to the site of lignin deposition. The glucose residue is then removed by a β-glucosidase at the correct time and location for lignin biosynthesis (121).

The deposition of lignin starts with the dehydrogenative polymerization of the monolignols by peroxidase enzymes in the presence of H_2O_2 (146). This creates a highly reactive phenoxy radical which possesses several mesomeric (transient) forms corresponding to the sites of greatest electron spin density at different instants in time (121). The formation of these phenoxy radicals results in near random radical coupling reactions in which the lignin monomers polymerize into the dense, branched structure typical of lignin (Figure 1.6). The frequency of the different bonds observed in lignin is dependent upon the reactivity of the various mesomeric forms; common bonds include β-

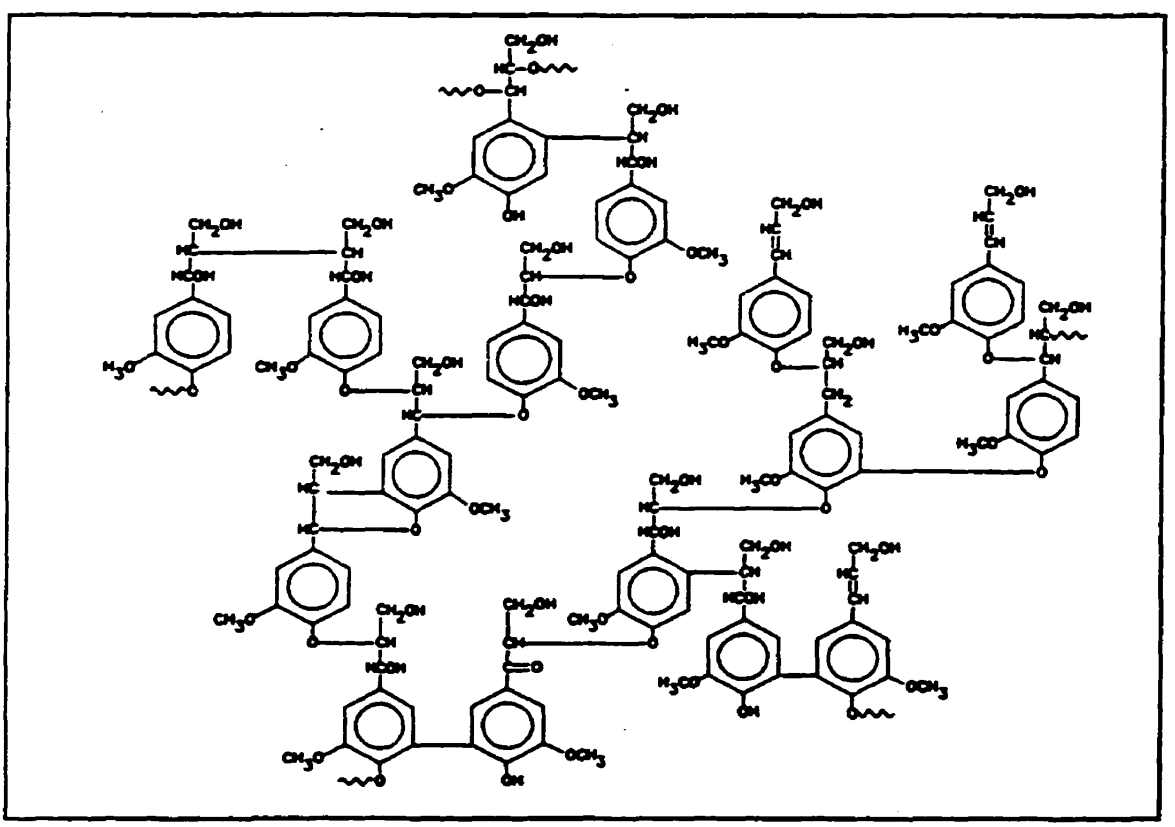

Figure 1.6. Depiction of a hypothetical fragment of a softwood lignin molecule in two of its three dimensions.

O-4, α-O-4 (ether linkages), and direct C-C coupling of aromatic rings. During the polymerization reaction, bonds are commonly formed with carbohydrates (hemicellulose) present at the site of lignification, producing a lignin-carbohydrate complex (LCC). These bonds are normally formed between the Cα of the monolignol and the primary alcohol or carboxyl group of the carbohydrate (121).

1.4. Biodegradation of Lignocellulose

Lignin is a difficult molecule to extract from natural sources, due to its large size and insolubility as well as to its extensive bonding with carbohydrates present in the woody tissue. Unmodified preparations of lignin from natural substrates are notoriously difficult (probably impossible) to obtain (57), and hampered early research in this field. To study lignin formation and biodegradation, scientists have utilized the propensity of the monolignols to undergo random polymerization upon oxidation to prepare synthetic lignins. These dehydrogenative polymerizates (DHPs) are made by oxidizing monolignols *in vitro* with oxidative enzymes, commonly laccase (polyphenol oxidase) or peroxidases. DHPs can be easily radiolabelled, either uniformly or on specific carbon atoms, are free from carbohydrate, and can be solubilized (252). All of these properties greatly facilitate the study of lignin degradation, although caution must be used in interpreting results obtained with such molecules since it has not been shown that they accurately model the structure of native lignin (252). To circumvent this problem, workers have devised a means for preparing radiolabelled natural lignins by feeding plants L-[U-^{14}C]-phenylalanine through cut stems (80). This allows precise monitoring of the degradation of a pseudo-natural lignocellulose by microorganisms.

In addition to DHPs and radiolabelled natural lignins, synthetic dimeric lignin model compounds (dilignols) have been used to study lignin degradation. These compounds possess a known chemical structure which is assumed to be representative of

common bonds found in native lignin[1]. Dilignols with the β-1 diaryl propane, β-O-4 aryl ether, and phenyl-coumaran structures have been the most extensively studied, since together they represent the majority of lignin substructures (57). Like the DHPs, however, dilignols are synthetic molecules and the real relevance of results obtained with these substrates remains to be conclusively demonstrated (252). Nevertheless, DHPs and dilignols have been very useful in identifying ligninolytic organisms and studying their mechanisms of delignification. In the following sections a summary of current knowledge of biological mechanisms of lignin degradation will be presented.

1.4.1. Organisms capable of lignin degradation

1.4.1.1. Bacteria

The degradation of lignin by bacteria is a subject that has not received widespread research attention, in part because bacteria in general are inefficient lignin degraders. Nevertheless, several reports exist of the mineralization of lignin and lignin model compounds by diverse genera, including *Nocardia*, *Pseudomonas*, *Alcaligenes*, and *Arthrobacter* (reviewed in (316)). In general, the amount of $^{14}CO_2$ released from radiolabelled DHPs is quite low (see (316) and references therein). However, there are exceptions. In one study, *Xanthomonas* sp. mineralized radiolabelled DHP, releasing 30% of the label as $^{14}CO_2$ over 20 days (174). Moreover, the bacteria were able to use the DHP as a sole carbon source, and incorporated part of the radiolabel into cellular macromolecules. Importantly, however, these organisms were only capable of degrading spontaneously dissociated oligomers of less than 1000 Da; they could not depolymerize the DHP. In another case, *Serratia marcescens* was shown to degrade extensively only a low molecular weight fraction of kraft pine lignin (242). Many bacterial genera will grow using various dilignols as a sole carbon source, indicating that they are capable of breaking many of the bonds found in lignin, but are inefficient lignin degraders. Vicuña

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[1]The random assembly via polymerization of the monolignols leads to the formation of a molecule which contains discrete chemical bonds in certain proportions, but contains no common recognition/binding sites potentially recognizeable by degradative enzymes. This fact separates lignin from all other biological macromolecules and, along with the condensed nature of the molecule which prevents the penetration of enzyme-sized molecules, is primarily responsible for its resistance to degradation by the vast majority of organisms in the biosphere.

(316) has suggested that the bacterial enzymes that break these bonds are intracellular, and that these bacteria cannot depolymerize lignin but can metabolize lignin fragments released by the activity of other organisms. Consistent with this hypothesis, Gooden et al. (123) grew six actinomycete strains on a β-aryl ether dilignol and found that two strains, *Streptomyces badius* and *Thermomonospora mesophilia*, that were able to use the dilignol as a sole carbon source produced a number of intracellular enzymes that were responsible for the degradation, including catechol 1,2-dioxygenase, protocatechuate 1,2-dioxygenase, and β-carboxymuconate decarboxylase. The production of extracellular peroxidases (123) and polyphenol oxidases (315) has also been demonstrated, but their involvement in lignin depolymerization has not been shown (123). Thus, although the products of lignin fragmentation are available to at least some bacteria, there appears to be little capacity for active high molecular weight lignin depolymerization among these organisms. In spite of this fact, it has been pointed out (316) that the ecological role of bacteria in metabolizing the fragments released by fungal ligninolytic activities may be significant in recycling the carbon present in the lignin. That bacteria perform this ecological function may be borne out by the difficulty researchers have had in obtaining bacteria-free isolates of the ligninolytic fungus *P. chrysosporium* (288), although this claim of commensalism has been disputed (154).

In contrast to their relatively minor role in lignin degradation in terrestrial environments, bacteria appear to play a more important role in aquatic habitats. In one study (222), both bacteria and microfungi were associated with the decay of submerged pine samples. Bacteria of unknown genera — known simply as "erosion", "cavitation", and "tunneling" bacteria — appear to be capable of extensive wood cell degradation in aquatic habitats (223). In addition, Benner et al. (30) analyzed the relative contributions of bacteria and fungi to lignin degradation in aquatic environments and determined that bacteria play a more important role than fungi in degrading lignocellulosic detritus in such habitats.

1.4.1.2. Fungi

The only organisms which can extensively degrade high molecular weight lignin are certain fungi. As discussed in Section 1.2.3, the fungi known to be associated with wood and lingocellulose degradation fall into three broad categories: soft-rot, brown-rot, and white-rot. The soft-rot fungi are mostly ascomycetes and conidial fungi, while the brown- and white-rot fungi are basidiomycetes. Major differences exist among these classes of fungi in their ability to degrade lignin, the ecological niches they fill, and the mechanisms they use to degrade lignocellulose. The main features of lignocellulose degradation carried out by each of these groups of fungi will be outlined below.

Soft-rot fungi in general show a very limited ability to release $^{14}CO_2$ from radiolabelled lignins, although all can degrade cellulose very well (57). This fact is consistent with their place in the succession of organisms flourishing on a decaying log (Section 1.2.3) — they establish themselves before the lignin degraders, which normally quickly out-compete them for nutrients in the log. Thus, lignin degradation by soft-rot fungi has been very little studied (252) and the true extent of their contribution to the total amount of lignin degradation in natural conditions remains unknown.

The basidiomycetes, then, are the only group of fungi that can extensively depolymerize and mineralize lignin, although this ability varies widely among taxonomic groups. Tanesaka et al. (299) analyzed the wood block degradative ability of 68 different species of basidiomycetes, including 41 wood decomposers (both white- and brown-rot), 22 litter decomposers, and 5 mycorrhizal symbionts. Interestingly, the litter decomposers showed the highest lignin loss ratio (percent lignin loss/percent wood block weight loss), followed by the white-rot fungi, which in turn had a much higher ratio than the brown-rot fungi. None of the mycorrhizal species analyzed were capable of degrading lignin. Thus the soil-inhabiting litter decomposing fungi appear to be the most effective lignin degraders, consistent with the large amount of plant detritus that is deposited on the forest floor (see Section 1.2.3). Even certain soil-inhabiting ascomycetes have been shown to mediate limited lignin transformations (263).

Among the true wood rotters, there appears to be a certain host specificity. Tuor et al. (310) have pointed out that brown-rot fungi appear predominantly on rotting gymnosperm wood, while white-rotters express a preference for deciduous wood; however the specificity among the white-rot fungi is not very high since approximately half of the species degrade hardwood only and half will degrade either hardwood or softwood. Furthermore, the white-rot fungi can be divided into two broad categories: the "simultaneous degraders", which degrade lignin and cellulose at the same time, and the "selective" lignin degraders (252). However, this classification scheme is wrought with problems since the same species can exhibit both behaviours on the same substrate; microenvironmental conditions appear to play an important role in the type of lignin degradation observed (57).

The brown-rot fungi do not degrade lignin, but preferentially degrade cellulose and hemicellulose (57). However, these organisms are capable of chemically modifying lignin as they digest the wood carbohydrates. These modifications include a decrease in the number of methoxyl groups (demethylation) and an increase in the number of phenolic hydroxyl groups. In addition, the oxidation of the propyl side chains of the lignin monomers forming α-carbonyl groups is commonly observed, although almost no cleavage of aromatic rings occurs and little depolymerization is noted (57). Macroscopically, lignin acted upon by these fungi is left as a brown, powdery mass, from which the brown-rot fungi derive their name. In contrast, the white-rot fungi are capable of degrading all of the components of woody tissue. Lignin that has been subjected to such degradation typically shows a large decrease in methoxyl group content, along with oxidative cleavage of the aromatic rings of the monolignols (57). In addition, hydroxylation reactions and the cleavage of aryl ether bonds (4-O-α, 4-O-β) are commonly observed. The major depolymerization reaction may be Cα-Cβ cleavage of the propyl side chain (57).

1.4.2. Physiology of lignin degradation by white-rot fungi

In an effort to optimize the rate and extent of lignin degradation by white-rot fungi, the culture conditions conducive to the phenomenon have been carefully examined. Although the ligninolytic system of *P. chrysosporium* was originally thought to be constitutive, Ulmer et al. (312) showed that pre-incubation of mycelia with various lignin preparations increased the subsequent rate of lignin degradation by these cultures. This suggested that an induction or activation mechanism was at work, and that lignin, or some component of lignin, is capable of inducing the expression of the ligninolytic system of this fungus.

The induction of at least some ligninolytic activities is, however, very sensitive to the concentration of certain nutrients in the fungal culture. For example, nutrient nitrogen limitation is required for the induction of LiP, MnP, and other components of the ligninolytic enzyme system of *P. chrysosporium* (58, 110, 253, 254) and *Ceriporiopsis subvermispora* (274). The induction of ligninolytic activities in these fungi appears to be associated with nutrient depletion and entry into "secondary" metabolism (110), and its supression upon addition of nutrient nitrogen seems to be associated with glutamate metabolism (109). Little is known of the control mechanisms that govern secondary metabolism and lignin degradation (57), and not all white-rot fungal lignin degradation is governed by nutrient nitrogen levels. Strains of *P. chrysosporium* have been found which degrade lignin under nitrogen-sufficient conditions (60), and ligninolysis by *Lentinus edodes* and *Pleurotus ostreatus* is known to be unaffected by nitrogen limitation (59, 194). Finally, the extensive delignification and brightening of kraft pulps (see section 1.5.2.2) observed with *T. versicolor* occurs under nitrogen-sufficient conditions (13).

Other culture parameters have been shown to affect lignin degradation. Jeffries et al. (155) found that carbon or sulfur limitations were also able to trigger lignin degradation in *P. chrysosporium*, probably as a result of a shift into secondary metabolism. In addition, the need for a carbon co-substrate was shown for *Phlebia tremellosa*; cellulose was shown to be a very efficient inducer (255). In this case, the co-

substrate probably serves as the energy source for the biosynthesis of the ligninolytic enzymes, since lignin itself is not generally used as a carbon source by white-rot fungi (57, 267). Sufficient oxygen appears to be an absolute requirement for lignin biodegradation, as almost no lignin degradation is seen under anaerobic conditions (133, 229); however above a certain concentration of O_2 no augmentation of ligninolysis is evident (57). Finally, for certain fungi, ligninolysis is only observed under solid-state fermentation conditions; culture agitation appears to inhibit this activity (115).

1.4.3. Enzymology of lignin degradation by white-rot fungi

The large size, impermeability, and insolubility of lignin necessitates that its initial depolymerization steps be extracellular. Enzymes and other species which mediate the depolymerization must therefore be excreted into the extracellular milieu in order to exert their effects. All of the extracellular enzymes that have been hypothesized to be involved in lignin biodegradation by white-rot fungi are oxidoreductases, and none recognize and cleave specific bonds found in lignin. This is hardly surprising, given that the highly condensed structure of lignin does not allow the penetration of enzyme-sized molecules (166), and given the lack of common enzyme recognition sites predicated by the random structure of the molecule. The initial steps of lignin depolymerization are thus apparently catalyzed by rather nonspecific oxidases; this initial oxidation leads to a chain of spontaneous (or at least non-enzymatic) reactions that result in lignin depolymerization. These enzymatically initiated but not enzymatically directed reactions have led researchers to term the process enzymatic "combustion" (182). Existing in a tight balance with such oxidative reactions are enzyme-catalyzed reductive reactions that may also be important in lignin depolymerization. A number of extracellular oxidoreductases have been isolated from culture supernatants which may be involved in ligninolysis. In the following sections the characteristics of these enzymes will be reviewed.

1.4.3.1. Lignin peroxidase

Lignin peroxidase (LiP; diarylpropane: hydrogen peroxide oxidoreductase; EC 1.11.1.14) is an extracellular glycoprotein with a molecular weight of approximately 40

kDa (57). The enzyme possesses one protoporphyrin IX prosthetic group per molecule, and has an unusually low pH optimum of around 3.0 (182). Originally described in *P. chrysosporium* (126, 303), lignin peroxidase has since been found to be nearly ubiquitous among white-rot fungi (231, 250) and has been isolated from cultures of brown-rot fungi (91).

The importance of LiP in lignin biodegradation has been inferred from the fact that the treatment of lignin model compounds with the enzyme results in the formation of products commonly seen in white-rotted lignin: Cα-Cβ cleavage of β-O-4 aryl ether and β-1 diaryl ether compounds; Cα oxidation of these same compounds to a corresponding ketone; oxidative aromatic ring cleavage between C3-C4; demethylation; and hydroxylation of benzylic methylene groups (see (57) and references therein). These observations led researchers to refer to LiP as "ligninase" (182), although that term is now rarely used. A large number of molecules serve as substrates for LiP oxidation, including a plethora of non-phenolic lignin model compounds (182). Importantly, LiP also oxidizes veratryl alcohol, a natural secondary nonphenolic aromatic metabolite of *P. chrysosporium*. Oxidation of this molecule serves as a standard assay for its activity (57) although other compounds have been used (17).

The catalytic cycle of LiP is that of a true peroxidase. The resting state of the enzyme is ferrous-LiP, which undergoes a two-electron oxidation to form compound I, an oxy-ferryl compound; concomitant with this oxidation is the reduction of H_2O_2 to H_2O. Compound I then performs a one-electron oxidation of a substrate (a lignin monomer or veratryl alcohol), forming compound II, which re-forms ferrous-LiP via another one-electron oxidation (57). The aryl cation radicals of the lignin monomers so formed are then thought to undergo subsequent nonenzymatic reactions leading to a multiplicity of products (182). In the presence of excess H_2O_2, LiP may be irreversibly inactivated. Wariishi and Gold (320) demonstrated that LiP compound III (LiPIII) is formed when excess H_2O_2 is present via the formation of LiPIII*, which combines with H_2O_2 and results in inactivation of the enzyme. LiPIII is an adduct of ferric LiP and superoxide,

which is formed when H_2O_2 is oxidized by LiPI. LiPIII spontaneously reverts to ferric LiP, unless excess H_2O_2 converts it to LiPIII* and inactivates it. Interestingly, veratryl alcohol may act to protect LiP from peroxide inactivation by binding to LiPIII* and returning the enzyme to its native state, releasing superoxide in the process (320). Thus the protection of LiP may be an important function of veratryl alcohol (182).

Other groups have postulated another role for veratryl alcohol: that of redox mediator. The first step in this process is presumed to be the formation by LiP compound I of veratryl cation radicals which diffuse away from the enzyme and oxidize nonphenolic lignin subunits. These oxidized lignin subunits then undergo further spontaneous reactions, accounting for the multiplicity of reaction products observed (284). In addition, Popp et al. (244) proposed a mechanism whereby the veratryl radicals that are formed by LiP-mediated oxidation react with oxalate and ultimately form Mn(III), a powerful oxidant which can initiate cleavage reactions in lignin. The mediator concept is attractive because it provides an explanation for the depolymerization of a molecule that is generally thought to be too condensed to allow the penetration of enzyme-sized molecules (166).

LiP is known to be produced by most white-rot fungi as a series of isozymes. Odier et al. (228) isolated 9 different isozymes of LiP from *P. chrysosporium*. These enzymes showed small differences in kinetic parameters, but overall the homology as shown by antibody cross reactions and amino-terminal sequencing was very high. Other studies (28, 181) have shown even higher numbers of LiP isozymes which generally differ slightly in pI, specific activity, and K_m for various substrates, but overall are very similar. The total number of LiP isozymes, and the total number of genes encoding them, is not known.

How important is LiP in fungal delignification? Although the isolated enzyme can catalyze *in vitro* the cleavage of lignin model compounds known to be representative of intact lignin (306), evidence has accumulated that casts doubt on its originally proposed role as the central ligninolytic enzyme. For example, an LiP-negative mutant of

P. chrysosporium was still able to delignify, albeit at a fraction of the wild-type level (43). Furthermore, investigations comparing the treatment of lignin with *P. chrysosporium* and with isolated LiP showed that the enzyme was not responsible for most of the depolymerization observed (175). However, observations with isolated enzymes may be misleading due to the absence of "natural mediators" such as veratryl alcohol, as described above. Perhaps more convincing is the observation both in *T. versicolor* (13) and in other white-rot fungi (231) that actively delignifying culture media potently inhibit LiP activity. Furthermore, the addition of vanadate ions, also inhibitors of LiP activity, has no effect on the delignification or lignin bleaching abilities of *T. versicolor* (13). The picture, then, is far from clear. LiP may well play a role in biological delignification, but it is not an important and central enzyme in all white-rot fungi. The reason for the multiplicity of only slightly different isozymes is unknown. Much research remains to be done this field.

1.4.3.2. Manganese peroxidase

Shortly after the discovery of LiP in culture supernatants of *P. chrysosporium*, another extracellular peroxidase was reported (192, 235). This enzyme is similar to LiP in molecular weight (approximately 46 kDa) and in containing one protoporphyrin IX-based heme prosthetic group per enzyme molecule. The enzyme activity was shown to be dependent upon the presence of manganese, specifically Mn(II), H_2O_2, and lactate or similar organic acids (192). The principal function of this enzyme was shown to be the peroxide-dependent oxidation of Mn(II) to Mn(III) (122), and hence the enzyme was called manganese peroxidase (MnP; Mn(II): hydrogen peroxide oxidoreductase; EC 1.11.1.13). The dependence of MnP on organic acids is explained by the necessity of chelating the unstable Mn(III) produced by its action (192). Chelated Mn(III) then acts as a diffusible oxidant, and directly oxidizes phenolic lignin subunits and crosslinks (81); thus MnP also uses a redox mediator to exert its effects on intact lignin. The small size of the Mn(III)-organic acid chelate facilitates the penetration of the dense lignin structure which is inaccessible to MnP and other enzymes. This has been clearly shown in experiments demonstrating polymeric dye oxidation by MnP/Mn(II)-lactate and H_2O_2 when the enzyme and the dye were separated by an ultrafiltration membrane (122).

Since its original isolation from cultures of *P. chrysosporium*, MnP has been found to be produced by a large number of white-rot fungi (61, 169, 273), including *T. versicolor* (156). Like LiP, MnP was shown to be expressed in cultures of both *P. chrysosporium* (197) and in *T. versicolor* (157, 158) as a series of at least 5 isozymes encoded by a family of structurally related genes. The isozymes of *P. chrysosporium* were shown to be highly homologous to one another (197), and, in *T. versicolor*, N-terminal sequence identity among the isozymes of over 70% was observed (158). Minor differences in isoelectric point and specific activity were observed, but essentially all the isozymes were very similar. Multiple isozymes thus seem to be a universal characteristic of fungal peroxidases, although the reasons for this multiplicity are unclear.

The catalytic cycle of MnP is very similar to that of LiP, but with one crucial difference: The reduction of MnP Compound II (the two-electron oxidized form) to the native ferric state only occurs in the presence of Mn(II) (318, 319). The rate-determining step in the redox cycle of MnP was found to be this second step (190). Moreover, Kuan et al. (190) demonstrated that MnP does not readily oxidize free (hexaquo) Mn(II); this species must be chelated. The managanese chelator that is used is therefore of central importance to the activity of MnP that is observed; an ideal chelator must both stabilize and facilitate the dissociation of Mn(III) from the enzyme, and must have a relatively low binding constant for Mn(II) (321). Kishi et al. (184) examined various organic acid chelators for *P. chrysosporium* MnP in an attempt to identify the *in vivo* chelator used by the fungus. Among the chelators lactate, oxalate, malonate, and succinate, only oxalate was found to support MnP compound II reduction at physiological concentrations; furthermore, unlike the other chelators, the reaction of oxalate with MnP compound II was irreversible (184). These observations implicated oxalate, a known metabolite of *P. chrysosporium* produced under secondary metabolic conditions, as the physiological chelator for MnP-produced Mn(III). Interestingly, Mn(III) chelated to oxalate causes the oxidation of the latter to CO_2 and $CO_2\bullet^-$, the formyl radical; the latter species in the presence of oxygen forms reactive oxygen radicals which may oxidize lignin subunits. Consistent with this, oxalate has been shown to support MnP-mediated oxidation in the

absence of exogenous H_2O_2, as the formyl radical formed from Mn(III)-oxalate reacts with atmospheric O_2 to form peroxide (191). Oxalate may in fact play a very important role in the degradation of wood components by both white-rot and brown-rot fungi (reviewed in (290)).

That MnP-mediated production of Mn(III) is important in biological delignification has been shown in numerous studies. Thus MnP, unlike LiP, can cause the depolymerization of high molecular weight lignin molecules, implying an important role for the enzyme in the initial steps of lignin degradation (193). More incidental evidence of a role for MnP in lignin depolymerization comes from studies of the positive effect of manganese ions on lignin degradation in *Pleurotus* spp., presumably due to the action of an MnP (61, 173). During the degradation of a solid wood substrate, *P. chrysosporium* produced an isozyme of MnP as the predominant peroxidase (85). Direct evidence has come from studies of purified *T. versicolor* MnP, which was shown to cause most of the demethylation and delignification observed by whole culture filtrates (234). Moreover, Addleman et al. (3) isolated a mutant of *T. versicolor* which is unable to secrete MnP; this strain was completely incapable of bleaching or delignifying industrial kraft pulp lignin, in sharp contrast to its wild-type parent. Partial restoration of these abilities was obtained by supplementing cultures with purified *T. versicolor* MnP (3). Other workers have also shown that purified MnP can cause delignification of kraft lignin and a brightening effect on the delignified material (138, 187).

1.4.3.3. Peroxide-generating enzymes

Clearly, fungal lignin and manganese peroxidases are involved in biological delignification by many different white-rot fungi, although their relative importance probably varies among the different species. Common to all peroxidases is the necessity for H_2O_2 in the catalytic cycle; thus a fungus using these enzymes to mediate extracellular lignin depolymerization must also provide, directly or indirectly, a source of hydrogen peroxide. Accordingly, candidate peroxide-generating systems have been found in *P. chrysosporium*. Kelley and Reddy (171) identified glucose oxidase as a probable

candidate for H_2O_2 production. This enzyme was found to be associated with the mycelia rather than free in the culture supernatants, implying that the activity of the extracellular peroxidases may be localized to the site of H_2O_2 generation. The importance of glucose oxidase in the degradation of lignin was dramatically shown by the fact that u.v.-induced glucose oxidase-negative mutants were unable to degrade lignin, while revertants regained this ability (251). However, other researchers have shown that glyoxal oxidase, which is an extracellular enzyme, may also be an important source of H_2O_2 for peroxidase reactions in *P. chrysosporium* (176).

1.4.3.4. Laccase

Although its presence in plants and fungi has been known for over a century, the physiological significance of laccase (benzenediol: oxygen oxidoreductase, or phenol oxidase; EC 1.10.3.2) remains largely speculative. Laccase is a member of a small group of enzymes known as the blue copper oxidases; other members of this family include ascorbate oxidase in higher plants, and the mammalian serum protein ceruloplasmin (reviewed in (213)). All members of this family are copper-containing glycoproteins which catalyze the one-electron oxidation of various substrates and the concurrent four-electron reduction of oxygen to water. Laccases are monomeric tetracopper proteins which consist of approximately 550 amino acids and have molecular weights around 64 kDa (302). The enzyme is found in a wide variety of plants and fungi. In plants, laccase may be involved in lignification (25), and may in some cases have a protective function. For example, laccase is found in the white latex of lacquer trees; upon exposure to air (plant injury), the laccase oxidizes the latex, causing polymerization and cauterization of the wound (213). In non-white-rot fungi, laccase has been observed to be responsible for the green colour of *Aspergillus* conidiospores, implying a role in spore maturation (70). Laccase is also nearly universal among wood-degrading fungi (302), and may be involved in delignification.

Copper enzymes contain several types of copper centres, which are distinguished by their spectral properties (168). A "type 1" Cu centre is mononuclear (1 Cu atom) and is

characterized by a high absorbance in the visible spectrum (λ_{max} ~600 nm). This feature imparts a distinctly blue colour to enzymes containing type 1 Cu centres (hence the "blue" copper oxidases posses a type 1 Cu centre). These Cu centres function primarily in electron transfer rather than in catalysis, which distinguishes them from the mononuclear type 2 Cu centres. Type 3 Cu centres consist of two closely situated Cu atoms. These centres also function in catalysis, specifically in O_2 binding and activation. Laccase contains four tightly bound Cu atoms, one of which acts as a type 1, one as a type 2, and two are involved in a type 3 Cu centre (213). The type 1 Cu centre is situated approximately 13 Å from the remaining three Cu atoms, which are closely grouped into a catalytic triad and are approximately equidistant from one another (3.4-5.1 Å apart) (168). A cysteine residue that is not involved in a disulfide bridge has been hypothesized to be involved in mediating the interaction between the type 1 Cu centre and the catalytic triad (53). In fungal laccases, the amino acids which coordinate the type 1 Cu atom are situated within the C-terminal region of the protein, and the typical methionine-Cu ligand is replaced by a leucine-Cu bond; this fact may account for the higher redox potential of fungal laccases compared to tree laccases (213). The catalytic cycle of laccase consists of a controlled electron flow from the substrate to the catalytic triad (and ultimately to oxygen) through the type 1 Cu centre (168). Laccase can be considered as a biological "battery", since it catalyzes the one-electron oxidation of a substrate along with the four-electron reduction of oxygen to water; electrons must be stored from sequential oxidations before their release in the formation of 2 H_2O from 1 O_2 (302).

The one-electron abstraction which is catalyzed by laccase results in the formation of an organic free radical. This species is generally unstable and undergoes further reactions, either spontaneous (for example, polymerization or fragmentation), or enzymatically catalyzed (re-reduction, or further oxidation to a quinone) (302). Although laccase is classified as a diphenol oxidase, its substrate range *in vitro* is quite wide; thus monophenols (including monolignols) and diamines are also oxidized, and the full substrate range of laccase is currently unknown (302). Attempts to demonstrate a role for laccase in biological delignification were stymied by the tendency of laccase to cause the

net polymerization of phenolic lignin-like substrates, not their fragmentation, in *in vitro* studies. However, like LiP and MnP, laccase can act via redox mediators or intermediates in order to exert effects on substrates that are not normally accessible to or oxidizeable by the enzyme. For example, several non-phenolic lignin model compounds with a redox potential too high to be directly oxidized by laccase are oxidized and degraded by laccase in the presence of 2,2'-azinobis-(3-ethylbenzthiazolate-6-sulfonate), or ABTS (49). This finding was important for *in vitro* delignification with a laccase/ABTS system, and also implied that laccase may have a role to play in delignification in fungal systems if a physiological equivalent of ABTS is present.

In the case of one efficiently ligninolytic white-rot fungus, *Pycnoporus cinnabarinus*, a low molecular weight compound suspected of being a physiological redox mediator has been found. In the wild, *P. cinnabarinus* is characterized by an orange to red basidiocarp, and produces a red pigment when grown in liquid cultures. Eggert et al. (98) identified the chromophore as cinnabarinic acid (CA), and further showed that CA is formed from its precursor, 3-hydroxyanthranilic acid (3-HAA) by the action of a laccase that is produced by the fungus. Further studies implicated 3-HAA as a physiological redox mediator for *P. cinnabarinus* laccase (97). Thus a non-phenolic dimeric lignin model compound was oxidatively cleaved in the presence of *P. cinnabarinus* laccase and 3-HAA. Furthermore, high molecular weight (>9600 Da) DHP was actively depolymerized by the laccase/3-HAA system, resulting in the formation of degradation products ranging from approximately 4 kDa to monomeric phenolics (97). The mediator effect of 3-HAA in these experiments was shown by separating the DHP and the laccase/3-HAA system by a membrane apparatus which only allowed the diffusion of small molecules (97). The fact that, in the *P. cinnabarinus* system, laccase is produced as the predominant extracellular enzyme and that LiP and MnP were undetectable in delignifying cultures lends further credence to the hypothesis that the laccase/3-HAA system is important in delignification (97). The laccase of *P. cinnabarinus* was shown to be a typical fungal laccase which is produced constitutively during primary metabolism, but, as in *T. versicolor* (74), is strongly induced by 2,5-

xylidine (99). Furthermore, N-terminal sequencing showed that *P. cinnabarinus* laccase is homologous to that produced by other white-rot fungi such as *T. versicolor* and *Coriolus hirsutus* (99). To date, however, a physiological redox mediator for laccase has been found only in this system.

Evidence for a role for laccase in delignification has also accumulated in other systems. An early and powerful demonstration was the work of Ander and Eriksson (8), who showed that a laccase-deficient mutant of *P. chrysosporium*, in contrast to wild-type and revertant strains, was unable to degrade kraft lignin or wood. In another study (130), lignosulfonates were shown to induce strongly the secretion of laccase by *Fomes annosus*, providing circumstantial evidence that laccase may be involved in lignin metabolism. Like MnP and LiP, laccase has been shown to produce lignin-oxidizing Mn(III) chelates (16). The mechanism is thought to be similar to that hypothesized by Popp et al. (244) for the LiP-mediated oxidation of Mn(II) (16), making the process of Mn(III) production peroxide-independent. Finally, a redox-cycling mechanism was proposed for *T. versicolor* laccase and glucose oxidase in which net depolymerization of high molecular weight lignins is observed only in the presence of both enzymes; glucose oxidase was hypothesized to re-reduce laccase-generated radicals, decreasing their tendency to polymerize and actually resulting in depolymerization (297).

1.4.3.5. Cellobiose dehydrogenase

An extracellular cellobiose-oxidizing, quinone-reducing enzyme was first detected in lignocellulolytic cultures of *T. versicolor* in 1974 (323, 324). Since that time, the enzyme has been referred to variously as cellobiose:quinone oxidoreductase, cellobiose oxidase, cellobiose:cytochrome c oxidoreductase, and more recently cellobiose dehydrogenase (CDH; EC 1.1.99.18). The latter name will be used in this thesis. CDH has been found in a large number of white-rot fungi, including *P. chrysosporium* (27), *Sporotrichum thermophile* (76), *F. annosus* (149), and in the brown-rot fungus *Coniophora puteana* (282). However, since the enzyme can be produced in large

quantities by optimized cultures of *P. chrysosporium* (24, 131), most of the biochemical information that has accrued to date is from the CDH produced by this fungus.

CDH is found in cellulolytic cultures of most white-rot fungi as a glycoprotein with a molecular weight around 90 kDa by SDS-PAGE analysis (27). Although in *P. chrysosporium* and most other fungi CDH appears as a monomer, some reports have identified dimers (282) or even tetrameric aggregates of CDH in some fungi (149). The enzyme contains one cytochrome b type heme and one flavin adenine dinucleotide (FAD) per molecule (27). CDH oxidizes cellobiose very readily, but will also accept electrons from cellotriose and other cellodextrins, including cellulose (27, 266), and reduces a wide range of substrates, including dichlorophenolindophenol (DCIP), cytochrome c, quinones, metal ions, and a host of others (see (11) and references therein). A second cellobiose-oxidizing quinone-reducing activity is commonly found in cellulolytic cultures. This enzyme, referred to as cellobiose:quinone oxidoreductase (CBQ; EC 1.1.5.1), has a smaller molecular weight than CDH (approximately 70 kDa), and contains a flavin cofactor. Both CDH and CBQ readily reduce certain substrates such as DCIP, but only CDH reduces horse heart cytochrome c (279); this fact can be used to distinguish the two enzymes. Evidence has accumulated which shows that CBQ is a proteolytic cleavage product of CDH. Henriksson et al. (143) showed that purified CDH can be cleaved with the plant protease papain into two discrete domains, a heme-containing domain and a flavin-containing domain; the latter protein possesses many of the properties of CBQ, including a greatly decreased ability to reduce cytochrome c (142). Even more convincingly, another group (327) showed that polyclonal antibodies raised to either protein cross-react with the other, and that partial digestion of both proteins with staphylococcal V8 protease or cyanogen bromide yields nearly identical bands by SDS-PAGE. Habu et al. (132) identified proteases produced by *P. chrysosporium* that cleave CDH, forming CBQ and an enzymatically inactive heme domain. All of this evidence points toward a two-domain model for CDH, with the heme and flavin domains separated by a protease-sensitive linker region. This model was confirmed by small-angle X-ray

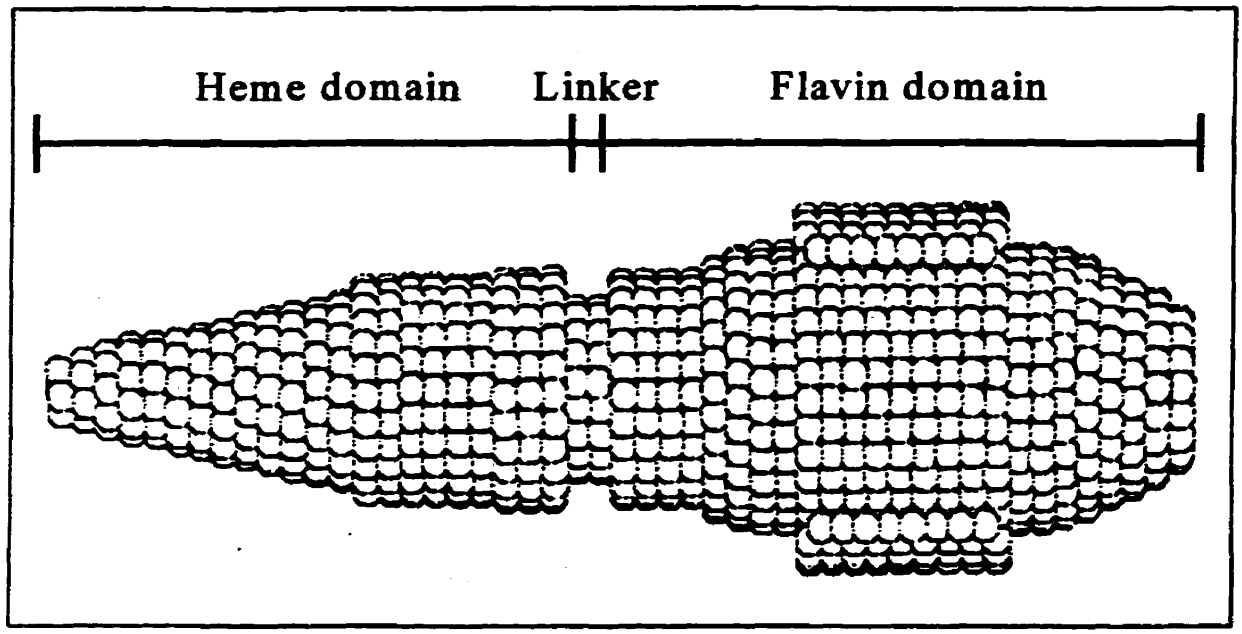


Figure 1.7. Model of *P. chrysosporium* CDH derived from small-angle X-ray scattering studies. Each of the two domains is approximately 9 nm in length, and the diameter at the widest point is 4.3-5.1 nm. Glycosylation is not shown. Figure taken from reference 196.

scattering studies of purified CDH (196), which showed CDH to have an oblong shape (Figure 1.7).

CDH is commonly thought to be involved in the degradation of cellulose. CDH is produced by a range of fungi which degrade cellulose, and is often coordinately regulated with their cellulase systems (265). Moreover, like most cellulases (see section 1.4.3.5.), CDH binds to cellulose (26, 260, 266). The cellulose-binding capacity of CDH resides in the flavin domain (143). The binding of CDH to cellulose is distinct from that of other fungal cellulases in that it is bound more strongly and more sparsely (144). For *P. chrysosporium* CDH, the cellulose/CDH interaction is not electrostatic, since it is unaffected by NaCl, but is more likely to be a charge transfer or hydrophobic interaction (144). Furthermore, CDH has been shown to bind preferentially to amorphous rather than crystalline cellulose (152, 280), and a cooperation with fungal cellulases in the breakdown of cellulose has been demonstrated (26). Presently, several families of cellulose-binding domains (CBDs) are recognized (see section 1.4.3.6), but the CBDs of both *P. chrysosporium* CDH (144) and of *T. versicolor* CDH (Chapter 3) do not appear to belong to any of them.

However, the simple binding of CDH to cellulose does not in itself implicate it in cellulose hydrolysis. Both the oxidative and reductive half-reactions catalyzed by CDH could be involved in cellulolysis. For example, the oxidation of cellobiose results in the production of cellobiono-1,5-lactone (145), an unstable species which hydrolyzes to cellobionic acid. This oxidation effectively decreases the concentration of cellobiose, which dampens the end-product inhibition of exo-cellulases that cleave cellobiose units from the ends of celluose chains. Furthermore, Kremer and Wood determined that among the many substrates reduced by CDH, Fe(III) is likely one of the most physiologically relevant (188). The reduction of Fe(III) to Fe(II) in a system containing hydrogen peroxide results in the formation of a highly reactive oxygen species, according to the Fenton reaction:

$$Fe(II) + H_2O_2 \rightarrow \bullet OH + OH^- + Fe(III)$$

The hydroxyl radical (•OH) is a powerful oxidant which can chemically cleave cellulose chains (136). Clearly, the CDH-mediated reduction of Fe(III) to Fe(II) will both promote Fenton's chemistry and sustain it by regenerating the Fe(II). Although *P. chrysosporium* CDH will also reduce atmospheric O_2 to hydrogen peroxide, so that it can generate both of the Fenton's reactants, oxygen is in general a very poor electron acceptor for CDH and will only function in the absence of other substrates (27, 279). Nevertheless, H_2O_2 is efficiently produced in the extracellular milieu by other enzymes, since it is required for MnP and LiP function (section 1.4.3.2). Thus, if CDH is secreted, Fenton's reagent (H_2O_2/Fe(II)) can clearly be produced by white-rot fungi (328). Cleavage of cellulose chains by this mechanism has been reported both in *P. chrysosporium* (208) and in brown-rot fungi (150). CBQ reduces Fe(III) much less efficiently than does CDH and so does not likely generate hydroxyl radicals under physiological conditions (142) (Chapter 2). Interestingly, the proteases produced by *P. chrysosporium* which cleave CDH to CBQ do so only when CDH is bound to cellulose (132); this implies not only that CDH undergoes a conformational change upon cellulose binding, but also that the fungus possesses a means for dampening the CDH-mediated production of hydroxyl radicals via

the proteolytic cleavage of the heme domain. Even more intriguingly, proteases have been isolated from cultures of *P. chrysosporium* which only cleave CDH to CBQ when cellobiose is present and CDH is in its reduced state; in other words, the oxidation state of CDH affects its susceptibility to proteolysis in addition to its binding to cellulose (100). These observations hint at a highly sophisticated mechanism for the mediation of CDH activity by the action of extracellular proteases and by variable rates of CDH secretion.

CDH may also be involved in lignin degradation through its capacity to produce hydroxyl radicals. In addition to causing the chemical cleavage of cellulose, hydroxyl radicals are known to cleave lignin model dimers (300). Henriksson et al. (141) investigated this phenomenon using CDH in a hydroxyl radical-producing system and found that the CDH/cellobiose/Fe(III)/H_2O_2 mixture was not only able to degrade cellulose and xylan, but synthetic lignin (DHP) was also depolymerized into fragments small enough to pass through membranes of 500 - 3000 Da cutoff. In all cases, hydroxyl radicals were demonstrated to be formed, strengthening the hypothesis that the degradation was mediated by Fenton's chemistry (141). In another study investigating the effect of Fe(III)/H_2O_2 and CDH on lignin degradation, Ander (7) demonstrated that guaiacyl/syringyl lignin (hardwood lignin) is more susceptible to CDH-generated •OH-mediated cleavage than is guaiacyl lignin (softwood lignin).

CDH may have another role in the degradation of lignin. Among the many substrates reduced by CDH are the phenoxy radicals generated by the actions of the ligninolytic peroxidases and laccases produced by the white-rot fungi. The isolated peroxidases commonly cause polymerization of lignin rather than degradation; however it has been found that CDH is capable of decreasing this polymerization, presumably by re-reducing the radicals generated by the oxidative enzyme, often to phenolics (12). The oxidation of the cation radical generated by LiP-mediated oxidation of veratryl alcohol was also strongly dampened by CDH and cellobiose. These results implied that CDH may function in a complete fungal system by helping to shift the polymerization/depolymerization balance in the direction of depolymerization (12).

Similarly, a range of interactions between CDH and oxidative ligninolytic enzymes was found in another study (270). The cellobionic acid that is ultimately formed by the oxidation of cellobiose by CDH was found to be an effective chelator for Mn(III) produced by MnP; furthermore, CDH was able to reduce insoluble $Mn(IV)O_2$ to Mn(II) or Mn(III), thereby making Mn available to the MnP catalytic cycle. These interactions are thought to be supplemented by the reduction of Mn(III)-generated quinones back to phenolic lignin subunits, which can then be re-oxidized by MnP-generated Mn(III). The cycle, however, is not futile since smaller, non-phenolic lignin fragments are produced which are not susceptible to reduction by CDH; this is proposed to result in a net depolymerization of lignin (270).

Thus, the biochemical versatility of CDH allows a number of hypotheses to be made regarding its true *in vivo* role in lignin and/or cellulose biodegradation. The true role and importance of CDH in these processes remains to be elucidated, and the enzyme may well function in both cellulose and lignin biodegradation. In a long-term study following the production of cellulases and CDH in a leaf litter decomposition system, CDH was found to peak much later than cellulases (205). These workers proposed a three-stage model for leaf litter decomposition by basidiomycetes in which CDH is produced during the third stage, when a shift from mostly polysaccharide degradation to the coupled degadation of lignin and polysaccharide occurs (205).

1.4.3.6. Other enzymes: Cellulases and proteases

The unique talent of the white-rot fungi is the ability to degrade and render bioavailable the vast amount of photosynthetically-derived carbon contained within lignin molecules. However, these fungi do not use the lignin as a major carbon source (57, 267), and remove the lignin only as a means to the end of exposing the cellulose and hemicellulose which can be used as a source of carbon and energy for their growth. Thus white-rot fungi possess an active cellulolytic system to liberate this carbon; however, a wide array of other organisms, including soft-rot fungi and a number of bacterial genera, are also cellulolytic. In order to avoid expending energy removing lignin only to expose

cellulose to degradation by opportunistic competitors, the white-rot fungi must rein in their ligninolytic activity. As the ligninolytic enzyme system is extracellular, one of the few means available to modulate its activity is with extracellular proteases that destroy the ligninolytic enzymes. The major properties of the cellulolytic and proteolytic enzymes produced by white-rot fungi will be reviewed below.

Organisms which are capable of degrading cellulose do so by secreting a complex mixture of cellulases that act synergistically to degrade the polymer (29). The fungi are no exception and produce three classes of cellulases: exo-1→4-β-glucanases (cellobiohydrolases (CBH)), which release cellobiose or glucose from the non-reducing end of a cellulose chain; endo-1→4-β-glucanases (endoglucanases (EG)), which cleave β-1→4 glycosidic bonds randomly along the length of the cellulose chain; and β-glucosidases (cellobiases), which hydrolyze cellobiose and low molecular weight glucodextrins to glucose (29). The EGs are thought to attack amorphous regions of cellulose fibres, thereby creating new sites for CBHs; the cellobiases prevent the accumulaton of cellobiose, an inhibitor of CBH synthesis (29). Among the fungi, the cellulase system of the soft-rot fungus *Trichoderma reesei* is by far the best studied (29); however all indications are that the cellulolytic systems of *P. chrysosporium* (103, 313) and other white-rot fungi (265) are similar. The cellulase system is repressed in the presence of glucose, and is induced by cellulose; the mode of induction appears to be the release of a soluble inducer from cellulose through the action of a low constitutive cellulase system (102).

Cellulases contain a set of common structural elements and are organized into domain structures (reviewed in reference (119)). The isolated domains commonly retain their independent function when isolated from the native protein. A cellulose-binding domain (CBD) is present on nearly all fungal cellulases. The CBD is not required for enzymatic activity of the cellulase, but modulates the interaction of the enzyme and the substrate. Several classes of CBD are known, including fungal (family I), and bacterial (family II), which are somewhat different in structure and appear to recognize distinct

structures on the cellulose surface (307). The fungal CBDs are approximately 30 amino acids long and are always located either at the amino or the carboxyl terminus of the protein (119). They contain four cysteine residues which form two disulfide bridges, and also contain a number of aromatic residues (tyrosine, tryptophan, and phenylalanine), which are thought to mediate the interaction with cellulose (119). In addition to the CBDs, cellulases possess a distinct catalytic domain. By primary structural homology, these domains are grouped into nine families of related sequences. A given organism may possess cellulases with catalytic domains from a number of different families, and the same family of catalytic domain can be found in widely divergent organisms. These observations have led to the conclusion that the cellulases evolved from a few ancient common sequences by domain shuffling and subsequent mutations (119). Connecting the CBD to the catalytic domain in many cellulases is a short (6-59 amino acid) linker domain which is enriched in proline and hydroxyamino acids (serine and threonine). These domains are thought to form a flexible hinge connecting the cellulase domains (119).

Proteases are an important class of extracellular enzyme produced by white-rot fungi. Eriksson and Pettersson (105) purified two acidic proteases from cellulolytic cultures of *P. chrysosporium* which stimulated the activity of EGs by an unknown mechanism. In addition, the decay of LiP activity in late secondary metabolic cultures of *P. chrysosporium* was shown to be due to the action of several proteases whose activities increase as LiP activity decreases (83, 94). The synthesis of the extracellular protease is modulated by the amount of glucose in the medium and the enzyme appears under conditions of carbon starvation (93). Datta (84) purified another endoprotease from *P. chrysosporium* which is distinct from previously described proteases from this fungus. In addition to their aforementioned roles in limiting and possibly regulating ligninolytic activity, the extracellular proteases have been proposed to be important in recycling nutrient nitrogen present in the amino acids which constitute extracellular proteins (83).

1.4.4. Molecular biology of lignin degradation by white-rot fungi

The wealth of biochemical information which has accumulated on the enzymes involved in lignocellulose degradation has been supplemented by molecular analyses in recent years. A large number of clones encoding various features of the ligninolytic system of different white-rot fungi have been isolated (Table 1.1). These sequences have in turn provided a great deal of new information regarding these enzymes, including their complete amino acid sequences, their intron/exon structure, and the regulation of expression of the genes encoding them. Some of the major findings of these molecular studies are outlined below.

The isolation of several clones encoding LiP (Table 1.1) has enabled the detailed genetic analysis of this enzyme. The *lip* genes appear to occur in clusters on one of the chromosomes (248), and have been found in tandem (163). Gaskell et al. (117) localized 10 different LiP-encoding genes to 3 distinct linkage groups in *P. chrysosporium*; one group contained 8 closely linked *lip* genes. In addition to their genomic distribution, the regulation of the expression of *lip* genes has been closely examined. In *P. chrysosporium*, the transcription of the gene encoding LiP isozyme H8 was shown to be regulated by nutrient nitrogen limitation in the same way that ligninolysis is regulated in this fungus, providing circumstantial evidence that LiP may be involved (203). However, other reports examining *lip* expression in the same fungus showed no regulation by nitrogen (160), as seen in another white-rot fungus, *Bjerkandera* spp. (167). In another report investigating the expression of *lip* in low nitrogen medium by *P. chrysosporium*, expression of different LiP-encoding genes was found to be time-dependent, such that different isozyme-encoding transcripts peaked separately; moreover, no precise correlation was found between transcript and isozyme levels (44). Similarly, Stewart et al. (294) found that the relative *lip* transcript level displayed complex regulation in response to both carbon and nitrogen nutrient limitation. This seeming complexity of *lip* regulation may be explained by an underlying regulation of all the *lip* genes by the intracellular cAMP levels (45). Thus increased levels of cAMP, known to rise sharply during the shift from primary to

Enzyme	Nature of clone	Organism	Comment	Reference
LiP	genomic	*P. chrysosporium*	*LG2*; 8 introns	262
LiP	genomic	*T. versicolor*	*LPG1*; 6 introns	38
LiP	4 cDNA	*P. chrysosporium*		333
LiP	cDNA	*P. chrysosporium*		304
LiP	genomic	*T. versicolor*	LPG1, LPG2; tandem genes	163
MnP	genomic	*P. chrysosporium*	*mnp*-1; 6 introns	124
MnP	cDNA	*P. chrysosporium*		236
MnP	cDNA	*P. chrysosporium*	*mnp*1	245
MnP	genomic, cDNA	*P. chrysosporium*	*mnp*2; 7 introns	210
Laccase	genomic	*P. radiata*	9 introns	277
Laccase	2 genomic, 2 cDNA	*Coriolus hirsutus*	allelic forms; 10 introns	186
Laccase	cDNA	*T. versicolor*		224
Laccase	2 genomic	*T. versicolor*	*CVLG1*, *CVL3*; 11 introns	153, 215
Laccase	genomic, cDNA	PM1[1]	*lac*1; 10 introns	71
Laccase	genomic, cDNA	*P. ostreatus*	*pox*1; 19 introns *pox*2	118
Laccase	2 genomic, 2 cDNA	*Trametes villosa*	*lcc*1, *lcc*2; 8, 10 introns	330
Laccase	3 genomic	*T. villosa*	*lcc*3, *lcc*4, *lcc*5	331
Laccase	2 cDNA, 1 genomic	*T. versicolor*	*lcc*I, *lcc*IV[2]	230
CDH	2 genomic	*P. chrysosporium*	alleles *cdh*-1, *cdh*-2; 13 introns	200
CDH	cDNA	*P. chrysosporium*	*cdh*-2	201
CDH	cDNA	*P. chrysosporium*		249
novel peroxidase	genomic	*T. versicolor*	*PG V*; 12 introns	162
cellobiohydrolase	3 genomic	*P. chrysosporium*	*cbh*1-1, *cbh*1-2, *cbh*1-3	78
cellobiohydrolase	2 genomic, 2 cDNA	*P. chrysosporium*	alleles of *cbh*II; 6 introns	301
AAD	cDNA	*P. chrysosporium*	aryl-alcohol dehydrogenase	259
glyoxal oxidase	cDNA	*P. chrysosporium*	glx-1c	177

[1]Basidiomycete PM1, deposited in Spanish type culture collection (CECT 2971), is an unidentified white-rot fungus isolated from paper mill watewater (71).

[2] cDNA clones isolated from the *T. versicolor* cDNA library prepared as described in Appendix 1.

Table 1.1. Clones encoding major lignocellulolytic enzymes from white-rot fungi. This list is incomplete, as a number of unpublished sequences for several ligninolytic enzymes are also available (see http://www.ncbi.nlm.nih.gov/Taxonomy/).

secondary metabolism and to be a carbon and energy starvation signal, are associated with transcription of *lip* genes, and as cAMP levels drop, transcription of all *lip* genes stops. In addition, the isolation of a u.v.-induced mutant of *P. chrysosporium* defective in the production of all LiP isozymes hints that the regulation of the entire *lip* gene family is controlled by one or only a few loci (43).

The expression of the *mnp* genes, like the *lip* genes, appears to be controlled in *P. chrysosporium* by the nutrient nitrogen level; thus *mnp* mRNA and MnP only accumulate under conditions of nitrogen limitation (55). This correlation with nutrient depletion is not observed in *T. versicolor*, however, and so is not universal among white-rot fungi (72, 73). Moreover, in *P. chrysosporium*, several other factors influence the synthesis of MnP. The appearance of MnP in nitrogen-limited cultures is critically dependent upon the presence of Mn(II) ions, which appear to regulate the transcription of the *mnp* genes (54, 55). Mn(II) ions strongly regulate the expression of *mnp* in *T. versicolor* as well (234). Pease and Tien (238) showed that the different isozymes of MnP display differential regulation, like the LiP isozymes; again, the significance of this not known. In addition to Mn regulation, the transcription of *mnp* genes is sensitive to heat shock induction, consistent with the existence of heat shock response elements (HSEs) upstream of *mnp* (56). The physiological relevance of this form of regulation is unclear, but the relief of oxidative or chemical stress may be important (202).

Laccase is known to be produced as a series of 6-10 isozymes in many white-rot fungi (39) which are commonly differentially regulated (116). The availability of a number of laccase clones has allowed researchers to analyze the regulation of transcription of the *lcc* genes. In *T. versicolor*, *lcc* transcription can be induced by either 1-hydroxybenzotriazole or 2,5-xylidine (74). Interestingly, transcription of the *T. versicolor lcc* genes is also induced by copper and nitrogen (74).

With the notable exception of CDH, the lignocellulolytic enzymes produced by white-rot fungi are encoded by gene families. Moreover, each isozyme tends to be differentially regulated, even though the gene products are highly similar. For example, at

least ten distinct *lip* sequences have been reported in *P. chrysosporium* (125), the similarities of which range from 71.5%-99.5%. A complicating factor in the identification of new isozymes from genetic sequences is that allelic variants of several of the *P. chrysosporium* LiP isozymes (125) as well as of CDH (200) and glyoxal oxidase (178) have been reported to occur. For CDH, two distinct alleles, *cdh*-1 and *cdh*-2, were identified based on RFLP patterns from single basidiospore progeny. The two genes are 97% identical in sequence, and their products have an identical amino acid sequence since the nucleotide changes are all in wobble bases or introns (200).

Another interesting finding of the gene cloning studies is that functionally related genes tend to occur in clusters. This has been observed not only for lignocellulolytic genes (78, 163), but also for other functionally related genes in many filamentous fungi (311). The direction of transcription within such gene clusters may be the same (163) or different (78). Since each gene has its own regulatory elements, and since polycistronic mRNAs are not observed in eukaryotes, the reason why such an arrangement has evolved is unclear (311).

The biochemical analysis of enzymes involved in lignocellulose biodegradation is commonly hampered by the existence of several related isozymes in a single preparation. Moreover, these enzymes are often produced in small amounts and are susceptible to degradation by endogenous proteases during purification. In an attempt to overcome these difficulties, researchers have developed means for expressing cloned ligninolytic genes from white-rot fungi in other organisms (heterologous expression) or under the control of a constitutive promoter in the same organism (homologous expression). Several such systems have been utilized successfully to express various components of the lignocellulolytic system of different white-rot fungi (Table 1.2). One of the most common approaches is to use species of the ascomycete genus *Aspergillus*, normally *A. oryzae* or *A. nidulans* (Table 1.2). The expression of heterologous proteins in aspergilli is attractive since these organisms stably integrate heterologous DNA into their chromosomes and are known to correctly process and excrete foreign proteins when the

genes possess appropriate regulatory regions (68). These fungi are prodigious producers of extracellular amylases when grown on a substrate containing maltodextrins, and the availability of *Aspergillus* amylase gene sequences (227, 298, 309) has enabled the design of efficient expression vectors for this fungus (68). In addition to their utility for producing purified recombinant protein using cloned genes, heterologous expression systems have been used in the reverse sense to isolate clones encoding cellulolytic genes from filamentous fungi (82).

Enzyme	Source	Host	Comment	Reference
Laccase	*T. villosa*	*A. oryzae*	amylase promoter	331
Laccase	*Myceliophthera thermophila*	*A. oryzae*	amylase promoter	32
Laccase	*C. hirsutus*	*Saccharomyces cerevisiae*		186
Laccase	*T. versicolor*	*Pichia pastoris*		164[1]
Laccase	*P. radiata*	*Trichoderma reesei*	cellulase promoter	278
MnP	*P. chrysosporium*	*A. oryzae*	amylase promoter	295
MnP	*P. chrysosporium*	*P. chrysosporium*	homologous expression	211
MnP	*P. chrysosporium*	*Spodoptera frugiperda* (Sf9 insect cells)	Baculovirus expression	237
LiP	*P. chrysosporium*	*Spodoptera frugiperda* (Sf9 insect cells)	Baculovirus expression	159
LiP	*P. chrysosporium*	*Spodoptera frugiperda* (Sf9 insect cells)	Baculovirus expression	204
glyoxal oxidase	*P. chrysosporium*	*A. nidulans*	glucoamylase promoter	178

[1]The *lcc*IV cDNA from *T. versicolor* has also been successfully expressed in *Pichia pastoris* (M. Brown, unpublished results).

Table 1.2. Production of ligninolytic enzymes in heterologous or homologous hosts.

1.5. Applications of White-Rot Fungi

The extracellular oxidative enzyme system produced by white-rot fungi to degrade lignin in nature is sufficiently nonspecific to catalyze the oxidation of a wide

range of lignin-related model compounds as well as an array of molecules that are not represented in lignin substructures. This fact has not been lost on researchers, who have been keen to take advantage of the nonspecific, powerful oxidative capacity of white-rot fungi and their enzyme systems in a variety of applications. Included among these are the oxidative degradation of recalcitrant organic pollutants, the treatment of effluents from pulp and paper mills, and the modification of wood pulp to improve brightness and fiber properties.

1.5.1. Bioremediation

The term "bioremediation" refers to the use of microorganisms or their enzyme systems to decrease the concentration, toxicity, or colour of pollutants in water or soils. White-rot fungi have been widely investigated as potential bioremediation agents. For example, Bezalel et al. examined the biodegradation of a range of polycyclic aromatic hydrocarbons (PAHs) by *P. ostreatus* (33-35). Up to 94% of the PAH species decreased to undetectable concentrations over an 11-day incubation period, with a proportion mineralized to CO_2. In addition, *P. chrysosporium* has been reported to degrade 2,4-dichlorophenol (314) and dibenzo-p-dioxin (165), and several white-rot fungi can degrade pentachlorophenol, a toxic wood preservative that has contaminated many sites worldwide (see (212), and references therein). Fungal inocula for pentachlorophenol-contaminated soils consisting of *Phanerochaete sordida*, *P. chrysosporium*, *T. versicolor*, and several other fungi removed up to 90% of the contaminant over a four week period (198). Most of the bioremediation studies have implicated MnP, LiP, or laccase as the enzymes responsible for the decrease in concentration and toxicity of the organopollutants (40, 41, 67, 314); however in at least one case, an intracellular enzyme of *P. chrysosporium* is involved in the breakdown of aromatic pollutants (261). In addition to organic pollutants, white-rot fungi are the only organisms known to degrade nylon (87), a material with a very long half-life in the environment due to its resistance to biodegradation. Interestingly, white-rot fungi are also known both to degrade and to produce a class of toxic compounds known as adsorbable organic halogens (AOX); in

fact, these organisms may be one of the most important sources of "natural" AOX in the environment (reviewed in reference (88)).

1.5.2. White-rot fungi in the pulp and paper industry

The pulp and paper industry produces cellulosic fibres, principally from wood substrates. Overall, the most important industrial process for the production of wood pulp is the kraft process (292). Kraft pulping involves "cooking" wood chips in a liquor composed of sodium hydroxide and sodium sulfide. The cook normally proceeds at very high temperatures (160°C-180°C) for several hours, and results in a pulp that is renowned for its great strength. Kraft pulps are produced using wood chips derived either from hardwoods (HWKP) or softwoods (SWKP). The process also generates valuable by-products such as tall oil and turpentine. However, the major disadvantage of kraft pulping processes is that the pulp produced is dark brown in colour due to chromophores present in the chemically modified residual lignin (292). The pulp is therefore bleached in order to obtain a whiter product with a high cellulose content and very little residual lignin. The increase in "brightness", or brightness gain, is measured using a standardized procedure (ISO brightness) which determines the reflectance of blue light of a sample expressed as a proportion of that of a standard reference (52). A fully bleached pulp possesses an ISO brightness of over 90. The bleaching of kraft pulp is a multi-stage procedure. In each stage, oxidizing chemicals are added which react with some of the residual lignin to form acid- or alkali-soluble degradation products. Each stage is therefore followed by an extraction stage, in which the residual lignin is dissolved in a solution of 1-2% NaOH. The bleached pulp is then moved into the following bleaching stage, and the alkaline extraction liquid is ultimately discharged into the mill's receiving waters (after treatment to remove toxicity). A number of different bleaching chemicals have been successfully used to bleach kraft pulp (Table 1.3). A typical chemical bleaching sequence, CEDED or CEHDED, results in a product with acceptable brightness levels. However, bleaching sequences using a C stage (Cl_2) result in environmental problems in the mill receiving waters due to the chlorinated organic compounds that are extracted in the E stage. Thus,

modern bleaching sequences have almost completely eliminated the use of Cl_2 as a bleaching chemical (elemental chlorine free, or ECF bleaching), and there is currently a push toward the elimination of chlorine dioxide in bleaching sequences (totally chlorine free, or TCF bleaching).

Given that the goal of bleaching processes is to selectively remove residual lignin, it is not surprising that white-rot fungi have been viewed as being potentially useful in this area. Biotechnological processes have primarily found application in two areas: the treatment of the alkaline extraction effluent to reduce colour, and the biological bleaching of pulps to reduce or eliminate altogether the need for chemicals in bleaching. In the following sections the progress made to date in these fields will be reviewed.

Stage	Chemical	Abbreviation
Chlorination	Cl_2	C
Alkaline extraction	NaOH	E
Hypochlorite	NaOCl + NaOH	H
Chlorine dioxide	ClO_2	D
Peroxide	H_2O_2	P or QP
Oxygen	O_2 + NaOH	O
Ozone	O_3	Z

Table 1.3. Common chemical bleaching stages. From (292).

1.5.2.1. Decolourization of kraft bleachery effluents

The effluent which emerges from the alkaline extraction stage of the bleaching process (called E_1 effluent) is dark in colour due to the presence of solubilized, modified lignins. These effluents are normally treated by aerobic or anaerobic biological (bacterial) treatment systems before being discharged into the mill's receiving waters; however, while such treatment systems are very effective at removing some of the toxicity and biological oxygen demand (BOD) of these effluents, they are notably ineffective at removing bleachery effluent colour or degrading the high molecular weight

chloroorganics. The ability of white-rot fungi to decolourize this effluent is well documented. One of the most well-studied and effective fungi is *T. versicolor*. Almost 30 years ago, it was shown that this fungus can remove over 70% of the colour from so-called "black liquor", the spent kraft pulping liquor (209). Later, Livernoche and Jurasek (206, 207) reported the isolation of a particularly effective strain of *T. versicolor* (strain 52, the dikaryotic parent of the strain used in the studies described in this thesis). This fungus was found growing on a rotting elm stump near the Paprican building in Pointe-Claire, Quebec. The treatment of kraft bleachery effluent with this strain removes >80% of the colour within three days. Other groups have reported similar results with *T. versicolor*. Bergbauer et al. (31) showed over 90% colour removal and a decrease of 45% in the concentration of chloroorganics over a three day treatment period. Furthermore, *T. versicolor* has been used in a batch reactor system to remove over 93% of the colour and 35% of the COD from bleachery effluents (22). In another study, over 60% of effluent toxicity was removed, as much as 10% of which was due to passive binding of the toxic compounds to the fungal mycelium rather than active degradation (317). These sorts of spectacular colour removal and detoxification results provided an impetus for the development and patenting of a rotating disk bioreactor — the MyCoR process (mycelial colour removal), which, using *P. chrysosporium*, can reportedly remove more than 75% of the colour in one day and decrease the concentration of toxic materials in effluents (240). One drawback to the use of white-rot fungi in the treatment of effluents is the need for a co-substrate, normally glucose, to enable fungal growth and metabolic activity. However, the use of inexpensive sugar refinery wastes (15), along with the finding that *Lentinus edodes* effectively decolourizes the effluent without the addition of a co-substrate (108), have offset this disadvantage.

1.5.2.2. Biobleaching of kraft pulps

Kraft pulping removes the majority of the lignin in wood chips, and what remains (kraft lignin) is a chemically modified, condensed, demethoxylated, hydroxylated form with only a limited resemblance to native lignin. Nevertheless, white-rot fungi have been investigated for their ability to degrade kraft lignin in pulps, in the hope that all or

part of the bleaching chemicals may be replaced by a biological process. In 1989, Paice et al. (233) screened several species of white-rot basidiomycetes and found that *T. versicolor* 52, the same strain found to be so effective in decolourizing effluents, increased the brightness of an unbleached hardwood kraft pulp by 15 points to 48% with a concomitant decrease in the pulp's lignin content. Although some undesirable cellulose degradation was also observed, the pulp strength was not adversely affected, probably due to improved hydrogen bonding as a result of "cleaner" or more lignin-free cellulose fibres (233). The fungus was later shown to also delignify softwood kraft pulp (258), but little brightening was seen until an alkaline extraction was run (258). Other groups have reported such "biobleaching" with other fungi, such as *P. sordida* (187), *P. chrysosporium* (308) and an unclassified basidiomycete IZU-154 (114). However, only the latter fungus shows large improvements in brightness and lignin removal comparable to *T. versicolor* strain 52. Given the importance of the *T. versicolor* system to our group at Paprican, the present discussion will be limited to this fungus (for a more detailed review, see (18)).

A subsequent screening of several monokaryotic and dikaryotic strains of *T. versicolor* showed that, in general, the monokaryotic strains were slightly more efficient (2). Furthermore, the original dikaryotic strain of *T. versicolor* 52 was separated into two mating-compatible monokaryons by protoplasting and compared to the parent strain (2). One of the monokaryons, *T. versicolor* 52J, consistently bleached pulp to a high brightness and was chosen for subsequent work (2), including the work described in this thesis.

The most important advantage of the biological bleaching of kraft pulps is the chemical savings: Delignification by fungi can reduce the amount of bleaching chemicals required by over 70% (257). The potential chemical and cost savings and the resultant decrease in undesirable emissions (especially of increasing, unacceptable AOX) from pulp mills has motivated researchers to find ways to apply a biological bleaching system as a stage in the kraft pulp bleaching process. The major barrier to be overcome is the time required to achieve a substantial brightening effect; *T. versicolor* and the other fungi

investigated require several days, which is far too long to be of any industrial use (257). In an effort to improve both the speed and the efficacy of the fungal pulp treatments, the mechanisms employed by the fungus to delignify and brighten kraft pulp have been investigated. Early experiments showed that, although a cell-free filtrate of a biobleaching culture had no effect on unbleached kraft pulp, the fungus was still able to delignify when separated from the pulp by a membrane filter (14). This demonstrated that direct contact between the pulp fibres and the fungal mycelium is not necessary for bleaching to proceed, and that certain components of the biobleaching system must be constantly renewed by the fungus. An investigation of the enzymatic activities produced by *T. vesicolor* during biobleaching showed that while LiP does not play a role (13), both MnP and laccase are produced during biobleaching (234). The importance of MnP in biobleaching was shown both by its chemical effects on the pulp (234) and by the lack of brightening by u.v.-induced MnP-negative mutants of *T. versicolor* (3). A system consisting of *T. versicolor* MnP, Mn(II)-malonate, and H_2O_2 can substantially delignify modern kraft pulps (232) and the treated pulp is easily bleached to commercially attractive brightness levels by a subsequent alkaline hydrogen peroxide bleaching stage. Enzymatic delignification with MnP is therefore a potentially useful bleaching step for kraft pulps; however its application is limited by the sensitivity of MnP to concentrations of H_2O_2 in excess of 0.1 mM (232). In addition, purified MnP, while an effective delignifying agent, is unable to reproduce the extensive brightening shown by the complete fungal system (234), and the MnP-negative mutant also shows decreased levels of laccase (3). Bourbonnais et al. subsequently showed that *T. versicolor* laccase, in the presence of the artificial mediator ABTS, is able to remove as much as 50% of the residual lignin in chemical pulps in a two-hour treatment (48, 50). However, the industrial application of this process is currently limited by the high cost of the ABTS (48), so other mediators are being sought which lack this disadvantage. Thus, although both MnP- and laccase-based enzymatic delignification stages work well enough to be commercially viable, impediments presently exist which limit their industrial application (232).

Experiments such as these have provided the impetus for a great deal of research in the field of biological delignification and have led to an improved understanding of the enzymatic mechanisms used by white-rot fungi to degrade lignin. However, none of the enzymes that have been isolated from biobleaching liquors to date have been able to reproduce the extensive brightening of kraft pulps which is observed using the complete fungal system (232). CDH has been hypothesized to play a key role in the degradation of lignin through its various interactions with the oxidative enzymes MnP and laccase (18). These interactions may explain, at least in part, why the complete fungal system is so much more effective than either purified enzyme. The studies described in this thesis are therefore aimed at providing some insight on the characteristics and role of CDH in the biodegradation of lignocellulose by white-rot fungi.

PREFACE TO CHAPTER 2.

Previous work in this laboratory (266) indicated that *T. versicolor* CDH may be involved in lignin degradation through a number of hypothesized interactions with the oxidative enzymes MnP and laccase. Moreover, *T. versicolor* CDH appeared to be distinct from the *P. chrysosporium* enzyme in a number of its features, including molecular weight (thought to be 57 kDa and 47 kDa for the heme/flavin and flavin-only isoforms), and in its ability to reduce glucose in addition to cellobiose. With a view to clarifying these characteristics of *T. versicolor* CDH, the purification of the enzyme was undertaken.

The purification protocol had previously been developed by B. Roy (266), a former student in this laboratory. The experiments described in this chapter supplement and complement the characterization undertaken by B. Roy, and were published essentially in the form presented here (269). The data presented here derive from the purification I undertook, and exclude for the most part the data which already appears in the thesis of B. Roy. However, where necessary for comparison, some of the data contributed by B. Roy is presented; this is clearly indicated by reference to his thesis.

CHAPTER 2. PURIFICATION AND CHARACTERIZATION OF CELLOBIOSE DEHYDROGENASES FROM *TRAMETES VERSICOLOR*

2.1. Abstract

The white-rot fungus *T. versicolor* degrades lignocellulosic material at least in part by oxidizing the lignin via a number of secreted oxidative and peroxidative enzymes. An extracellular reductive enzyme, cellobiose dehydrogenase (CDH), oxidizes cellobiose and reduces insoluble $Mn(IV)O_2$, commonly found as dark deposits in decaying wood, to form Mn(III). The latter species, in many chelates and complexes, is a powerful lignin oxidizing agent. CDH also reduces *ortho*-quinones and produces sugar acids which can promote manganese peroxidase-mediated oxidation and therefore ligninolytic activity. To understand better the role of CDH in lignin degradation, proteins exhibiting cellobiose-dependent quinone-reducing activity were isolated and purified from cultures of *T. versicolor*. Two distinct proteins were isolated, with apparent molecular weights of 97,000 and 81,000 and isoelectric points of 4.2 and 6.4 respectively. The larger CDH (CDH 4.2) contained both flavin and heme cofactors, whereas the smaller contained only a flavin (CDH 6.4). These CDH enzymes were rapidly reduced by cellobiose and lactose, and somewhat more slowly by cellulose and certain cello-oligosaccharides. Both glycoproteins were able to reduce a very wide range of quinones and organic radical species but differed in their ability to reduce metal ion complexes. Temperature and pH optima for CDH 4.2 were affected by the reduced substrate. Although CDH 4.2 showed rather high substrate specificity among the *ortho*-quinones, it could also rapidly reduce a structurally very diverse collection of other species, from negatively charged triiodide ions to positively charged hexaquo ferric ions. CDH 6.4 showed a higher K_m and a lower V_{max} and turnover number than did CDH 4.2 for all substrates tested. Furthermore, CDH 6.4 did not reduce the transition metals Fe(III), Cu(II), and Mn(III) at concentrations likely to be physiologically relevant, while CDH 4.2 was able to rapidly reduce even very low concentrations of these ions. The reduction of Fe(III) and Cu(II) by CDH 4.2 may be important in sustaining a Fenton's-type reaction, which produces hydroxyl radicals that

can cleave both lignin and cellulose. Unlike the CDH proteins from *P. chrysosporium*, CDH 4.2 and 6.4 are unable to produce hydrogen peroxide.

2.2. Introduction

Over a period of several days, the white rot fungus *T. versicolor* 52J can bleach and delignify both hardwood and softwood kraft pulps (2, 18, 233, 257, 267). Intimate contact between the fungus and pulp fibres is not required for this to occur: the culture liquor contains everything necessary to bleach and degrade kraft lignin, although constant regeneration or renewal of liquor components is necessary for delignification to continue (14). During pulp biobleaching *T. versicolor* secretes both low molecular weight metabolites and a number of lytic enzymes (267). Two of these enzymes, manganese-dependent peroxidase (MnP) (234) and laccase (47) can, in the presence of appropriate cofactors, oxidize, demethylate, and delignify kraft pulps, although not nearly as extensively as a complete *T. versicolor* culture.

Cellobiose dehydrogenase (CDH) has been proposed as an enzyme important in both lignin (104, 106, 324) and cellulose biodegradation (26, 188). CDH is produced by many white-rot fungi (10) including *T. versicolor* (324), and is also secreted by a number of non white-rot fungi (76, 89, 275, 282). The enzyme has reportedly been isolated from the white-rot fungi *P. chrysosporium* (324), and *F. annosus* (149). It should be noted that CDH activity may easily be underestimated or missed, as laccases can mask its presence in culture supernatants by rapid re-oxidation of the hydroquinones produced by CDHs from quinone assay substrates (268). To counter this, a new CDH assay has been developed (268). CDHs can reportedly use electrons from the oxidation of cello-oligosaccharides (26, 188, 270) to reduce free radicals to phenolics (12, 267, 279, 324), quinones (76, 90, 323), Fe(III) (76, 188, 189), Mn(III) (27), and Mn(IV) (266, 270). Thus, CDH-mediated reduction produces many lignin-based structures which are good substrates for the laccases and MnPs commonly secreted by white-rot fungi during delignification. The reduction of O_2 to H_2O_2 via superoxide is also reportedly catalyzed by *P. chrysosporium* CDH *in vitro* (21, 76, 142, 220); however, O_2 reduction rates are low

compared to those of many other CDH substrates (326). A consequence of the wide range of reductive reactions catalyzed by this enzyme has been that the nomenclature for this enzyme is in a state of flux. The term "cellobiose dehydrogenase" (CDH) is used in this report to describe both flavin only and heme-flavin cofactored proteins isolated from *T. versicolor*. The heme-flavin cofactored enzyme with a similar activity from *P. chrysosporium*, formerly referred to as cellobiose oxidase (CBO), is also referred to as cellobiose dehydrogenase (5). The term "cellobiose:quinone oxidoreductase" (CBQ) is used here only for the non-heme enzyme from *P. chrysosporium*.

Based on its broad substrate range and interaction with laccases, peroxidases, cellulose, and lignins, a wide variety of roles for CDH in wood and industrial pulp delignification have been proposed (6, 18, 104, 270). CDH may be an essential component of the delignification system in white-rot fungi. The objective of this study was to determine some of the structural and catalytic properties of the *T. versicolor* CDHs in order to understand better their role in *T. versicolor*-mediated kraft pulp biobleaching and delignification.

2.3. Materials and Methods

2.3.1. Fungal Cultures

T. versicolor strain 52J was stored at -80°C and cultured on mycological broth (MB) plates as previously described (2). For the production of CDH, the complex spore germination medium described by Canevascini (63) (Medium A; 0.5 g/L KCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L yeast extract, 1 g/L KH_2PO_4,0.4 g/L NH_4Cl), and 1 mL of a trace metals solution (267) was inoculated with 4 agar plugs (1 cm dia.) taken from the periphery of a growing colony of *T. versicolor*. Inocula for production cultures were prepared in a 500 mL plastic Erlenmeyer flask containing 200 mL of Medium A, 5 g/L glucose and incubated at 27°C for 48 h with shaking (r = 4.5 mm) at 200 rpm, followed by transfer to 800 mL of medium A plus 5 g/L glucose to a 3.8 L Fernbach flask that was in turn incubated for 48 h agitated at 100 rpm (r = 4.5 mm). CDH production was done in 14 L of Medium A with 5 g/L cellulose (Solka Floc) in aerated 20 L Nalgene carboys

inoculated with 1 L of a growing *T. versicolor* culture and shaken (75 rpm) at 27°C. The culture was sampled every 12 h, clarified by centrifugation (10,000 x g; 5 min) and CDH activity assayed.

2.3.2. Assays

CDH activity in column fractions was monitored using the cellobiose-dependent reduction of 3,5-di-*tert*-butylbenzoquinone(1,2) (TBBQ) at 420 nm (324). The assay mixture contained Na- acetate (100 mM; pH 4.5), ethanol (20% v/v), cellobiose (2 mM), and TBBQ (0.33 mM). Otherwise, CDH activity was measured using the chlorpromazine radical reduction assay, as described elsewhere (268). The use of this assay overcomes the problem of laccase-mediated re-oxidation of the TBBQ reduced by CDH, which masks the true extent of enzyme activity.

The reductions of 2,6-dichlorophenolindophenol (DCIP), cytochrome c, ferricyanide ion, and triiodide ion were measured spectrophotometrically in a 1 mL assay volume and enzyme activity was calculated using molar extinction coefficients of 7100, 33700, 1040, and 26200 respectively. The kinetics of cellobiose oxidation were measured using 0.2 mM DCIP in 50 mM Na-acetate, pH 4.5. Fe(III) reduction was measured in the presence of 0.1% phenanthroline; the appearance of Fe(II)-phenanthroline was indicated by an increase in absorbance at 510 nm and quantified using an experimentally determined molar extinction coefficient of 16200. The reduction of Cu(II) to Cu(I) was measured using 1 mM 2,9-dimethyl-4,7-diphenyl-1,10 phenanthrolinedisulfonic acid (bathocuproinedisulfonic acid). Reduction to the Cu(I)-chelate was measured at 483 nm using a molar extinction coefficient of 12250 (92). Mn(III)-malonate was prepared by mixing 100 mM Mn(III)-acetate with 100 mM Na-malonate, stirring for 4 h at 23°C, and filtering (0.22 μm). The concentration of Mn(III)-malonate obtained was determined at 270 nm (molar extinction coefficient of 8000). Unless otherwise stated, all enzymatic assays were performed at 23°C in 50 mM Na-acetate, pH 4.5, and 2 mM cellobiose.

Cellobiose-dependent O_2 uptake by CDH was measured with a polarographic oxygen electrode (Rank Brothers, Cambridge, U.K.) at 25°C in a 3 mL closed cell. The reaction mixture contained 10 U/mL CDH and 2 mM cellobiose in 100 mM Na-acetate pH 4.5. Assays under air were performed after flushing the reaction mixtures with 30 mL of air from a syringe, while determinations under O_2 were made after bubbling pure O_2 through the assay mixtures for 1 min. H_2O_2 was determined by the addition of 600 U/mL catalase to the reaction mixture and measuring the O_2 produced. As a positive control, H_2O_2 was added to the reaction mixtures to a final concentration of 0.0125% and the burst of O_2 produced was measured. Both short term (5 min) and longer term (overnight) measurements of O_2 concentration and H_2O_2 production were made.

2.3.3. Purification Procedure

CDH was purified from cellulolytic cultures of *T. versicolor* 52J exactly as described elsewhere (266, 269). Briefly, CDH-containing culture liquors were subjected to column chromatography in the following sequence: DEAE-Sepharose, Sephacryl S-300, and MonoQ (performed twice). Between each step, fractions displaying CDH activity were pooled and washed before being loaded onto the next column.

2.3.4. Enzyme kinetics

The Michaelis-Menten K_m and V_{max} values were determined for a number of CDH substrates using Eadie-Hofstee plots of kinetic data. TBBQ or DCIP were used as the electron acceptor with various electron donors while cellobiose was used as the electron donor for electron acceptor trials. All determinations were at pH 4.5 in 100 mM Na-acetate. Temperature optima of the two CDH enzymes were determined using a thermo-jacketed cuvette holder and using TBBQ or DCIP and cellobiose as substrates at pH 5 in 100 mM Na-acetate. The optimal pH of CDH 4.2 was determined using 50 mM Na-acetate (pH 4.0, 4.5, 5.0), Na-succinate (pH 5.5), K-phosphate (pH 6.0, 6.5, 7.0) and Tris-HCl (pH 7.5, 8.0, 8.5).

2.3.5. Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using a Pharmacia Multiphor apparatus with 8-18% gradient acrylamide gels against molecular weight standards. Isoelectric focusing was performed with the Phast Gel IEF apparatus with pH ranges of 3-9 and 2.5-6 in conjunction with appropriate IEF isoelectric point (pI) standards (Pharmacia). Protein bands were visualized by staining with Coomassie blue and CDH activity detected using 2 mM DCIP (pH 6) and 2 mM cellobiose.

2.3.6. Spectroscopy

Absorption spectra were recorded on a Perkin-Elmer Lambda 3 spectrophotometer at room temperature in a cuvette with a 1 cm path length. Enzyme spectra were recorded in 20 mM Na-acetate buffer (pH 4.5). Enzymes were reduced using cellobiose (2 mM).

2.3.7. Western blot

Purified CDH was separated by SDS-PAGE using ExcelGel SDS 8-18 and the Multiphor electorphoresis system (Pharmacia) and the procedures recommended by the manufacturer. Samples were prepared by diluting them 2-fold in 2x SDS-PAGE sample buffer (0.5 M Tris-Cl/0.4% SDS/20% glycerol/3% DTT/0.001% bromophenol blue), placing them in a boiling water bath for 5 min, then setting the samples on ice for 5 min. Electrophoresis was carried out at 600 V for 60 minutes at 10°C. Following electrophoresis, proteins were transferred to a polyvinyldienefluoride (PVDF) membrane (Immobilon-P; Millipore) using a Nova-blot semi-dry transfer system (Pharmacia) and the recommended procedures. Transfer was effected using a current of 0.8 mA/cm^2 of membrane for 60 min. Proteins transferred to the PVDF membrane were visualized by staining with Ponceau-S Red and the locations of the molecular weight markers was noted. The membrane was destained in 0.1 N NaOH and rinsed thoroughly with water. The membrane was dried by placing it at 37°C for 60 min. Proteins reacting with a rabbit anti-*P. chrysosporium* CDH polyclonal antibody (polyAb) were detected using a

chemiluminescent Western blotting kit (Boeheringer-Mannheim). The anti-CDH polyAb was used at a dilution of 1:20,000 for detection.

2.4. Results

2.4.1. Purification of CDH

CDH synthesis was found to be strongly induced in *T. versicolor* by cellobiose and cellulose (data not shown), and the latter was used as a carbon source for the growth of the fungus for enzyme production. Under these conditions, each 15 L culture contained at harvest homogeneous 3-4 mm spherical white fungal colonies filling the lower third of the carboy. Nearly all of the CDH enzyme activity was found extracellularly as two proteins easily separable on non-denaturing polyacrylamide gels (266). The time of harvest was critical as, after peaking, medium CDH activity would fall in a few hours to virtually zero. The CDHs secreted by *T. versicolor* 52J during growth on Solka Floc cellulose were isolated and purified from the culture medium in four steps. The initial separation on DEAE-Sephacryl gave three activity peaks (Figure 2.1).

Fractions containing the first CDH activity peak (Figure 2.1A, peak 1) were pooled and subsequently purified. The fractions containing the second CDH activity peak (Figure 2.1A, peak 2) were also pooled and further purified (Figure 2.1B, C). The smallest peak from the DEAE column (Figure 2.1A, peak 3), had a higher CDH activity than implied by the graph since it co-eluted with a laccase activity (not shown) that re-oxidized the TBBQ. These fractions were pooled and purification was attempted, but the CDH activity declined to zero. The instablility of the activity of this peak may have been due to proteolytic activity that co-eluted with the CDH. Indeed, instability of activity was a problem throughout the purification, although the pooled fractions from the MonoQ column remained stable when stored at 4°C.

Unfortunately, this procedure often gave very low yields and high losses, especially in the ion-exchange steps. More recently, both the yield and stability of CDH have been improved by harvesting early, when there is only about 5 chlorpromazine units

per mL of CDH in the supernatant, and by changing the buffer used throughout to 10 mM ammonium acetate, pH 5.0 and using linear 10-500 mM gradients of the same buffer to elute the DEAE and MonoQ columns. Post-harvest CDH activity losses appear to be due largely to proteolytic cleavage with active CDH fragments of 47,000 and 57,000 kDa often found (266).

Purification of CDH peak 1 activity gave a pale yellow solution. The molecular weight of the protein was estimated to be 81 kDa by SDS-PAGE, although multiple bands were observed that were probably due to proteolytic degradation (Figure 2.2). By isoelectric focusing, the pI of CDH peak 1 was determined to be 6.4 (266). Therefore CDH peak 1 was designated CDH 6.4.

Purification of CDH peak 2 yielded a red-brown protein producing a band on SDS-PAGE gels with an estimated molecular weight of 97 kDa (Figure 2.2). The protein also appeared to be degraded, since multiple bands were observed all of which reacted with anti-*P. chrysosporium* CDH polyAb (Figure 2.2). Furthermore, when a non-denaturing PAGE gradient gel (10-25%) was stained for activity using DCIP or for protein with Coomassie blue, a single diffuse band was observed (not shown). Isoelectric focusing gave a pI of 4.2 for CDH peak 2 (266) and so in subsequent discussion, it is designated CDH 4.2.

CDH 4.2 showed maximal activity with TBBQ in acetate buffer at pH 5.0. (Figure 2.3). However, when DCIP was used as substrate, the pH optimum shifted to 4.5 and the relative activity was higher at most pHs assayed. With TBBQ as substrate, enzyme activity was optimal at 50°C, and was rapidly lost at temperatures >60°C (50% inactivation in 30 s at 60°C). However, with DCIP as substrate, a shift in the temperature optimum to 55°C was observed. Furthermore, CDH activity was greatly enhanced at the higher temperatures assayed (55-65°C) with DCIP as substrate compared to TBBQ. DCIP reductive activity was completely lost at 70°C. The temperature profiles of CDH 4.2 and CDH 6.4 were identical using DCIP as substrate (Figure 2.3). Thus, the nature of the

substrate reduced by CDH appeared to have a large effect on the useful pH and temperature ranges of the enzyme.

The purified CDHs (3 mg/mL) were stored at 4°C in sterile buffer for over a year without substantial loss of enzymatic activity, but lost 30-50% of their initial activity when frozen and thawed. Lyophilization resulted in complete inactivation of the CDH proteins. Full activity was maintained by storage of the enzymes in 50% glycerol/20 mM Bis-Tris pH 4.5 at -20°C.

2.4.2. Cofactors of CDH 4.2 and 6.4

FAD was previously determined to be a cofactor of CDH 4.2 and CDH 6.4, with approximately one FAD molecule per protein molecule (266). Subsequent extraction of CDH 4.2 with acidified acetone and preparation of a pyridine hemochromogen extract yielded a solution with a strong absorbance at 555 nm (266), demonstrating that the enzyme's heme was of the cytochrome *b* type, similar to *P. chrysosporium* CDH (27, 220).

2.4.3. Spectrophotometric analysis of CDH reduction

CDH 6.4 displayed absorption peaks at 457 and 496 nm (266), as has been observed for the CBQ from *P. chrysosporium* (221, 325). Addition of Na_2SO_3 or cellobiose eliminated these peaks, presumably by reduction of the FAD (266). CDH 4.2 had an A_{420}/A_{280} ratio of 0.53-0.59, which is similar to values previously reported for the *P. chrysosporium* CDH (140).

Oxidized CDH 4.2 differed from oxidized CDH 6.4 in having absorption peaks at 410 nm and 530 nm (Figure 2.4). When CDH 4.2 was reduced to the ferrous form with cellobiose, the absorption maxima were shifted to 418 and 548 nm and the molar absorptivities of the peaks were increased (Figure 2.4). The observed decrease in absorption between 500 and 450 nm upon reduction by cellobiose (Figure 2.4) has been associated with the reduction of the flavin cofactor in *P. chrysosporium* CDH (27, 220).

2.4.4. Catalytic properties of *T. versicolor* CDHs

With TBBQ as the electron acceptor, both CDHs oxidized cellobiose as their preferred substrate (Table 2.1), though lactose was used at lower but very significant rates. Previous work has shown that cellotriose is oxidized by CDH at rates similar to lactose (266). The enzyme will not significantly oxidize glucose or any other of the monosaccharides tested at realistic concentrations, although very slow oxidation was observed in 1.5 M glucose (data not shown). Both CDH isoforms are quite specific for the ß-1,4 glycosidic bond, since of the disaccharides tested, only cellobiose (glc-ß-1,4-glc) and lactose (gal-ß-1,4-glc) were measurably oxidized (Table 2.1). The specificity of CDH for glucose-containing saccharides is revealed by the fact that lactose, which differs from cellobiose only at the epimeric C-4 of the galactose residue, is oxidized at a much lower rate than cellobiose (Table 2.1). Though oxidation of the cello-oligosaccharides by CDH was not detectable in a short (5-10 min) assay, TBBQ reduction by CDH was detectable with longer (6-24 h) incubation periods (266). CDH 4.2-mediated oxidation of cellulose was also detected using a more sensitive assay which showed a marked CDH-dependent increase in the number of carboxylic acid groups on insoluble cellulose (268).

Consistent with the low reductive specificity of similar enzymes isolated from *P. chrysosporium*, CDH 4.2 from *T. versicolor* 52J readily reduced a wide range of substrates, including positively and negatively charged ions, quinones, and various cation radicals (Table 2.2; (266)). However, *T. versicolor* CDH was evidently unable to reduce O_2 to H_2O_2 (Table 2.2), a capability which has been suggested to be a function of *P. chrysosporium* CDH (21). In short term (5 min) assays in pure oxygen, and in longer term (overnight) assays in air, neither a decrease in dissolved oxygen concentration nor the presence of newly produced H_2O_2 was detectable. In determining the detection limit for this assay, we assumed that a change in oxygen concentration corresponding to 2% of the full scale (set to 240 µM under air and 1200 µM under oxygen) would be detectable. Therefore, we should have been unable to detect a change in O_2 concentration of less than 4.8 µM under air and 24 µM under oxygen; these values were used to calculate detection

limits for the assay shown in Table 2.2. Thus these observations do not absolutely rule out oxygen reduction by *T. versicolor* CDH, but the rate of O_2 reduction must be very slow, especially since it was undetectable after 16 hours of incubation. Furthermore, all O_2 reduction assay mixtures were checked after the O_2 assay for CDH activity by removing the assay mixture to a tube containing 100 μM DCIP; in each case, decolourization of the DCIP was noted, verifying that the reaction mixture could carry out CDH-dependent reduction.

A comparison of the reductive activities of CDH 4.2 and CDH 6.4 with various substrates revealed that the two enzymes possess similar substrate specificity among non-metallic substrates, but CDH 6.4 had a higher K_m and lower turnover number (k_{cat}) and maximum velocity (V_{max}) for all of the one-electron acceptors tested (Table 2.2). Among transition metals the differences were very pronounced. Oxidized forms of the three transition metals (Fe, Mn, and Cu) suspected of involvement in fungal delignification were far more readily reduced by CDH 4.2 than by CDH 6.4. In fact, the latter enzyme was entirely unable to reduce cytochrome c or ferricyanide ions, substrates rapidly reduced by CDH 4.2 (Table 2.2, Figure 2.5).

2.4.5. Stability of CDH

It was observed during production of CDH in the carboys that early CDH production was largely CDH 4.2 (as determined by the cytochrome c reduction assay) but that by day 6 or 7 of culture growth it was mostly CDH 6.4, and that the latter enzyme activity was much less stable through the purification procedure. From days 3 to 7 or 8 of the carboy culture growth of 52J, CDH activity rose at an increasing rate, peaking at 13-17 chlorpromazine units per mL, followed by a "crash" in which virtually all CDH activity disappeared within 8-10 h. All of this is consistent with proteolytic degradation of CDH 4.2 to CDH 6.4 and finally to inactivity. The studies of Habu *et al.* (132), Wood and Wood (327) and Eggert *et al.* (100) have shown that in the *P. chrysosporium* system secreted proteases cleave the heme/flavin CDH (analogous to *T. versicolor* CDH 4.2) to the flavin-only CBQ (analogous to *T. versicolor* CDH 6.4) and an inactive heme domain.

2.5. Discussion

Archibald has demonstrated that all of the essential components of the *T. versicolor* biobleaching system are present in the extracellular supernatant of actively bleaching cultures (14). Though oxidative enzymes such as MnP and laccase do carry out a useful level of hardwood and softwood kraft pulp delignification, their effect is not as extensive as that observed when whole fungal cultures are used (47, 234). One hypothesis posits that a catalytic oxidative and reductive cycling of lignin substrates is established by ligninolytic cultures of *T. versicolor* and which favours a net depolymerization of lignin (270). Similar catalytic schemes have been proposed by other investigators for the *in vitro* depolymerization of lignosulfonates by peroxidases and glucose oxidase (129, 235).

CDH is the only extracellular enzyme known in *T. versicolor* biobleaching cultures that can reduce aromatic substrates and organic free radicals. *T. versicolor* 52J secreted CDH isoforms into the culture medium under nitrogen-sufficient conditions when either cellulose or cellobiose was the carbon source, conditions similar to those under which biobleaching is observed (2, 233, 256, 267).

The reductive activities of these two enzymes were similar to the equivalent enzymes isolated from *P. chrysosporium* (Table 2.3; (21, 27, 220, 221, 279, 324). A notable difference of the *T. versicolor* heme-flavin enzyme from those of other fungi is its inability to reduce oxygen to hydrogen peroxide. Henriksson et al. (142) hypothesized that the function of the heme group in *P. chrysosporium* CDH is to augment the reaction rate with 1-electron acceptors, since only the reaction rate with 1-electron acceptors was significantly affected by the removal of the heme domain. We did not specifically test this hypothesis with the *T. versicolor* CDHs. In addition, there is some difference in the apparent MW of the CDH proteins isolated from *T. versicolor* and *P. chrysosporium*. CDH 4.2 from *T. versicolor* gave a MW of 97 kDa on SDS-PAGE gels (Figure 2.2), somewhat larger than most reports of the equivalent enzyme from *P. chrysosporium*, which generally give an apparent MW of around 90,000 (107, 143, 327). However, other groups have reported the MW of *P. chrysosporium* CDH to be 98,000 (279) and as high

as 102,000 by SDS-PAGE (21). Recently, Li *et al.* (201) have reported a calculated molecular wight of 80,115 Da for *P. chrysosporium* CDH based on a cDNA sequence of 2319 base pairs (773 amino acids, including an 18 amino acid signal peptide). The discrepancy between the apparent MW of the CDH enzyme of *P. chrysosporium* as measured by SDS-PAGE and as calculated from the cDNA sequence is probably due to glycosylation. A range of MW values for the CBQ (flavin only) enzyme of *P. chrysosporium* has also been reported, from about 60,000 (107, 327) to 75,000 (279). The equivalent enzyme from *T. versicolor*, the flavin-only CDH 6.4, migrated on SDS-PAGE gels with an apparent MW of 81,000 (Figure 2.2).

Purified preparations of the two quinone-reducing enzymes yielded two proteins with a ten-fold difference in their specific activities when TBBQ was used as the quinone substrate. The smaller, flavin-only enzyme, CDH 6.4, showed lower activity toward all substrates tested compared to the larger, heme-flavin CDH 4.2. Moreover, CDH 6.4 was unable to reduce cytochrome c or ferricyanide ions under the conditions tested and reduced Cu(II), Fe(III), or Mn(III) only very slowly, while CDH 4.2 rapidly and efficiently reduced all of them. When CDH 6.4 reduced Fe(III) and Cu(II), the K_m values were in the millimolar range and rates of catalysis low, compared to micromolar K_m values and rapid catalysis by CDH 4.2 (Table 2.2). The concentrations of these ions are likely to be in the micromolar range under physiological conditions. Thus the presence of CDH 4.2 may render all three metals more bioavailable, produce lignin-oxidizing Mn(III) directly, and promote Fe(II) and Cu(I)-driven Fenton's chemistry. In a Fenton's reaction, Fe(II) or Cu(I) (135) reacts rapidly with H_2O_2 to form Fe(III) or Cu(II) and the hydroxyl free radical (•OH). This radical has been implicated in cleavage reactions of both cellulose (136, 293) and lignin (300). Although CDH from *T. versicolor* has been shown to not generate hydrogen peroxide, even under extended exposure to pure O_2, some peroxide is certainly present under delignifying conditions since it is required to sustain MnP activity. The reduction of Mn(III)-malonate by CDH may be important in dampening Mn(III) oxidative activity on lignin; thus, in addition to supporting MnP

activity by providing Mn(II) from insoluble Mn(IV) (270), CDH may dampen the effects of MnP activity.

The lower specific activity, yield, and molecular weight of CDH 6.4 compared to CDH 4.2 may be because CDH 6.4, like the *P. chrysosporium* flavin only CBQ (132), is formed from CDH 4.2 by proteolysis and heme loss. CDH 4.2 may fragment because: (a) the heme domain of the protein is not covalently linked to the rest of the protein and spontaneously dissociates; (b) the protein has an autoproteolytic activity; or (c) CDH 4.2 is partially degraded by a protease. The first two are not likely, since purified CDH 4.2 is quite stable at 4°C in dilute buffer. In *P. chrysosporium*, the heme and flavin domains are reportedly joined by a protease-sensitive region since not only proteases from this fungus (132), but also staphylococcal V8 protease (327) and papain (143) cleave the protein to yield heme- and flavin-containing fragments. Recently Eggert *et al.* (100) have shown that at least three proteases from *P. chrysosporium* can cleave cellulose-bound CDH. Two of these proteases (designated II and III), cleave CDH in the presence of cellobiose alone, which suggests that the enzyme is susceptible to proteolysis only in its reduced form when its quinone or free radical substrates are absent. If in the *T. versicolor* system CDH 6.4 is indeed a proteolysis product of CDH 4.2, then this cleavage could serve to modulate the activity of CDH 4.2. This could be especially relevant in terms of the Fenton's reaction; the ability of CDH to sustain a Fenton's reaction by reducing ferric and cupric ions to ferrous and cuprous ions would be greatly decreased under physiological conditions by the cleavage of CDH 4.2 to CDH 6.4. If, however, CDH 6.4 is transcribed from a separate gene, then the smaller enzyme may be playing another role in lignocellulose degradation. The possible proteolytic cleavage of CDH 4.2 to CDH 6.4 is currently under investigation.

The heme-containing CDH of *P. chrysosporium* has been thought to serve primarily as a generator of H_2O_2 (21); however our results (Table 2.2) for the *T. versicolor* enzyme and those of others for *P. chrysosporium* (326) are not consistent with this hypothesis. Both CDH 4.2 and 6.4 reduced quinones, organic radicals, and Fe, Cu, and

Mn complexes, but neither reduces O_2 to O_2^- or to H_2O_2 (Table 2.2). Though the list of quinones reduced by *T. versicolor* CDH was extensive, many *para*-quinones are only very slowly reduced, unlike most *ortho*-quinones, cation radicals, and Fe(III), Cu(II) and Mn(III)-complexes which were readily attacked (Table 2.2; (266)).

2.6. Conclusions

(1) *T. versicolor* CDH 4.2 is similar to the CDH proteins reported from *P. chrysosporium* in having FAD and heme cofactors, being of similar size, binding to cellulose, and being able to reduce many quinones, organic radicals, and metal ions. It differs at least in its inability to reduce O_2 to H_2O_2 in the presence of a suitable electron donor.

(2) CDHs 4.2 and 6.4 specifically oxidize β1→4 linked glucose polymers. Using one of these as an electron source, CDH can reduce a broad range of quinone and non-quinone substrates. These CDH proteins are however unable to rapidly reduce some bulky *para*-quinones.

(3) CDH 6.4 has an FAD cofactor only and is less efficient at catalyzing all substrates tested than is CDH 4.2. Compared to CDH 4.2, CDH 6.4 is almost inactive in reducing Fe(III), Cu(II), and Mn(III) complexes. As appears to be the case with the *P. chrysosporium* flavin-only CDH, CDH 6.4 may be a proteolysis product of CDH 4.2.

(4) Neither protein is a useful generator of hydrogen peroxide, but both bind to, oxidize, and are induced by cellulose and kraft pulp.

(5) *T. versicolor* CDH 4.2 may complement MnP activities in delignification by: (a) oxidizing cellobiose, cellooligomers and cellulose to effective Mn-complexing agents; (b) reducing MnO_2 to Mn(II) and Mn(III), thus rendering Mn available to MnP as well as inducing MnP; (c) producing Mn(III) complexes directly, and; (d) producing phenolic substrates for MnP. Moreover, CDH 4.2 may dampen Mn(III) oxidative activity toward lignin by reducing the ion to Mn(II).

(6) By reducing Mn(IV) and Mn(III) to Mn(II), and Cu(II) and Fe(III) to Cu(I) and Fe(II) respectively, CDH 4.2 can effectively solubilize and render more available these essential metals in the extracellular milieu.

(7) CDH 4.2 may promote cellulose and hemicellulose polymer degradation by: (a) reducing end oxidation; and (b) by promotion of •OH-mediated polymer cleavage via Fe(III) and Cu(II) reduction in the presence of hydrogen peroxide.

(8) The temperature and pH ranges of CDH 4.2 are dependent to a significant extent on the nature of the substrate which is reduced by the enzyme.

Substrate	CDH 6.4 activity (peak 1) U/mg	CDH 4.2 activity (peak 2) U/mg
Cellobiose (glcß1,4-glc)	3.6 ± 0.51	25 ± 0.4
Cellotriose[1]	1.6 ± 0.14	17 ± 0.4
Lactose (glcß1,4-gal)	1.8 ± 0.17	3.1 ± 0.0
Mannitol[1]	0.34 ± 0.03	0
Sorbose[1]	0.36 ± 0.06	0.1 ± 0.004
Raffinose[1]	0.49 ± 0.11	0
Cellulose oligomers[1]	0	ND[2]
Bacterial cellulose[1]	0	0
Whatman cellulose[1]	0	0
Solka Floc cellulose[1]	0	0

[1] Data from (266)

[2]ND = not determined

Table 2.1 CDH activity using various sugars as electron donors. The electron acceptor was TBBQ or DCIP in 100 mM Na-acetate, pH 4.5. Each assay was monitored for less than ten min (short-term assays). Results are the means of duplicate determinations ± standard deviations. In addition to the substrates shown, D-glucose, L-arabinose, D-fructose, D-galactose, D-gentobiose, D-gluconate, glycerol, glucuronate, sorbitol, D-xylose, sucrose, maltose, and D-mannose were tried and could not serve as electron donors for CDH in this short-term assay.

		CDH 4.2			CDH 6.4		
Substrate	λ (nm)	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (s^{-1})	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (s^{-1})
Cellobiose[1]	520	120	6.3	6.1	220	1.9	1.5
DCIP	520	7.8	5.0	4.8	30.8	1.8	1.4
Cytochrome c	415	7.8	10.5	10.2	---	0	0
$Fe(CN)_6^{3-}$	420	110	5.4	5.2	---	0	0
Fe(III)	510	2.4	2.3	2.2	480	0.18	0.2
Cu(II)	483	0.89	2.6	2.5	123	0.81	0.7
O_2	---	---	<0.08	<0.07	---	<0.08	<0.07

[1]Used DCIP (0.2 mM) as the electron acceptor.

Table 2.2 Comparison of the reductive activites of CDH 4.2 and CDH 6.4. Reaction mixtures contained 2 mM cellobiose and 50 mM Na acetate, pH 4.5, to which various concentrations of substrate were added.

Characteristic	*T. versicolor*[1]		*P. chrysosporium*[2]	
	CDH 4.2 (heme/FAD)	CDH 6.4 (FAD)	CDH/CBO (heme/FAD)	CBQ (FAD)
MW	97 kDa[3]	81 kDa[3]	90 kDa	55 kDa
A_{420}/A_{280}	0.57	--	0.63	--
pI	4.2	6.4	4.18	5.45
T (optimum)	50-60°C	50-60°C	50°C	NR[4]
pH (optimum)	4.5-5.0	ND[5]	5	NR
Reduction of: I_3^-				
K_m (μM)	850	ND	0.2	0.3
k_{cat} (s^{-1})	2.2	ND	17	14
Cytochrome c				
K_m (μM)	7.8	0	0.3	0.3?
k_{cat} (s^{-1})	10.2	0	13	0.07
$Fe(CN)_6^{3-}$				
K_m (μM)	110	0	7000	4000
k_{cat} (s^{-1})	5.2	0	5.5	1.0

[1]All assays were done at 23°C in 50 mM Na-acetate buffer, pH 4.5 in a volume of 1 mL

[2]From: Henriksson *et al.* (142)

[3]MW measured by SDS-PAGE

[4]NR, not reported

[5]ND, not determined

Table 2.3 Comparison of *T. versicolor* CDH to the analogous *P. chrysosporium* proteins.

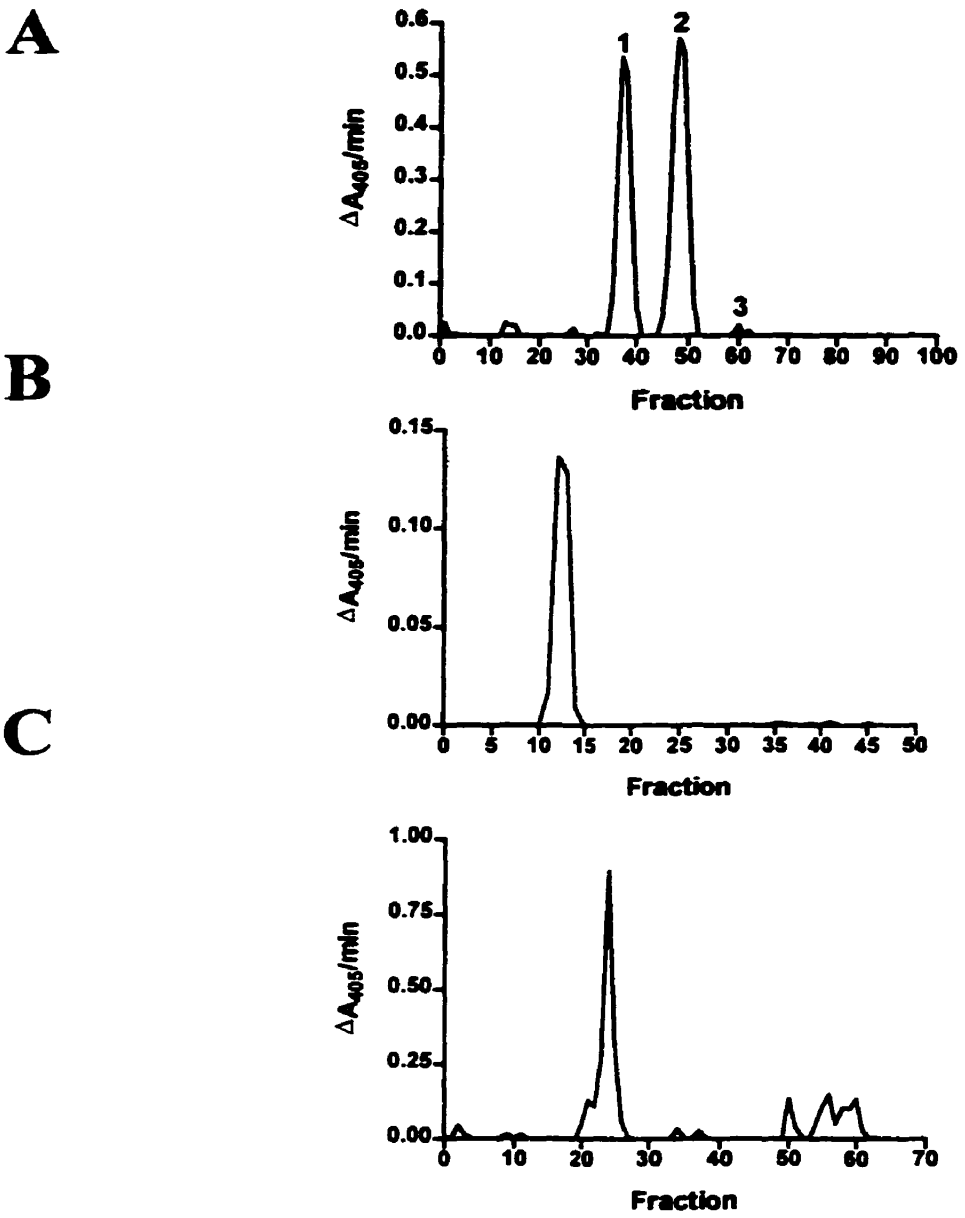


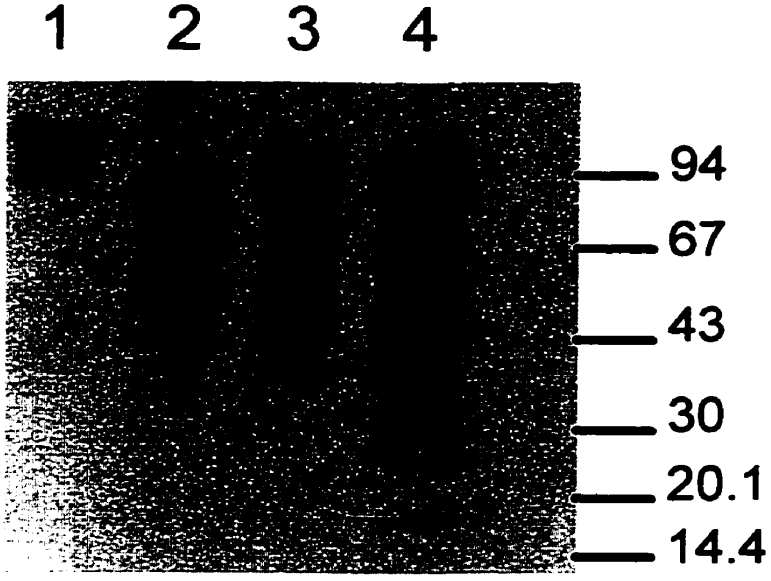
Figure 2.1. Purification of CDH. Concentrated and dialyzed culture supernatant was passed through DEAE-Sephacryl (**A**) equilibrated with Bis-Tris buffer (20 mM, pH 6.5), washed with 1 liter of the same buffer and eluted with a linear gradient of NaCl (0-1 M in 2 liters) (266). Pooled fractions displaying TBBQ reducing activity were concentrated using Amicon YM-10 membranes, washed with 20 mM Bis-Tris pH 6.5, and loaded onto a Sephacryl S-300 column (**B**) which was eluted with the same buffer. Pooled Sephacryl fractions were again concentrated and washed, then loaded onto a MonoQ column (Pharmacia) (**C**) and eluted with a linear NaCl gradient (0-400 mM). Active fractions were pooled, concentrated, and washed with 20 mM Bis-Tris pH 6.5. The graphs in (**B**) and (**C**) show the purification of CDH peak 2 (CDH 4.2). Each fraction was assayed for CDH activity by measuring the rate of change of absorbance at 405 nm in the presence of TBBQ and cellobiose.

Figure 2.2. SDS-PAGE and Western blot of *T. versicolor* CDH 4.2 and CDH 6.4. **A.** SDS-PAGE. Aliquots of CDH 4.2 and CDH 6.4 purified as described in Materials and Methods were subjected to SDS-PAGE in the presence of dithiothreitol and stained with Coomassie blue. Lanes: 1, *T. versicolor* CDH 4.2; 2, *P. chrysosporium* CDH; 3, another preparation of *T. versicolor* CDH 4.2; 4, *T. versicolor* CDH 6.4; M, Molecular weight markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa). **B.** Western blot of purified CDHs. An SDS-PAGE gel similar to that shown in A was blotted to a PVDF membrane and probed using anti-*P. chrysosporium* CDH polyclonal Ab as described in Materials and Methods. Lanes: 1, *T. versicolor* CDH 4.2; 2, *T. versicolor* CDH 6.4; 3, *T. versicolor* CDH 4.2 (corresponds to A, lane 3); 4, *P. chrysosporium* CDH. The locations of the molecular weight marker bands (same as in A) are indicated. **C.** Determination of the molecular weights of CDH 4.2 (*) and CDH 6.4 (+) by SDS-PAGE. The relative migration (R_f) of the highest MW antibody staining bands from each preparation was measured and compared to standards (■).

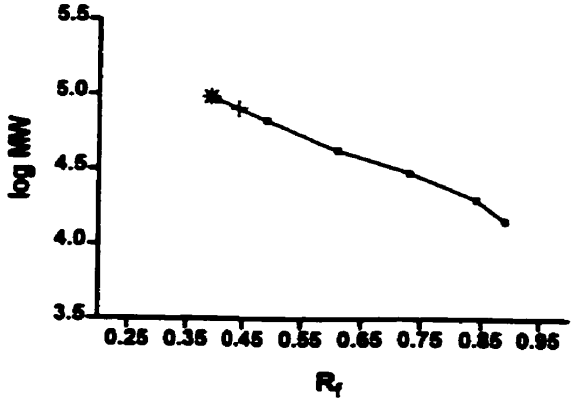
A


1
2
M
3
4

B


1
2
3
4
94
67
43
30
20.1
14.4

C


5.5
5.0
4.5
4.0
3.5
log MW
0.25
0.35
0.45
0.55
0.65
0.75
0.85
0.95
R_f

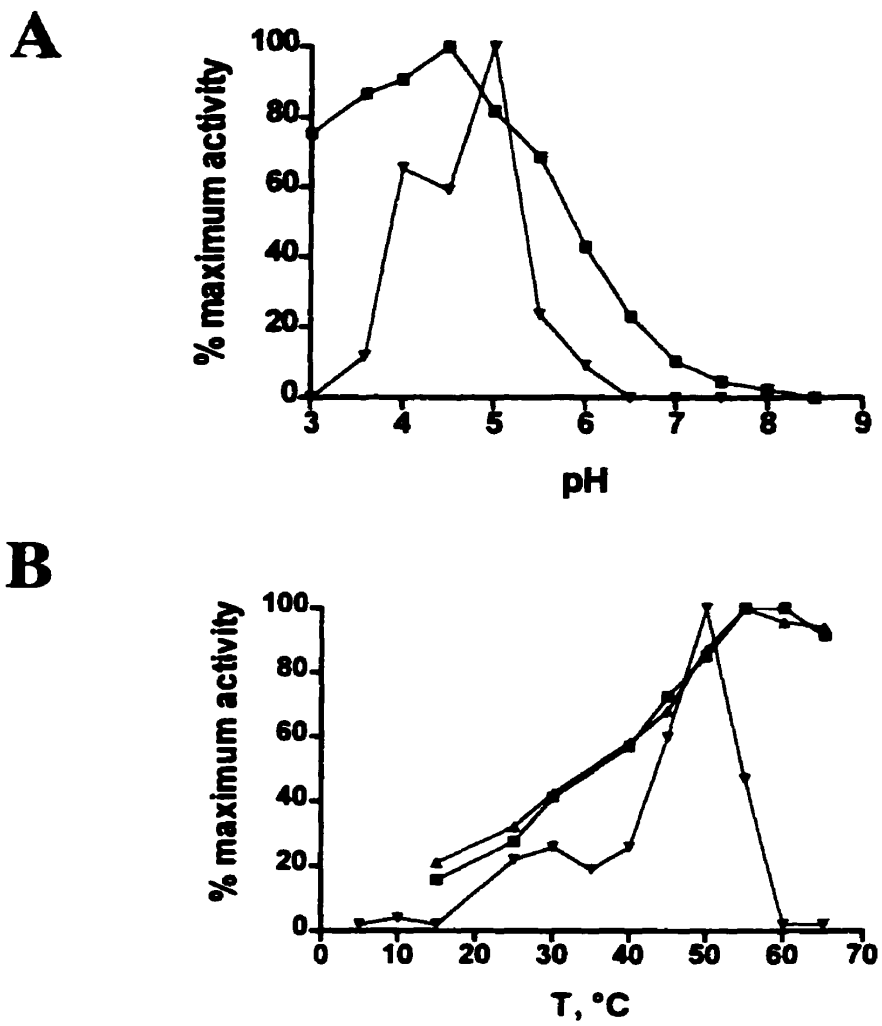


Figure 2.3. pH and temperature profiles of CDH 4.2 and 6.4. **A.** The CDH 4.2 pH range was determined using 0.33 mM TBBQ (▼) (266) or 0.2 mM DCIP (■) as substrate and cellobiose (2 mM) in 50 mM Na citrate (pH 3.0, 3.5), Na acetate (pH 4.0,4.5, 5.0), Na succinate (pH 5.5), K-phosphate (pH 6.0, 6.5, 7.0, 7.5) and Tris-HCl (pH 8.0, 8.5) buffers as described in Materials and Methods. Values shown are the means of triplicate determinations. **B.** Reducing activity of CDH 4.2 and CDH 6.4 at various temperatures. Activity of CDH 4.2 was measured in 50 mM Na acetate buffer (pH 5.0 for TBBQ; pH 4.5 with DCIP) with 0.33 mM TBBQ (▼) (266) or 0.2 mM DCIP (■) and 2 mM cellobiose as substrates. The temperature dependance of CDH 6.4 (▲) was measured using 0.2 mM DCIP as the electron acceptor under the same conditions as CDH 4.2. Values shown are the means of duplicate determinations.

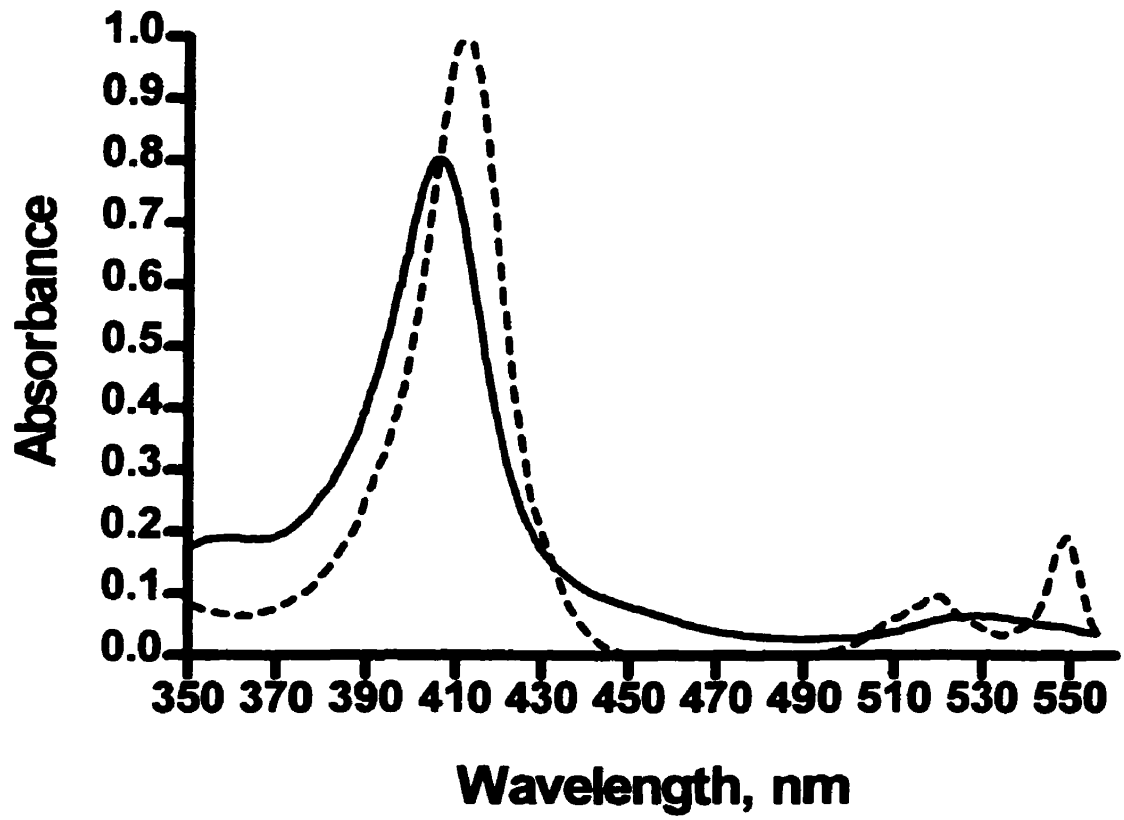


Figure 2.4. Visible spectrum of oxidized (—) and reduced (----) CDH 4.2 in 50 mM Na-acetate buffer (pH 4.5). The enzyme was purified as described and the absorbance profile recorded. The enzyme was reduced by adding 2 mM cellobiose to the enzyme preparation.

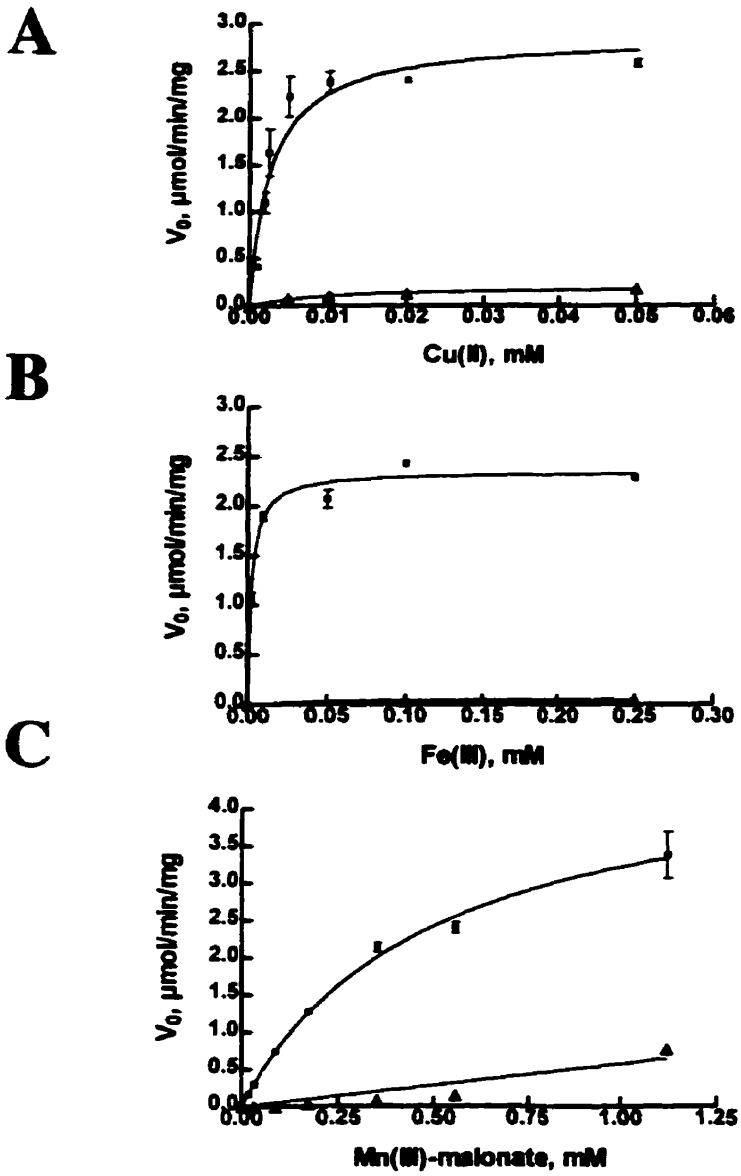


Figure 2.5. Reduction of ferric, cupric, and manganic ions by CDH 4.2 and CDH 6.4. **A.** Reduction of Cu(II) by CDH 4.2 and CDH 6.4. Assays were performed as described in Materials and Methods. To each mixture was added 5 μg CDH 4.2 (■) or 10 μg of CDH 6.4 (▲), and the appearance of Cu(I) was monitored at 483 nm. Results were calculated using an extinction coefficient of 12.25 mM^{-1}. **B.** Reduction of Fe(III) by CDH 4.2 and CDH 6.4. Assays were performed as described in Materials and Methods. To each mixture was added 10 μg of CDH 4.2 (■) or 30 μg of CDH 6.4 (▲), and the appearance of Fe(II) was monitored at 510 nm. Results were calculated using an experimentally determined extinction coefficient of 16.2 mM^{-1}. **C.** Reduction of Mn(III)-malonate by CDH 4.2 and CDH 6.4. Each reaction contained: 50 mM Na acetate, pH 4.5; 2 mM cellobiose; and Mn(III)-malonate (prepared as described in Materials and Methods). To each mixture was added 10 μg of CDH 4.2 (■) or 20 μg of CDH 6.4 (▲). The reduction of Mn(III)-malonate was monitored at 270 nm.

PREFACE TO CHAPTER 3

The purification and characterization of *T. versicolor* CDH as described in Chapter 2 revealed a number of interesting features. The enzyme appeared to be more similar both catalytically and structurally to *P. chrysosporium* CDH than was previously thought. In addition, a number of its catalytic capabilities supported hypotheses previously formulated to explain its role in the degradation of both lignin and cellulose. For example, the reduction of Fe(III) and Cu(II) could support the production of hydroxyl radicals, which can cleave both lignin and cellulose. To probe further the molecular features of this enzyme, and to acquire the tools required for a molecular analysis of *T. versicolor cdh*, we undertook the cloning and sequencing of the gene encoding CDH in *T. versicolor*. Since neither genomic nor cDNA libraries were available from our strain, the first step involved the construction of such libraries (described in Appendices 1 and 2). To probe the genomic library, we obtained a cDNA clone encoding CDH from *P. chrysosporium* (201), which was generously provided by Dr. V. Renganathan. The results, described in the following chapter, have been published elsewhere (96). K. Bartholomew helped with the production of the genomic library by producing the size-selected *Sau*3A-digesed genomic DNA. I performed all other experiments, with the guidance of K. Bartholomew, S. Moukha, T. Charles, and F. Archibald. The sequencing of the *cdh* cDNA clone isolated by RT-PCR was done commercially.

CHAPTER 3. CLONING AND SEQUENCING OF A GENE ENCODING CELLOBIOSE DEHYDROGENASE FROM *TRAMETES VERSICOLOR*

3.1. Abstract

Cellobiose dehydrogenase (CDH) is an enzyme produced under lignocellulose-degrading conditions by *T. versicolor* strain 52J and several other wood-degrading fungi including *P. chrysosporium*. In order to understand better the nature and properties of this enzyme, we isolated a genomic clone of *T. versicolor cdh* using heterologous probes derived from the sequence of *P. chrysosporium cdh*. DNA sequence analysis revealed that *T. versicolor cdh* consists of 3091 bp of coding sequence interrupted by 14 introns. Southern blotting showed that the gene was present in a single copy in the strain of *T. versicolor* analyzed. *T. versicolor cdh* was shown by Northern blot analysis to be expressed as a single transcript under cellulolytic conditions. RT-PCR of poly(A)$^+$ RNA isolated under cellulolytic conditions was used to generate a near full-length cDNA copy of the *cdh* mRNA. The deduced protein encoded by *T. versicolor cdh* consists of 768 amino acids (aa), including a predicted 19 aa signal peptide. The protein had 73% identity to the corresponding protein from *P. chrysosporium*, which is the only other CDH-encoding gene that has been cloned. Based upon its deduced primary structure and alignment to similar sequences, *T. versicolor* CDH shares a general structural organization with *P. chrysosporium* CDH and other hemoflavoenzymes. Amino acid residues H109 and M61 in the N-terminal heme domain are hypothesized to function in heme binding; the C-terminal flavin domain contained a consensus sequence for flavin binding between residues 217-222. Although the protein is known to bind to cellulose, no obvious homology to bacterial or fungal cellulose binding domains was observed. However, strong homology was observed to a region of *P. chrysosporium* CDH that is hypothesized to be involved in cellulose binding.

3.2. Introduction

T. versicolor is a white-rot basidiomycete fungus known to secrete at least three classes of oxidoreductases, namely laccase, manganese peroxidase (MnP), and cellobiose

dehydrogenase (CDH), during growth under lignocellulolytic conditions (18, 233). Purified laccase and MnP derived from *T. versicolor* strain 52J have been shown to be capable of industrial kraft pulp delignification (18). *In vivo*, these enzymes are thought to interact extensively in order to bring about the degradation of lignin and cellulose during wood biodegradation (12, 18). CDH is a hemoflavoenzyme which preferentially oxidizes cellobiose and reduces a wide range of substrates (reviewed in (11, 12, 107). *In vitro*, the enzyme has a number of interesting properties that could contribute *in vivo* to the degradation of both lignin and cellulose; it reduces phenoxy radicals generated by laccase and prevents their repolymerization, it generates cellobionic acid which can chelate Mn(III) produced by MnP, and it reduces insoluble MnO_2 and thereby renders Mn(II) available to the MnP catalytic cycle (270). Furthermore, CDH has the ability to reduce Fe(III) and Cu(II) which can sustain a Fenton's reaction in which Fe(II) or Cu(I) react with H_2O_2 to produce highly reactive hydroxyl radicals (•OH) that can chemically attack both lignin and cellulose (6, 7, 188, 189, 269, 328). Although the *in vitro* biochemical abilities of CDH allow many hypotheses to be made regarding its true *in vivo* role in lignocellulose degradation, the exact nature of that role remains unclear (6, 141).

CDH is found in cellulolytic supernatants of *T. versicolor* cultures predominantly in two isoforms: a large (97 kDa, measured on SDS-PAGE gels) isoform with both heme and flavin cofactors, and a smaller (81 kDa) protein with a flavin cofactor (269). The larger protein possesses all of the biochemical capabilities of the smaller enzyme along with several unique properties, including the ability to reduce ferric and cupric ions (269). This led to the hypothesis that the flavin-only isoform is a proteolytic cleavage product of the larger heme-flavin enzyme, as is the case for *P. chrysosporium* CDH (132, 327). CDH may be cleaved as a means of regulating its activity (100); for example the cleavage of the heme-flavin isoform to the flavin isoform would eliminate the ability of CDH to sustain a Fenton's reaction (269), thereby reducing the •OH-mediated cleavage of lignin and cellulose. Proteolysis is one of only a few mechanisms by which *T. versicolor* could control the activity of its extracellular enzymes in order to reduce the degradation of lignocellulose in areas distant from the fungal mycelium.

In order to gain more information about the structure, function, and regulation of *T. versicolor* CDH, and eventually to attain further insight into its role in lignocellulose biodegradation, we cloned and sequenced a CDH-encoding gene from *T. versicolor* strain 52J.

3.3. Materials and methods

3.3.1. Organism and culture conditions

The fungal strain employed in this study, *T. versicolor* 52J (ATCC 20869) is a monokaryon which was derived from a wild dikaryotic isolate by protoplasting (2). This strain has been shown previously to effectively biobleach and delignify unbleached industrial kraft (chemical) pulp (18). *T. versicolor* 52J produces CDH when grown on a defined medium containing cellulose as the carbon source (269).

3.3.2. Construction of a genomic library

High molecular weight DNA was isolated from *T. versicolor* strain 52J, partially digested with *Sau*3A, and size fractionated on a 10%-40% sucrose gradient using standard procedures (20). Fragments of approximately 12-18 kb were pooled, dephosphorylated, and ligated into *Bam*HI-digested λ GEM-12 arms (Promega). An aliquot (0.5 μg) of the ligation mixture was packaged using a GigaPack Gold packaging extract (Stratagene) and plated on *E. coli* NM539 (Promega). The packaged phage was amplified once in *E. coli* NM539.

3.3.3. Isolation of *T. versicolor cdh*

Duplicate plaque lifts of 20,000 clones from the genomic library were screened with two probes derived from the *P. chrysosporium cdh* cDNA (201). The 5' end probe was a 1 kb *Sac*I-generated fragment encoding the entire heme domain and the N-terminus of the flavin domain, and the 0.5 kb 3' end probe was a *Bgl*II-*Apa*I digestion product of the *P. chrysosporium cdh* cDNA encoding the C-terminus of *P. chrysosporium* CDH. Hybridization was effected using 5x SSC/5x Denhardt's solution (100x = 2% BSA, 2%

polyvinylpyrrolidone, 2% Ficoll 400)/0.5% SDS/20 μg/mL denatured sheared salmon sperm DNA (Pharmacia) at 56°C for hybridization and 2x SSC/0.5% SDS at 56°C for the most stringent wash. Clones that hybridized to both probes were picked and subjected to two successive rounds of screening at lower plaque densities. Three clones binding both probes were purified, and one of these, containing a 13 kb insert, was subjected to further analysis.

Southern analysis (not shown) of this 13 kb DNA fragment using each of the probes and the same hybridization and wash conditions indicated that the entire coding region of *cdh* was located on a 5 kb *Eco*RI-generated fragment. This fragment was subcloned into pBluescript KS$^+$ (Stratagene), restriction mapped, and appropriate fragments were subcloned (Figure 3.1).

3.3.4. DNA sequencing

The nt sequence of both strands of each subclone of the 5 kb *Eco*RI fragment was determined, and the identity of their terminal restriction sites was confirmed by sequencing through them in the 5 kb *Eco*RI fragment using synthetic oligonucleotides as primers. Sequencing reactions were performed using an AmpliTaq cycle sequencing kit (Perkin-Elmer) or a cycle sequencing kit (BRL). ^{33}P-labelled products were separated on a 6% polyacrylamide gel with 7 M urea and visualized by autoradiography. Some of the sequence was determined using an ABI 373A automated sequencer (MOBIX, McMaster University, Hamilton, Canada). The sequence was compiled and analyzed using Gene Works 2.5.1.

3.3.5. RT-PCR

T. versicolor poly(A)$^+$ RNA was isolated from total RNA (Section 3.3.7) using magnetic bead-coupled oligo dT (Boehringer-Mannheim), and reverse transcribed using Expand RT (Boehringer-Mannheim) and an oligo dT primer to generate a cDNA pool. The cDNA was used as a template in a PCR using *cdh*-specific primers (Figure 3.2), *Taq* polymerase (Boeheringer-Mannheim) and *Taq* extender (Stratagene). Thermocycling

conditions: 94°C x 1 min, 55°C x 45 s, 72°C x 3 min (5 cycles), then 94°C x 1 min, 55°C x 30 s, 72°C x 3 min (35 cycles), followed by 72°C for 5 min. A 5 μL aliquot of the initial amplification was used as a template for a second amplification using the same primers and *Pfu* polymerase (Stratagene). Cycling conditions for this re-amplification were the same as above, except that an extension time of 6 min was used. The product of the *Pfu* reamplification was separated from the reaction mixture using QiaQuick columns (Qiagen), then incubated with *Taq* polymerase and 0.2 mM dNTP mix (no primers) at 72°C for 60 min. The products of this reaction were again separated from the reaction mixture using QiaQuick columns, then used directly in a ligation reaction with pMOSBlue T-vector (Amersham) according to the manufacturer's directions. Clones containing a cDNA insert of the appropriate size were screened using standard procedures.

3.3.6. Southern blot

Total *T. versicolor* genomic DNA (10 μg) was digested with various restriction enzymes and fractionated on a 1% agarose-TBE gel electrophoresed at 30V overnight. The DNA was blotted to a Hybond N^+ (Amersham) membrane and probed using a ^{33}P-labelled probe consisting of a 1.2 kb *Xho*I fragment of *T. versicolor cdh* (Figure 3.1). Hybridization was carried out at 65°C using the same hybridization solution as used for the library screen. The most stringent post-hybridization washes consisted of 0.1X SSC/0.1% SDS at 65°C for 10 minutes (repeated once). The blot was exposed to X-ray film (Kodak X-OMAT AR) for 3 days at ambient temperature.

3.3.7. Northern blot

T. versicolor was grown in a defined medium consisting of 0.5g/L KCl, 0.1g/L $MgSO_4 \bullet 7\ H_2O$, 0.1 g/L yeast extract, 1 g/L KH_2PO_4 0.4 g/L NH_4Cl, 5 g/L cellulose, and a trace metal solution (267). Total RNA was isolated at various times from biomass aliquots of *T. versicolor* by following the procedure of Wendland *et al.* (322). Total RNA (5 μg) was loaded on a 1.4% agarose gel prepared in 10 mM Na phosphate buffer at pH

6.8 according to the procedure of Pellé and Murphy (239). RNA was blotted to a Hybond N^+ membrane (Amersham) in 20x SSPE and hybridized overnight at 51°C to a P^{33} labelled *Xho*I fragment of *T. versicolor cdh* (Figure 3.1) in 5x SSPE/5x Denhardt's solution/50% formamide/0.1% SDS/20 µg/mL denatured sheared salmon sperm DNA. The most stringent post-hybridization wash consisted of 0.1x SSPE/0.1% SDS at 65°C (repeated once). The washed blot was exposed to X-ray film (Kodak X-OMAT AR) for 2 days at ambient temperature.

3.3.8. Enzyme assays

The supernatant of culture aliquots taken for RNA harvesting was assayed for CDH activity. The heme/flavin isoform was assayed by measuring the cellobiose-dependent reduction of cytochrome c at 415 nm (269), and the activity of both isoforms (heme/flavin and flavin only) was determined using the chlorpromazine radical reduction assay described by Roy and Archibald (268).

3.4. Results and discussion

3.4.1. Cloning and sequencing of *T. versicolor cdh*

In order to isolate clone(s) containing the gene encoding CDH, a genomic library consisting of *T. versicolor* genomic DNA partially digested with *Sau*3A was produced in the vector λ GEM-12 and screened using probes derived from *P. chrysosporium cdh*. The gene was localized to a 5 kb *Eco*RI fragment by Southern hybridization, and the nt sequence surrounding *cdh* was determined. The region sequenced consisted of 3.6 kb of DNA, including 3091 bp which encompassed the entire coding sequence, 262 nt upstream of the gene, and 262 nt downstream of the gene (Figure 3.2). The region upstream of the methionine (M) start codon contained a putative TATAA box consensus sequence at -55 bp and a possible CAAT box at -88 bp (311). No polyadenylation signal that was consistent with mammalian or yeast poly(A) sites (311) was found in the 3' untranslated

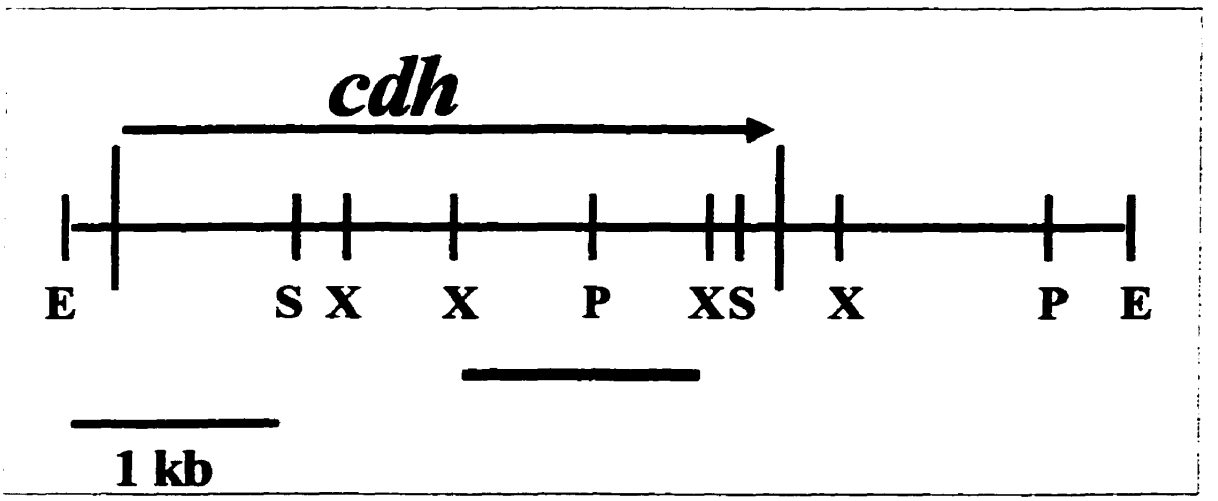


Figure 3.1. Physical map of *T. versicolor cdh* and the surrounding region. This restriction map was derived from the 13 kb λ clone which hybridized to both *P. chrysosporium cdh* probes. The location of the coding region of *cdh* is shown, as is the 1.2 kb internal *Xho*I fragment used as a hybridization probe. E, *Eco*RI; S, *Sac*I; X, *Xho*I; P, *Pst*I.

region (UTR).

The coding region of *T. versicolor cdh* was interrupted by 14 introns. The locations of the introns were inferred by alignment of the *T. versicolor* sequence to the known *P. chrysosporium* cDNA and genomic *cdh* sequences (200, 201) as well as by translating the open reading frame and comparing the resultant protein sequence to that of *P. chrysosporium* CDH. The intron assignments were subsequently confirmed by sequencing the *cdh* cDNA (Section 3.3.2). All of the introns contained 5' and 3' splice sites that corresponded to those of other basidiomycetous introns (286). The consensus sequence of the introns of the *T. versicolor cdh* gene was 5'GTRMGT (32) GCTRA (12) YAG3' where the numbers in parentheses correspond to the average number of nt. The average intron length was 56, with a range from 50-67. This is very similar to the consensus intron structure for basidiomycetes found by Schuren (286). The positions of the introns of *T. versicolor cdh* were very similar to that of the corresponding *P.*

Figure 3.2. The 3615-nt sequence of *cdh* from *T. versicolor* 52J and its deduced aa sequence. Introns are shown in lower-case lettering. The putative TATA and CAAT elements are underlined. Oligonucleotide primers used for RT-PCR are indicated by underlines. The predicted first amino acid of the mature protein (Q-1) is marked by a double underline. The hypothesized heme-chelating M and H residues in the heme domain are underlined. The flavin binding consensus sequence is shown in bold. The stop codon is indicated by an asterisk (*).

```
gaattctcgccgtcgcgcgcgctggcgtccgcccgtcatcgcctgccccttgttgccagt -203
atcatcgacagcatataggcgaccgtacgtccgcgtaatagctacacatcggtacacgcc -143
                                                        -88
atatgggtcgcgtaatctcgctatccgcgtccttgaatgttcggctatttctcgcaatac -83
                                  -55
cggaatgctgggcatcccggggaggcgtataaagagctgtttagaccttcttgcacggtg -23
agcgcaacactacgaccgccgcATGAAGTTCAAGAGCCTGTTGTTGTCTCTGCTGCCATT 38
                        M  K  F  K  S  L  L  L  S  L  L  P  L -7
GGTCGGCTCCGgtgcgatgcatttttcgcgtcagcagctagagtgctaaacgatgcgta 98
 V  G  S  V                                                  -3
ttgaacagTGTACTCTCAAGTCGCCGCACCCTACGTAGACTCCGGGAATGGCTTCGTCTT 158
          Y  S  Q  V  A  A  P  Y  V  D  S  G  N  G  F  V  F 15
CGACGGCGTCACCGACCCGGTGCACAGCGTCACCTATGGAATCGTCCTCCCTCAAGCATC 218
 D  G  V  T  D  P  V  H  S  V  T  Y  G  I  V  L  P  Q  A  S 35
CACCAGCACAGAGTTTATCGGCGAGTTCGTTGCGCCCAACGAGGCGCAATGGATTGGATT 278
 T  S  T  E  F  I  G  E  F  V  A  P  N  E  A  Q  W  I  G  L 55
GGCTCTCGGAGGAGCCATGATCGGCAACCTTCTTCTCGTCGCATGGCCGAATGGGAACAA 338
 A  L  G  G  A  M  I  G  N  L  L  L  V  A  W  P  N  G  N  K 75
AATTGTGTCTTCGCCTCGCTACGCGACgtaagtgacattccaatataaatgacgatgtac 398
 I  V  S  S  P  R  Y  A  T                                   84
aaaactgaatgcggggactcacaaagCGGATACACATTGCCGGCGGCGTACGCAGGACCA 458
                           G  Y  T  L  P  A  A  Y  A  G  P  95
ACCATTACCCAGCTGCCGTCGAGTTCAGTCAACTCAACCCACTGGAAGTTTGTGTTCCGC 518
T  I  T  Q  L  P  S  S  S  V  N  S  T  H  W  K  F  V  F  R  115
TGCCAGAACTGCACAGgtacgtgatcttcacttcgttaggatcacgtcgttgacgcctcc 578
C  Q  N  C  T  A                                            121
ctcgacagCCTGGAACGGCGGAAGCATCGACCCCTCCGGCACTGGCGTCTTCGCCTGGGC 638
          W  N  G  G  S  I  D  P  S  G  T  G  V  F  A  W  A 138
CTTCTCGAACGTCGCGGTAGACGACCCCTCCGACCCCAACAGCAGCAATGCTGAGCACAC 698
 F  S  N  V  A  V  D  D  P  S  D  P  N  S  S  N  A  E  H  T 158
TGACTgtgagtggccaaactcacctgcgatcgccagcgtgctgatgaaacattccagTCG 758
 D  F                                                        G 161
GGTTCTTCGGCATCAACTTCCCCGACGCTCAGAGCTCGAACTACCAGAACTACCTCGCAG 818
  F  F  G  I  N  F  P  D  A  Q  S  S  N  Y  Q  N  Y  L  A  G 181
GCAACGCGGGGACTCCCCCTCCCACGTCCGTTCCTAGCGGCCCGTCCAGCACTACCACTA 878
  N  A  G  T  P  P  P  T  S  V  P  S  G  P  S  S  T  T  T  T 201
CCACCGGTCCTACGGCTACTgtgaggatcttctttcagcgctgcctgacccgtagctgac 938
  T  G  P  T  A  T                                           207
gattttgtagGCTACGCCGTTTGACTATATCGTCGTCGGCGCTGGCCCTGGTGGTCTCGT 998
          A  T  P  F  D  Y  I  V  V  G  A  G  P  G  G  L  V 224
```

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CACTGCCGATCGCTTGTCTGAAGCTGGCAAGAAAGTCCTACTTCTCGAGCGTGGCGGACC 1058
 T  A  D  R  L  S  E  A  G  K  K  V  L  L  L  E  R  G  G  P  244
CTCGACTGCGGAGACCGGTGGCACGTATGACGCCACATGGGCCAAGTCTGCTAACgtgag 1118
 S  T  A  E  T  G  G  T  Y  D  A  T  W  A  K  S  A  N        262
tccataatccgcgcatatagccagggtccatgcgacggagctgaccatcgtccaatccag 1178

CTTACAAAATTCGACGTCCCGGGATTGTTCGAGACTTTGTTCACCGACACGAACCCATTC 1238
L  T  K  F  D  V  P  G  L  F  E  T  L  F  T  D  T  N  P  F   282
TGGTGGTGCAAGGgtgagttttggtcgtagccgtgcctcacccctactgaagaagcctt 1298
W  W  C  K  D                                                 287
ccagACACCAACTTCTTCGCGGGATGCCTTCTGGGTGGCGGTACCTCGGTCAACGGAGCg 1358
      T  N  F  F  A  G  C  L  L  G  G  G  T  S  V  N  G  A   305
taagtccggttccctagtgcggccgccggtggttctaacgcttcttcccagTCTCTACTG 1418
                                                    L  Y  W   308
GTACCCTAACAGCCGCGACTTCTCCACTGCGAGCGGGTGGCCAAGCAGCTGGAGCAACCA 1478
 Y  P  N  S  R  D  F  S  T  A  S  G  W  P  S  S  W  S  N  H  328
CCAGCCGTTCACCGACAAGCTGAAGCAGCGCCTACCGAGCACGGACCACCCCTCCGCCGA 1538
 Q  P  F  T  D  K  L  K  Q  R  L  P  S  T  D  H  P  S  A  D  348
TGGCCAACGTTATCTCGAGCAGTCGGCCACCGTCGTCCAGCAGCTGCTCTCAGGCCAGGG 1598
 G  Q  R  Y  L  E  Q  S  A  T  V  V  Q  Q  L  L  S  G  Q  G  368
ATACTCGCAAATCACTATCAACGACAACCCCGACTCGAAGGACCACGTGTTCGGATTCAG 1658
 Y  S  Q  I  T  I  N  D  N  P  D  S  K  D  H  V  F  G  F  S  388
CGCCTTCGACTTCCTCAACGGCCAGCGTGCAGGCCCCGTCGCGACATACTTCGAGACCGC 1718
 A  F  D  F  L  N  G  Q  R  A  G  P  V  A  T  Y  F  E  T  A  408
GCTTGCGCGCAAGAACTTCGTGTACAAGGACAACGTGCTTGTCACGCAGGTTATCCGCAA 1778
 L  A  R  K  N  F  V  Y  K  D  N  V  L  V  T  Q  V  I  R  N  428
CGGGTCGACGATCCTCGGTGTCCGCACGAACGACAACACGCTCGGACCGGATGGGATCGT 1838
 G  S  T  I  L  G  V  R  T  N  D  N  T  L  G  P  D  G  I  V  448
GCCCCTGAACCCGAACGGCCGTGTCATCCTCTCTGGTGGTTCCTTCGGCACTCCCCGTAT 1898
 P  L  N  P  N  G  R  V  I  L  S  G  G  S  F  G  T  P  R  I  468
CCTCTTCCAGAGCGGTATCGGGCCGACGGACATGCTCCAGACCGTGCAGAGCAACGCCCA 1958
 L  F  Q  S  G  I  G  P  T  D  M  L  Q  T  V  Q  S  N  A  Q  488
GGCGGCGGCGAACCTTCCCCCGCAGAGCGAGTGGATTGACCTGCCCGTCGGACAGTCGGT 2018
 A  A  A  N  L  P  P  Q  S  E  W  I  D  L  P  V  G  Q  S  V  508
GTCCGACAACCCGTCGATCAACgtaagtgttcatgcatgcggtcgtaactattgcggaag 2078
 S  D  N  P  S  I  N                                          515
gctgatgtaacgtatagCTCGTGTTCACGCACCCGAGCATCGACGCCTACGACAACTGGG 2138
                 L  V  F  T  H  P  S  I  D  A  Y  D  N  W  A  530
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CTGATGTGTGGTCAAACCCCAGGCCGGCGGATGCCCAGCAGTACCTGCAGAGCCGCTCCG 2198
  D  V  W  S  N  P  R  P  A  D  A  Q  Q  Y  L  Q  S  R  S  G 550
GCGTGTTGGCCGGTGCATCCCCTAAgtacgtcctgcatcattggccatgaggtcttctaa 2258
  V  L  A  G  A  S  P  K                                     558
cgactgacatgtgctactacagGCTGAACTTCTGGAGAGCATACGGTGGCAGCGATGGCA 2318
                        L  N  F  W  R  A  Y  G  G  S  D  G  I 571
TCACCCGCTACgtgcgttgggcattctcatcacctcagaagtgcgatgtttgatacgaac 2378
  T  R  Y                                                    574
tacagGCGCAAGGAACTGTTCGCCCTGGTGCAGCGTCCGTCAACACATCCGTTGCGTACA 2438
     A  Q  G  T  V  R  P  G  A  A  S  V  N  T  S  V  A  Y  N 593
ACGCGAGCGAGATCTTCACAATCACTCTCTACCTGTCCAACGGTATTCAGTCCCGCGGCC 2498
  A  S  E  I  F  T  I  T  L  Y  L  S  N  G  I  Q  S  R  G  R 613
GCATTGGTGTCGATGCTGCCTTGAACGCGAAGGCCCTCGTCAACCCGTGGCTCACCAACT 2558
  I  G  V  D  A  A  L  N  A  K  A  L  V  N  P  W  L  T  N  S 633
CCGTCGACAAGACGGTCCTGCTGCAAGCCCTGCATGACGTGACGTCCACCATGAAGAACG 2618
  V  D  K  T  V  L  L  Q  A  L  H  D  V  T  S  T  M  K  N  V 653
gtaggttccccgtactcgcgatgttggacacggctcacgtatcgttttcagTGCCTGGCT 2678
                                                    P  G  L 656
TGACGATGATCACCCCCGACAACACGATGACGCTCGAGCAATACGTCGCCGCCTACGATC 2738
  T  M  I  T  P  D  N  T  M  T  L  E  Q  Y  V  A  A  Y  D  P 676
CGgtgcgtagcttctgctgcggtaccaccgcagcctgaaacagatgctgatgcaaacgct 2798

gcccacagGCGACAATGTGCTCCAACCACTGGGTCGGCGCCGCGAAGATGGGCACGAGCT 2858
        A  T  M  C  S  N  H  W  V  G  A  A  K  M  G  T  S  S 694
CGTCCACCGCAGTTGTCGACGAAAACGCGAAGGTGTTCAACACGGACAACCTGgtgagcg 2918
  S  T  A  V  V  D  E  N  A  K  V  F  N  T  D  N  L        711
ctgtggcgcctcatttccgacgcgcgcgggcactgacgaaattgacgtttgtgcagTTCA 2978
                                                    F  I 713
TCGTGGATGCGTCCATCATTCCGTCGCTGCCGATAGGGAACCCGCAGGGAGTGCTGATGT 3038
  V  D  A  S  I  I  P  S  L  P  I  G  N  P  Q  G  V  L  M  S 733
CGGCGGCGGAGCAGGCGGTTTCGCGCATTCTGGCGCTTGCTGGGGGTCCTTGAggcggcg 3098
  A  A  E  Q  A  V  S  R  I  L  A  L  A  G  G  P  *        749
caaaagcattttggatgtcgggctgcgtgggtggccccgtcgtgtgctgtacttgtattc 3158
ggtaaacggaggtactaatatcggagtatatgcttagtctcaggtctgtgagagtgtgag 3218
tgacattcaatggtattgcatcattgatacgctcgtgaaccctgccattattgtggacat 3278
gtatccgtacgcaattgctgattgatgaaatcaacccgccgagcaggctatcgtgcaagt 3338
gttgacatgctcgag                                             3353
```

chrysosporium gene (Figure 3.3). However, the region corresponding to exon 9 of the *T. versicolor* sequence was interrupted by an intron in the *P. chrysosporium* sequence and there were two introns in the C-terminus-encoding region of the *T. versicolor* sequence with no homologues in the *P. chrysosporium* sequence. Thus, overall, there was one additional intron in *T. versicolor cdh* (Figure 3.3).

T. versicolor, like *P. chrysosporium* and other white-rot basidiomycetes, has multiple genes possessing high but not complete homology encoding laccases (50, 218, 219), MnPs (72), and lignin peroxidases (163). To determine if *T. versicolor* CDH is also encoded by multiple genes, genomic DNA from *T. versicolor* was digested with various restriction enzymes and analyzed by Southern blot using an internal 1.2 kb *Xho*I fragment of *T. versicolor cdh* as a probe (Figure 3.4). The appearance of only one band in each of the lanes suggested that there was only one copy of *cdh* in the strain of *T. versicolor* analyzed. This finding supports the hypothesis that the two active isoforms of the enzyme found in culture supernatants, heme/flavin and flavin-only (269) are encoded by the same gene and that the flavin-only isoform is a proteolytic product of the "complete" enzyme. Both of these isoforms bind to anti-*P. chrysosporium* CDH polyclonal antibodies (Ab) in their SDS-denatured forms (not shown), suggesting that they contain common sequence elements. The appearance of the flavin-only isoform is not likely explained by alternative splicing of the mRNA since only one size of *cdh* mRNA is observed (Figure 3.5; Section 3.3.2). Whether *T. versicolor* CDH is encoded by allelic variants, as has been shown for *P. chrysosporium* CDH (200), is unknown since the strain analyzed here is monokaryotic.

3.4.2. Expression of *cdh* in *T. versicolor*

CDH is normally produced by *T. versicolor* under lignocellulolytic (primary growth) conditions (269). In order to isolate RNA which contained copies of *cdh* mRNA, *T. versicolor* was grown on a defined medium containing cellulose as the carbon source (269). RNA was isolated from biomass samples that were shown by enzyme assay to be producing CDH (Figure 3.5). This RNA was subjected to Northern analysis using a 1.2 kb *Xho*I fragment of *T. versicolor cdh* as a probe (Figure 3.5). The results showed that *cdh*

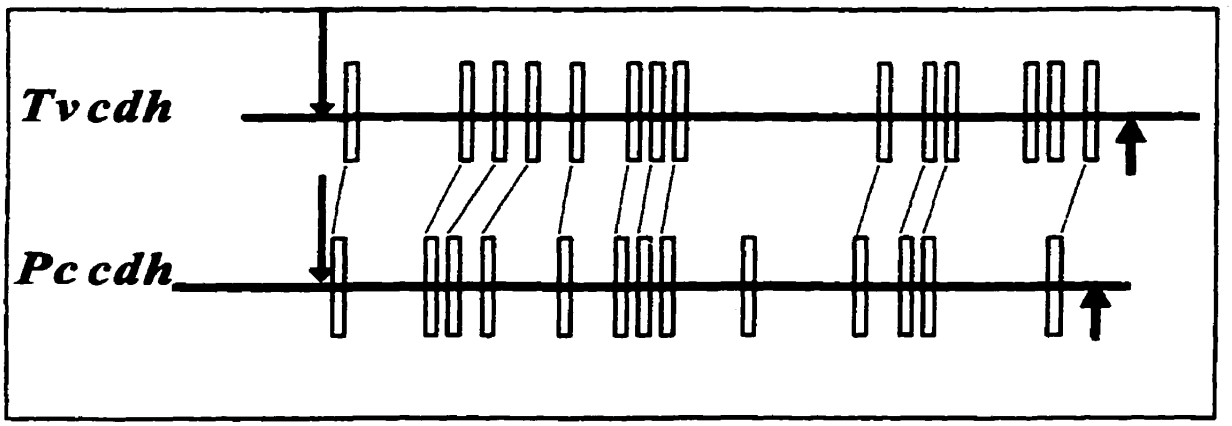


Figure 3.3. Locations of the *T. versicolor* and *P. chrysosporium cdh* introns. The sequences are aligned by their respective ATG start codons, shown by a downward arrow. Corresponding introns are connected by lines. The upward arrow indicates the locations of the respective stop codons. The *P. chrysosporium* sequence is the *cdh*2 allele (200), deposited in GenBank under accession number U65888.

was transcribed under cellulolytic conditions as a single mRNA species, lending further credence to the hypothesis that both isoforms are encoded by one gene. Furthermore it appears that the expression of *cdh* is controlled at the level of transcription since the transcript was detected at the same time as the active protein appeared in the supernatant (Figure 3.5). Two assays were used to measure the total activity contributed by both isoforms of CDH. The reduction of cytochrome c is catalyzed only by the heme/flavin isoform, while chlorpromazine reduction is catalyzed by both isoforms (269). The fact that cytochrome c-reducing activity declined at the same time as the level of transcript, while chlorpromazine-reducing activity remained high when little or no transcript was detectable, was consistent with the hypothesis that the initial translation product is the heme/flavin isoform and that the flavin-only isoform is formed by proteolytic cleavage.

The RNA population used in these experiments, which was shown to contain copies of *cdh* mRNA, was reverse transcribed into cDNA using a poly (T) primer and reverse transcriptase. The cDNA was used as a template in a PCR using *cdh*-specific primers (Figure 3.2), resulting in the amplification of a 2.2 kb cDNA corresponding to most of the deduced protein coding region of *cdh*. This cDNA was cloned into a pMOSBlue T-vector (Amersham) according to the manufacturer's instructions and the complete sequence was determined. The cDNA sequence confirmed the assignments of

all of the introns except for intron 1, which is located within the signal peptide-encoding region of the gene. The cDNA that was obtained is not full length and is missing the signal peptide-encoding region and 5' untranslated region (UTR), along with the DNA encoding the final four amino acids (aa), stop codon, and 3' UTR. The sequence of the cDNA is 99.9% identical to the corresponding coding region of the genomic sequence, but there are 3 discrepancies (a C-G transversion at base 13, a C-T transition at base 1647, and a A-G transition at base 2309), each of which result in aa changes in the deduced protein. These may correspond to base misincorporations by the *Taq* or *Pfu* polymerases used to amplify the *cdh* cDNA.

3.4.3. The *T. versicolor* CDH protein

The deduced protein encoded by *T. versicolor cdh* consists of 768 aa, including a 19-aa signal peptide. The site of the signal peptide cleavage was predicted using the method of Neilsen et al. (225), since an N-terminal aa sequence could not be obtained from the purified protein (269). The predicted start of the mature *T. versicolor* CDH is a glutamine (Q) residue at position 20 relative to the M residue (Figure 3.2). Overall, the homology of the *T. versicolor* apoprotein to the *P. chrysosporium* apoprotein was very high, with 73% identity. Polyclonal rabbit Ab raised against *P. chrysosporium* CDH strongly cross-reacted with *T. versicolor* CDH (not shown). Based on its homology with the *P. chrysosporium* protein and the organization of other hemoflavoenzymes, *T. versicolor* CDH appears to be divided into two domains: an N-terminal heme-binding domain and a C-terminal flavin-binding domain. A putative hydroxyamino acid-enriched "linker" region joining the two domains is found in *T. versicolor* CDH between residues 189-202; a corresponding region is found in *P. chrysosporium* CDH (201). The mature *T. versicolor* heme/flavin protein consists of 749 aa and has a MW of 79324 Da while the flavin domain has 548 aa and a MW of 58393 Da. Earlier observations on SDS-PAGE gels showed an apparent MW of 97 kDa for the heme/flavin protein and 81 kDa for the flavin domain (269). The presence of 4 predicted N-glycosylation sites on the protein, 3 in

the flavin domain and 1 in the heme domain, may explain these differences in MW if these sites are indeed glycosylated.

T. versicolor CDH displayed an FAD binding motif (G-X-G-X-X-G) between residues 217 and 222 (Figure 3.2). The N-terminus of the flavin domain of *P. chrysosporium* CDH displays a similar motif (201). In addition, residues 202-228 of *T. versicolor* CDH were highly homologous to the biochemically determined N-terminus of the flavin domain of the *P. chrysosporium* protein (201); thus residue 202 was presumed to be the first aa of the flavin domain of the *T. versicolor* protein. Furthermore, the C-terminal region of *T. versicolor* CDH was very similar to that seen in the *P. chrysosporium* protein which has been shown (201) to be homologous to other FAD-dependent enzymes. Cox et al. (79) showed that the axial ligands of the heme iron of CDH are M and histidine (H). In the heme domain of *P. chrysosporium* CDH, an M residue at position 65 is thought to be involved in binding the heme (201, 249); this M residue was conserved at position 61 in the *T. versicolor* sequence and extensive homology to the *P. chrysosporium* sequence was observed in this region (74% identity between aa 61-79 of *T. versicolor* and 65-83 of *P. chrysosporium* CDH). Moreover, the conserved locations of three H residues at positions 23, 109, and 157 in the *T. versicolor* protein (positions 23, 114, and 163 in the *P. chrysosporium* protein) implicated these residues as possible axial ligands of the hexacoordinate heme iron. Two of the three H residues (corresponding to H109 and H157 in *T. versicolor* and H114 and H163 in *P. chrysosporium*) were also found to be conserved in a CDH-encoding gene fragment amplified from another white-rot fungus, *Pycnoporus cinnabarinus* (Chapter 5). The full-length *P. cinnabarinus* CDH-encoding clone subsequently showed that only these two H residues are conserved among all three proteins (S. Moukha, unpublished observation), excluding H23 of *T. versicolor* CDH as a likely heme ligand. Furthermore, the recent report of a CDH-encoding sequence[2] from the ascomycete *Thielavia heterothallica* (*Sporotrichum thermophile*) shows that only the H residue analogous to H109 of *T.*

[2]B. Li et al., deposited in GenBank under accession number AF074951

versicolor CDH is positionally conserved among all four known CDH proteins. All of these observations suggest that H109 of *T. versicolor* CDH and H114 of *P. chrysosporium* CDH may be involved in the binding of the heme iron.

T. versicolor CDH binds to cellulose (269), as has been observed for the *P. chrysosporium* protein (144, 260). The cellulose binding domain (CBD) of *P. chrysosporium* CDH is probably located in the flavin domain (201). Furthermore, recent studies by Henriksson et al. (144) indicate that the cellulose-binding site of *P. chrysosporium* CDH is located within residues 251-299. Supporting this hypothesis, the corresponding region of *T. versicolor* CDH (residues 246-294; Figure 3.6) displayed very high homology, including the positional conservation of all 9 aromatic residues which may be involved in cellulose interaction (119). However, comparing the putative *T. versicolor* and *P. chrysosporium* CDH cellulose binding sequences to the known CBD consensus sequences for bacterial and fungal cellulases (119, 307) revealed no obvious homology. Thus the putative *P. chrysosporium* CDH CBD identified by Henriksson *et al.* (144) and the analogous *T. versicolor* sequence reported here appear to be structurally unique CBDs.

3.5 Conclusions

(1) Using two heterologous probes derived from *P. chrysosporium cdh*, a genomic clone of *T. versicolor cdh* was isolated.

(2) A total of 3.6 kb of DNA surrounding *cdh* was sequenced, revealing a protein coding region of 3091 nt with 262 nt upstream and 262 nt downstream. The coding sequence is interrupted by 14 introns ranging in size from 50-67 nt which are highly similar in structure to other basidiomycetous introns.

(3) *T. versicolor cdh* is highly homologous to *P. chrysosporium cdh* and displays a similar intron/exon structure. There is one more intron in the *T. versicolor* sequence than the *P. chrysosporium* sequence and the positions of two introns are different between the two genes.

(4) Southern analysis of the *T. versicolor* genome using a homologous *cdh* probe indicates that the gene is present in a single copy.

(5) *T. versicolor cdh* is probably transcribed as a single transcript under cellulolytic conditions and appears to be regulated at the level of transcription. Northern analysis indicates that RNA isolated when active CDH protein is measured contains *cdh* mRNA, a near full-length cDNA copy of which was isolated by RT-PCR.

(6) *T. versicolor* CDH is highly homologous to the corresponding *P. chrysosporium* protein and consists of 768 aa, including a 19 aa signal peptide. Similar to other hemoflavoenzymes, *T. versicolor* CDH appears to be composed of two domains, an N-terminal heme domain and a C-terminal flavin domain linked together by a hydroxyamino acid-enriched linker region. The flavin-only isoform of *T. versicolor* CDH is likely produced by proteolytic cleavage and not alternative mRNA splicing since only a single size of *cdh* mRNA is observed.

(7) Based on positional conservation and sequence homology to CDH sequences from other fungi, aa M61 and H109 are likely axial ligands for the heme iron. The flavin domain contains an FAD-binding motif, GAGPGG, between residues 217-222 and shares sequence similarity with other flavin enzymes.

(8) By homology to the putative *P. chrysosporium* CDH CBD identified by Henriksson et al. (144), residues 248-293 of *T. versicolor* CDH may be implicated in cellulose binding.

The sequence described in this report has been deposited in GenBank with accession no. AF029668.

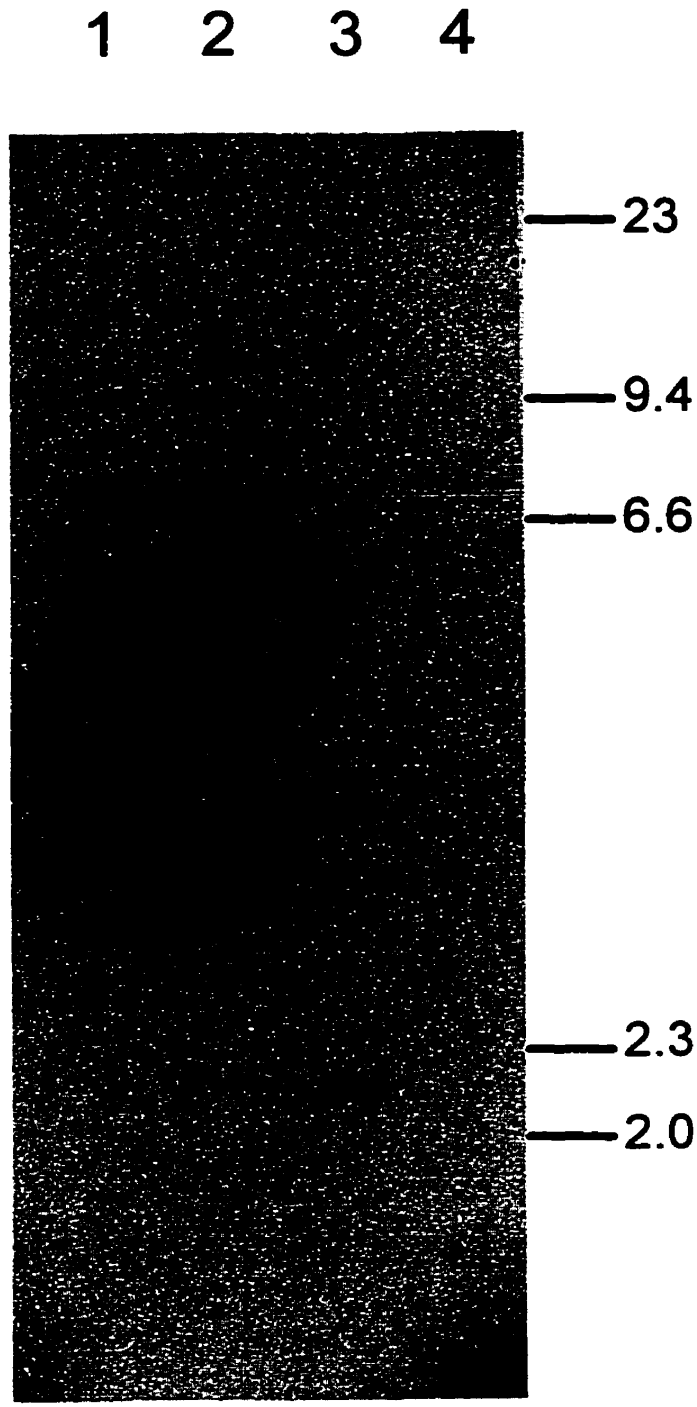
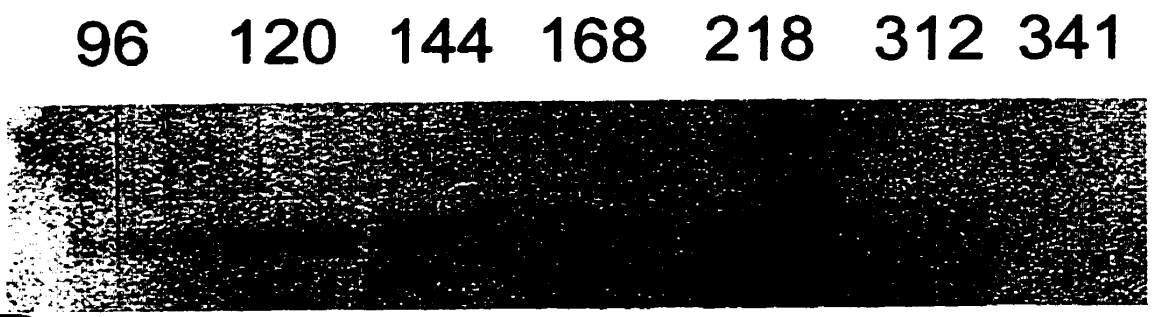


Figure 3.4. Southern blot of *T. versicolor* DNA probed using a homologous *cdh* probe. Lane 1: *Eco*RI; Lane 2, *Hin*dIII; Lane 3, *Sac*I; Lane 4, *Xho*I. The positions of the molecular weight markers are indicated, and their sizes (in kb) are shown.

A



B

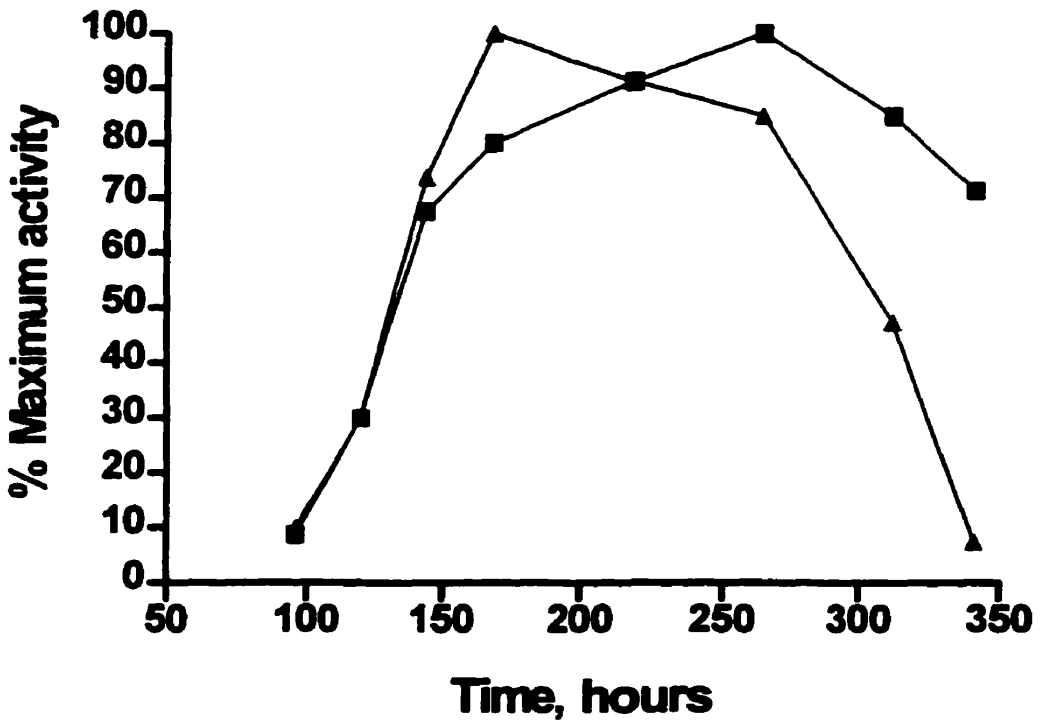


Figure 3.5. Northern blot of *T. versicolor* RNA (A) and concomitant assays for CDH (B). A. Northern blot. The gel was run at 3V/cm for 2.5 h with buffer recirculation. Lanes correspond to RNA harvested at t = 96, 120, 144, 168, 218, 264, 312, and 341 h after inoculation. B. Enzyme assays for CDH taken from the supernatant of the *T. versicolor* cultures at the same time as biomass aliquots were harvested for RNA isolation. Heme/flavin isoform (cytochrome c assay): ▲; both isoforms (chlorpromazine assay): ■.

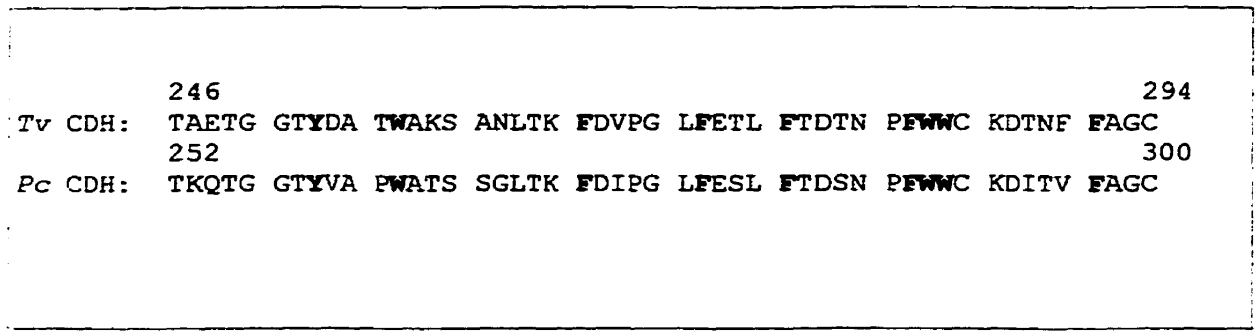


Figure 3.6. Alignment of the putative CBDs of *T. versicolor* and *P. chrysosporium* CDH. The CBD of *P. chrysosporium* CDH was predicted by Henriksson *et al.* (144). The conserved aromatic residues are boldfaced. The cysteine residues at positions 291 and 300 of the *P. chrysosporium* sequence (corresponding to residues 285 and 294 of the *T. versicolor* sequence) are thought to be involved in the formation of a disulfide bond (144).

PREFACE TO CHAPTER 4

One of the major problems encountered in the purification of *T. versicolor* CDH is the difficulty in isolating adequate quantities of non-degraded enzyme (Chapter 2). This fact hampered study of the biochemical features of CDH, and led to a search for ways to avoid this problem. The isolation of a cDNA clone encoding *T. versicolor* CDH (Chapter 3) provided us with the possibility of producing *T. versicolor* CDH in a heterologous host, thereby avoiding endogenous *cdh* regulatory systems (at the levels of gene expression and proteolysis). Furthermore, the study of structure/function relationships of the amino acids of CDH may be facilitated by the availability of a tool enabling site-directed mutagenesis studies. For these reasons, we undertook the expression of *T. versicolor* CDH in *Aspergillus oryzae*.

CHAPTER 4 CONSTRUCTION OF VECTORS FOR THE HETEROLOGOUS EXPRESSION OF *TRAMETES VERSICOLOR* CELLOBIOSE DEHYDROGENASE AND LACCASE IN *ASPERGILLUS ORYZAE*

4.1. Abstract

Five separate constructs for the heterologous expression of *T. versicolor* cellobiose dehydrogenase (CDH) and laccase in the filamentous hyphomycete *Aspergillus oryzae* were generated and cloned. Using the gene fusion technique of splicing by overlap extension (SOE-PCR), we spliced the control regions of *A. oryzae* Taka-amylase (promoter/ signal peptide and terminator DNAs) to cDNAs encoding three versions of *T. versicolor* CDH (the complete protein, heme domain alone, and flavin domain alone) and two *T. versicolor* laccases (I and IV). The results of the SOE-PCRs indicated that for the reaction to work well, a molar ratio of close to 1:1 of the DNA fragments to be spliced is essential. All five constructs were verified by restriction mapping, PCR, and DNA sequencing. They were then used to co-transform protoplasts of *A. oryzae* using growth on acetamide as sole nitrogen source as a selectable marker with the plasmid p3SR2, which contains the *A. nidulans amdS* gene for acetamidase. Several putative transformants were obtained for each of the five contructs. None of the laccase transformants appeared to produce a functional recombinant protein. Four of the CDH transformants, one cdhF (flavin only) and three cdhHF (heme/flavin), appeared to express functional recombinant CDH by a plate assay using the green $ABTS^{+}$ cation radical as an activity indicator. One of these strains, cdhF, was unequivocally demonstrated to be a true co-transformant by PCR analysis of genomic DNA.

4.2. Introduction

As discussed in Chapter 2, CDH is typically produced in low yields by *T. versicolor*, and extensive degradation of the protein purified from homologous sources is commonly observed (302). One promising means of obviating these difficulties is to produce the protein in a heterologous host. The filamentous hyphomycete genus *Aspergillus* includes well-studied organisms which have been used for many years for

the expression of foreign proteins (128, 180). Interest has centred on these fungi as hosts for the expression of foreign proteins because, among other reasons, of their ability to secrete copious quantities of their own proteins (grams per liter are not uncommon), and because their long history of use in the food industry (for example, in the production of soy sauce) assures that efficient fermentative processes are already in place (180). However, the production of heterologous proteins in aspergilli can be limited at several levels: transcriptional, translational, or post-translational (128), so researchers have focussed on using homologous transcriptional control regions to facilitate the expression of the heterologous gene. A common strategy is the fusion of the heterologous gene to the control regions of a known, well-expressed fungal gene such as amylase (128). This strategy has been successfully applied to the expression of various heterologous fungal proteins in *Aspergillus* (32, 69, 295) However, this approach necessitates that in-frame fusions be made between the homologous control regions and the gene in question.

One difficulty with performing in-frame fusions between DNA molecules is the necessity for convenient restriction sites within the molecules, which often must be engineered into the sequences. Horton et al. (148) have described a means for splicing genes without the use of restriction enzymes and ligase. The method, known as splicing by overlap extension (SOE-PCR) is based upon the polymerase chain reaction (Figure 4.1). SOE-PCR provides the researcher with precise control over the splice junction of two DNA molecules, and affords an extremely powerful method for fusing two genes at any desired point.

Herein we describe the application of SOE-PCR to the construction of vectors for the heterologous expression of *T. versicolor* proteins in *Aspergillus oryzae*. The recent isolation of cDNA clones encoding laccase (230) and CDH (Chapter 3) allowed us to expand the range of tools available for the study of these proteins.

4.3. Materials and Methods

4.3.1. Strains and plasmids

The *Aspergillus oryzae* strain used in these studies was from the GB culture collection (GB France SA No. 1135 strain RP). This is a derivative of a wild strain of *A. oryzae* derived by several rounds of u.v. mutagenesis followed by screening for amylase overproduction. Plasmid manipulations were done using the *E. coli* host strains JM109 (329) (Promega, Charbonnières, France) or MOSBlue (Amersham). Plasmid pMOSBlue was obtained from Amersham, and plasmid pBluescript KS^+ was from Stratagene (La Jolla, California). Plasmid p3SR2 containing the *amdS* gene of *Aspergillus nidulans* was obtained from the Fungal Genetics Stock Center (Kansas City, KS, USA).

4.3.2. DNA extraction and purification

Total genomic DNA was isolated from *A. oryzae* according to standard procedures (46). Plasmid DNA was prepared from cultures of *E. coli* by CsCl density gradient centrifugation using previously described methods (20).

4.3.3. PCRs to amplify products to be used in splicing reactions

The PCR primers which were used to generate products with termini modified appropriately for gene splicing by SOE-PCR are given in Tables 4.1 and 4.2. Primers were synthesized by Pharmacia. The primer pairs that were used to amplify each individual product are given in Table 4.3. Thermocycling was performed using a Perkin Elmer GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, USA).

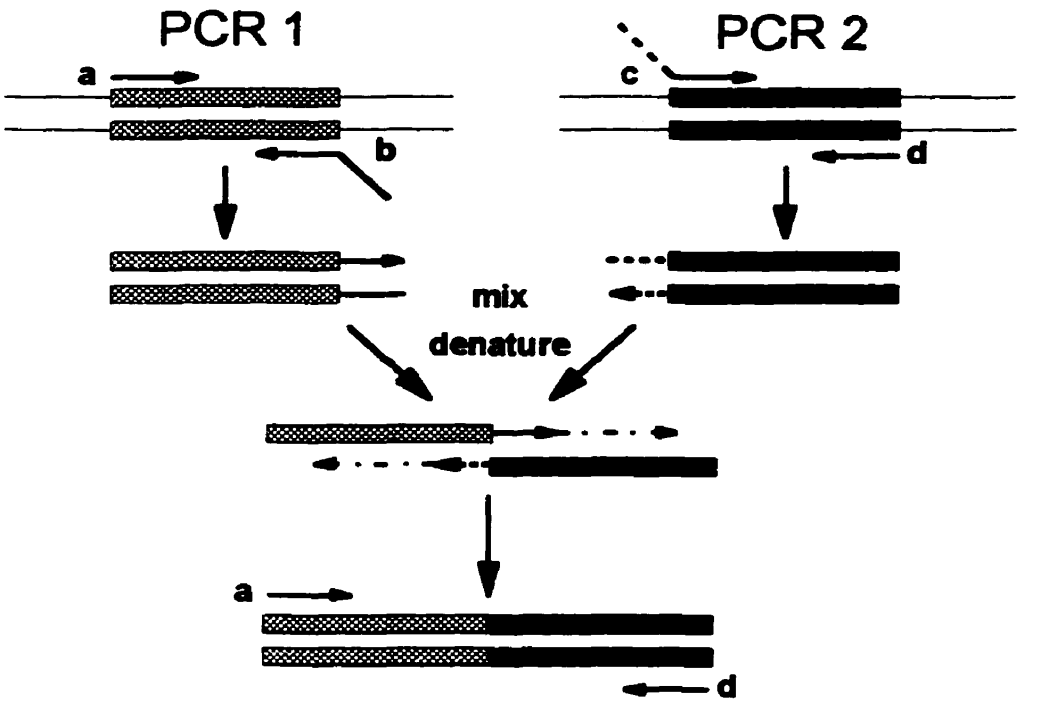


Figure 4.1. General scheme for splicing by overlap extension (SOE-PCR) as described by Horton et al. (148). The hatched box represents a DNA fragment (X) which is to be spliced to the DNA fragment represented by the filled box (Y). Primers b and c are designed so that their 5' ends are complementary to the end of DNA fragments Y and X respectively, which results in two PCR products with modified termini that are complementary to one another. These two PCR products are mixed, denatured, and allowed to re-anneal; among the possible duplexes that form is one in which the complementary termini anneal and prime one another. The 3' ends of these termini are extended by polymerase and the spliced product is the only DNA molecule in the reaction which is amplifiable using primers a and d.

Primer code	Primer sequence (5'-3')	Comment
L22	TCTAGAATTCTGGCTGTGGTGTACAGG	Upstream primer for Taka-amylase promoter amplifications. Amylase-specific region underlined; also contains *Xba*I and *Eco*RI sites.
L26	GGATCCGAATTCGGTGGAATATAGCTCG	Downstream primer for Taka-amylase terminator amplifications. Amylase-specific region underlined; also contains *Bam*HI and *Eco*RI sites.
TaP-cdhHF	GTGCGGCGACTTGAGCCAAAGCAGGTG	Downstream primer for amplification of amylase promoter cohesive for complete coding region of *cdh* cDNA.
cdhHF-TaP	ACCTGCTTTGGCTCAAGTCGCCGCAC	Upstream primer for amplification of complete coding region of *cdh* cDNA cohesive for amylase promoter.
cdhHF-TaT	TCAAGGACCCCCAGCAAGCGCCAGAATG	Downstream primer for amplification of complete coding region of *cdh* cDNA cohesive for amylase terminator.
TaT-cdhHF	GCTGGGGGTCCTTGAAGGGTGGAGAGTATA	Upstream primer for amplification of amylase terminator cohesive for complete coding region of *cdh* cDNA.
cdhH-TaT	TCTCCACCCTTCAGGTAGTGGTAGTG	Downstream primer for amplification of the heme domain-encoding region of *cdh* cDNA cohesive for amylase terminator.
TaT-cdhH	ACTACCACTACCTGAAGGGTGGAGAG	Upstream primer for amplification of amylase terminator cohesive for the heme domain-encoding region of *cdh* cDNA.
TaP-cdhF	CGTAGGACCGGTAGCCAAAGCAGG	Downstream primer for amplification of amylase promoter cohesive for the flavin domain-encoding region of *cdh* cDNA.
cdhF-TaP	CCTGCTTTGGCTACCGGTCCTACG	Upstream primer for amplification of the flavin domain-encoding region of *cdh* cDNA cohesive for amylase promoter.

Table 4.1. Primers used to amplify products used for SOE-PCR for the three CDH-encoding constructs.

The Taka-amylase promoter and terminator amplifications used *A. oryzae* genomic DNA (100 ng) as template. The reaction mixture contained 200 μM each dNTP (Boehringer-Mannheim), 25 pmol each primer, 1x *Pfu* polymerase buffer (Stratagene), and 1.9 U *Pfu* polymerase (Stratagene) in a 100 μL reaction volume. For most of the amplifications, cycling conditions were as follows: 94°C, 5 min followed by 5 cycles of 94°C, 1 min; 57°C, 30s; and 72°C, 1.5 min; then 35 cycles of 94°C, 30s; 57°C, 30s; and 72°C, 1.5 min; then 1 cycle of 72°C, 20 min. The primers for the *lcc*IV-cohesive amylase promoter and terminator amplifications required an annealing temperature of 52°C.

Primer code	Primer sequence (5'-3')	Comment
TaP-lccI	CGGCCCGATGGCAGCCAAAGCAGG	Downstream primer for amplification of amylase promoter cohesive for *lcc*I cDNA.
lccI-TaP	CCTGCTTTGGCTGCCATCGGGCCG	Upstream primer for amplification of *lcc*I cDNA cohesive for amylase promoter.
lccI-TaT	CTCCACCCTTCACTGGTTAGCCTC	Downstream primer for amplification of *lcc*I cDNA cohesive for amylase terminator.
TaT-lccI	GAGGCTAACCAGTGAAGGGTGGAG	Upstream primer for amplification of amylase terminator cohesive for *lcc*I cDNA.
TaP-lccIV	GGGCCCAATGGCAGCCAAAGCAGG	Downstream primer for amplification of amylase promoter cohesive for *lcc*IV cDNA.
lccIV-TaP	CACCTGCTTTGGCTGCCATTGGGCCCGT	Upstream primer for amplification of *lcc*IV cDNA cohesive for amylase promoter.
lccIV-TaT	TCTCCACCCTTCAGAGGTCGGACGAG	Downstream primer for amplification of *lcc*IV cDNA cohesive for amylase terminator.
TaT-lccIV	TCGTCCGACCTCTGAAGGGTGGAG	Upstream primer for amplification of amylase terminator cohesive for *lcc*IV cDNA.

Table 4.2. Primers used to amplify products used for SOE-PCR for the two laccase-encoding constructs.

For the cDNA amplifications, template DNA was as follows: CDH: plasmid pMB-cdh514, which was isolated by RT-PCR from *T. versicolor* mRNA (Chapter 3);

laccase: lccI, plasmid pBK117; lccIV, plasmid pBK116. The laccase cDNAs were isolated by Ong et al. (230) from the *T. versicolor* cDNA library prepared as described in Appendix 1. Aliquots containing 100 ng of plasmid were used in the cDNA amplifications. The same conditions were used as described above, except that the extension time was increased to 5 min. For amplification of the complete coding region of the *cdh* cDNA an annealing temperature of 65°C was used.

After amplification, a 10 µL aliquot of each reaction was analyzed by agarose gel electrophoresis (1% agarose-TAE). Products greater than 500 bp in length were purified from agarose gels using Gene Clean (Bio101), while smaller products (Taka-amylase terminator amplifications) were concentrated and washed using QiaQuick affinity columns (Qiagen). Aliquots of each purified PCR product were again analyzed by agarose gel electrophoresis as above with ethidium bromide (5 µg/mL) in the gel and running buffer. The bands were visualized under u.v. light and photographed using a digital camera. The amount of DNA in each band was calculated by determining its fluorescence intensity *vs.* known standards (Boehringer-Mannheim molecular weight marker sets III/VI).

Construct	Primer pairs used for product		
	Amylase promoter	cDNA	Amylase terminator
Tam-cdhHF	L22/TaP-cdhHF	cdhHF-TaP/cdhHF-TaT	TaT-cdhHF/L26
Tam-cdhH[1]	L22/TaP-cdhHF	cdhHF-TaP/cdhH-TaT	TaT-cdhH/L26
Tam-cdhF[2]	L22/TaP-cdhF	cdhF-TaP/cdhHF-TaT	TaT-cdhHF/L26
Tam-lccI	L22/TaP-lccI	lccI-TaP/lccI-TaT	TaT-lccI/L26
Tam-lccIV	L22/TaP-lccIV	lccIV-TaP/lccIV-TaT	TaT-lccIV/L26

[1]The cDNA portions of constructs Tam-cdhHF and Tam-cdhH are identical at the 5' end.
[2]The cDNA portions of constructs Tam-cdhHF and Tam-cdhF are identical at the 3' end.

Table 4.3. Primer pairs used for amplification of products to be spliced by SOE-PCR. Primer codes are the same as in Tables 4.1 and 4.2.

4.3.4. SOE-PCR

For the splicing reactions, equimolar amounts of PCR products to be spliced were added to the reaction. The molarity of each PCR product was determined based upon its known size and its calculated concentration. The total amount of DNA in each splicing reaction was 100 ng. The reaction (100 µL total volume) was prepared as two separate 50 µL reaction mixtures: mix 1 contained dNTPs (350 µM final concentration) and 100 ng DNA, while mix 2 contained ExPand polymerase buffer (Boehringer-Mannheim; 1x final concentration) and 2.6 U ExPand enzyme mix (Boehringer-Mannheim). The thermocycler was heated to the denaturing temperature, then mix 1 and mix 2 were combined on ice and quickly placed in the thermocycler. Conditions for SOE-PCR: 94°C, 2 min; followed by 5 cycles of 94°C, 10 s; 60°C, 30 s; and 68°C, 3 min. After these 5 cycles, primers were added to a final concentration of 600 nM, then cycling proceeded as follows: 5 cycles of 94°C, 10 s; 60°C, 30 s; and 68°C, 3 min; then 20 cycles of 94°C, 10 s; 60°C, 30 s; and 68°C, 6 min. Products of SOE-PCR were analyzed by agarose gel electrophoresis. Bands of the predicted size were gel purified using Gene Clean, then verified by re-amplification using primers L22 and L26 (Table 4.1). The re-amplification used 0.2 mM dNTPs, 25 pmol each primer, 5 µL of the putative SOE product, 1x *Pfu* polymerase buffer, and 1.9 U *Pfu* polymerase. Cycling conditions: 94°C, 5 min; followed by 5 cycles of 94°C, 1 min; 60°C, 30 s; and 72°C, 6 min; then 35 cycles of 94°C, 30 s; 60°C, 30 s; and 72°C, 6 min. A final 20 min incubation at 72°C was performed. SOE-PCR products which were successfully re-amplified in this reaction were selected for cloning.

The construct Tam-cdhHF, which contained the full coding region of *cdh*, was constructed in several steps (see Figure 4.2): A PCR primer, cdh1695 (5'-TGCTCTCCAGAAGTTCAG-3') was designed to pair with primer cdhHF-TaP (Table 4.1). PCR with these primers proceeded as follows: 0.2 mM dNTPs, 100 ng pMB-cdh514, 25 pmol each primer, 1x *Pfu* buffer, and 1.9U *Pfu* polymerase were mixed in a 100 µL final volume. Cycling conditions were the same as described for the other cDNA amplifications, except that an annealing temperature of 65°C and an extension time of 4

min were used. This was designed to give a product of 1.7 kb cohesive for Taka-amylase promoter DNA which encoded the heme domain and part of the flavin domain of CDH. The PCR product included a unique *Nru*I restriction site corresponding to an *Nru*I site contained within construct Tam-cdhF. This PCR product was spliced to the Taka-amylase promoter DNA as described above. The spliced product was cloned into plasmid pMOSBlue (see below) and digested with *Hin*dIII and *Nru*I. A product of 4.8 kb consisting of pMOSBlue with an insert of Taka-amylase promoter spliced to the 5' end of the *cdh* cDNA up to the *Nru*I site was then ligated to a 1.25 kb *Hin*dIII/*Nru*I fragment of cloned construct Tam-cdhF. The latter fragment encoded the 3' end of the *cdh* cDNA from the *Nru*I site spliced to the Taka-amylase terminator DNA. Products of this ligation were transformed into *E. coli* MOSBlue.

4.3.5. Cloning the spliced products

The termini of the SOE-PCR products were repaired and adenylated by incubation in a solution containing 0.2 mM dNTPs, 1x *Taq* polymerase buffer (Boehringer-Mannheim), and 2.5 U *Taq* polymerase (Boehringer-Mannheim). The reaction proceeded at 72°C for 60 min, then the spliced product was washed and concentrated using a QiaQuick column. Most of the spliced products were then ligated to the pMOSBlue T-vector according to the manufacturers' instructions and a 1 μL aliquot of the ligation reaction mixture was used to transform competent *E. coli* MOSBlue cells. Transformed cells were selected on LB plates containing 50 μg/mL ampicillin, 15 μg/mL tetracycline, 20 μg/mL isopropylthio-β-D-galactoside (IPTG), and 32 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal).

The laccase constructs, Tam-lccI and Tam-lccIV, were cloned into a T-vector prepared from pBluescript KS^+ (pBS-T) according to the procedure described by Hadjeb and Berkowitz (134) (see section 5.3.5). The ligation reaction contained 1x ligase buffer (Promega), 3 U of T4 DNA ligase (Promega), and a 3-fold molar excess of spliced product DNA over pBS-T DNA. The reaction was incubated at 16°C overnight, then

used to transform competent *E. coli* JM109. Transformants were selected on LB plates as above, except that no tetracycline was used.

Plasmid DNA obtained from the transformation reactions was analyzed by restriction enzyme digestion and agarose gel electrophoresis. Plasmids containing inserts of the appropriate size and restriction pattern were verified by re-amplification using primers L22 and L26 as described above.

4.3.6. Transformation of *Aspergillus oryzae*

Protoplasts were prepared from *A. oryzae* as follows: flasks containing 100 mL of LB with 200 μM EDTA were inoculated with a suspension of conidia to a final concentration of 1.7 x 10^6 conidia/mL. Flasks were incubated at 30°C 16 h, then germinating conidia were harvested by filtration through cheesecloth and washed with isotonic solution (0.8 M $MgSO_4$/50 mM malate pH 5.8). The washed mycelia were transferred to a 50 mL polypropylene tube (2 g wet weight mycelium/tube), and suspended in 6 mL of 10 mg/mL Novozyme 234 (InterSpex Products Inc., Foster City, CA, USA) in isotonic solution. The tubes were incubated in a horizontal position at 30°C with gentle shaking for several h and observed microscopically at regular intervals. When large amounts of protoplasts were evident, the solution was transferred to a glass homogenizer and the plunger was gently pressed down to liberate trapped protoplasts from the mycelium. The solution was then transferred to a 15 mL polypropylene tube, and 1 vol 0.6 M sorbitol/100 mM Tris-Cl pH 7 was layered on top. The solution was centrifuged at 2500 x g (Beckman RC-3 centrifuge, HG-4L rotor) for 30 min, and the protoplasts were aspirated from the $MgSO_4$/sorbitol interface with a Pasteur pipette. The protoplasts were washed twice with 1M sorbitol/10 mM Tris pH 7.5, and finally suspended in 1.2 M sorbitol/50 mM $CaCl_2$/10 mM Tris pH 7.5 to a final concentration of 10^8-10^9/mL. The protoplasts were stored on ice and used within 1 week.

Transformations were performed essentially according to the procedures of Yelton et al. (332). Briefly, 0.1 mL of the protoplast suspension was incubated with co-

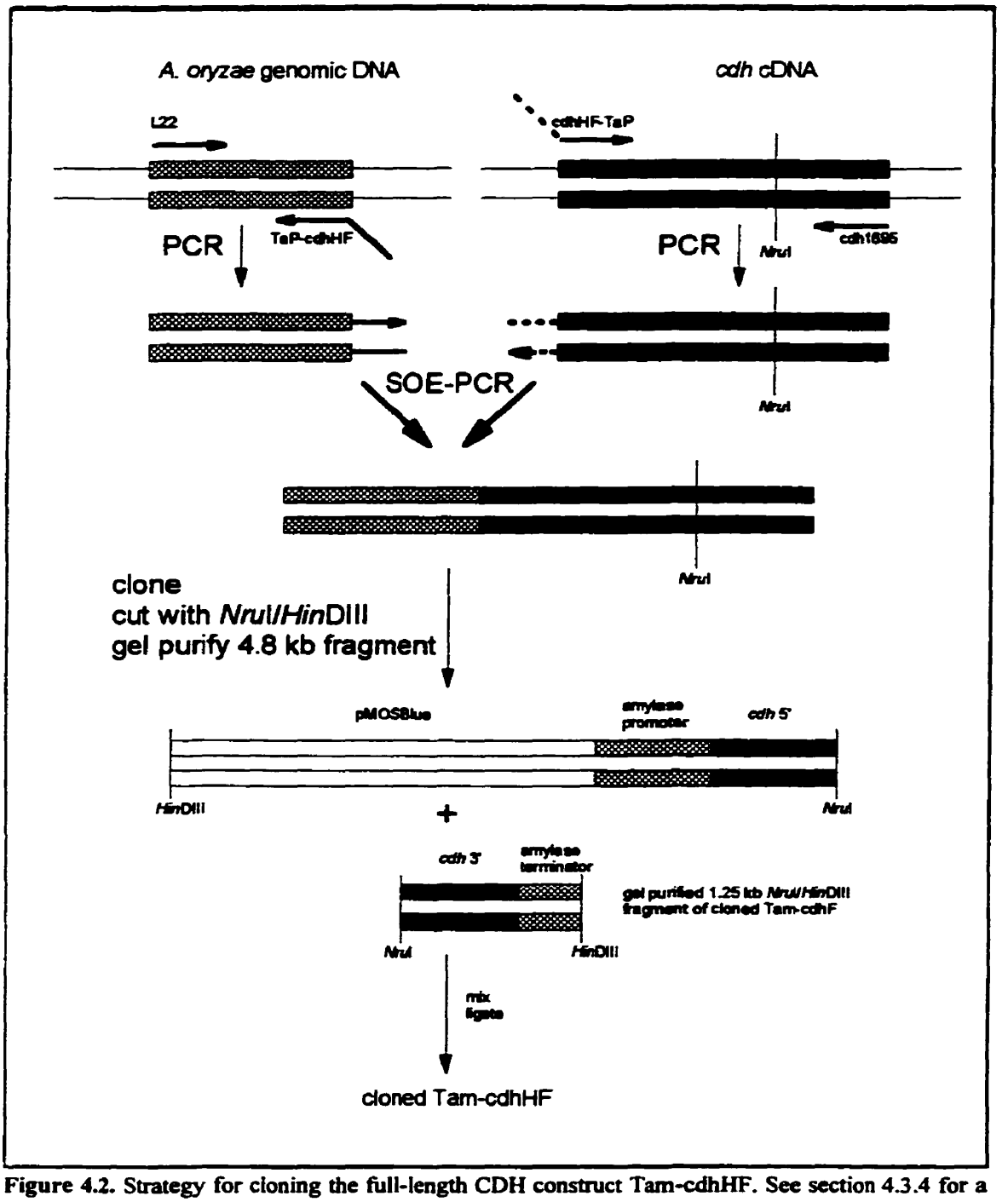


Figure 4.2. Strategy for cloning the full-length CDH construct Tam-cdhHF. See section 4.3.4 for a description.

transforming DNA (5 μg each of plasmid p3SR2 and the expression construct) for 25 min at room temperature, then 1.25 mL of PEG 3350/10 mM $CaCl_2$/10 mM Tris-Cl pH 7.5 was added and the suspension was incubated at room temperature for another 20

min. Protoplasts were collected by centrifugation (8000 x g, 5 min, Hermle Z230MR microcentrifuge), and resuspended in 0.5 mL STC buffer (1.2 M sorbitol/10 mM $CaCl_2$/10 mM Tris-Cl pH 7.5). Transformants were selected on minimal medium (77) with 10 mM acetamide, 20 mM CsCl, and 1.0 M sucrose, as described by Christensen et al. (69).

4.3.7. Analysis of transformants

Colonies of *A. oryzae* were transferred from the initial selection plates to plates containing minimal medium (77) with 0.5% glucose, 10 mM acetamide, and 20 mM CsCl. Strains which grew on these plates were maintained thereafter on YM plates (3 g malt extract, 3 g yeast extract, 5 g peptone, and 10 g glucose per liter).

The production of functional recombinant CDH was analyzed by subculturing transformants to minimal plates with 0.15% glutamine, 0.5% maltose, 0.2 mM cellobiose, and 0.1 vol of $ABTS^+$ radical solution. The $ABTS^+$ radical solution was prepared by oxidizing 10 mM ABTS with laccase until the solution turned dark green, then removing the laccase by ultrafiltration (Amicon YM-10 membranes). Production of functional CDH was inferred by the appearance of decolorized zones of reduced ABTS around the growing colonies. Recombinant laccase was assayed by growing transformants on minimal plates with 0.15% glutamine, 0.5% maltose, and 10 mM ABTS and observing the plates for green halos of oxidized $ABTS^+$ surrounding the colonies.

Genomic DNA was prepared from transformants according to the procedure of Wendland et al. (322). An aliquot of approximately 100 - 500 ng of the DNA was used in a PCR to amplify the expression construct. For amplification of the Tam-cdhHF constuct, primers cdh1695 and cdhHF-TaP were used, while the Tam-cdhF construct was amplified using primers cdhF-TaP and cdhHF-TaT. The PCRs contained 0.2 mM dNTPs, 25 pmol each primer, 1x *Taq* extender buffer (Stratagene), 1 U *Taq* extender (Stratagene), and 1 U *Taq* polymerase (Pharmacia) in a 100 µL final volume. A positive control for both of these amplifications contained 100 ng of pMB-cdh514 as template,

and a negative control contained 100 ng of untransformed *A. oryzae* genomic DNA. Cycling conditions: 94°C, 5 min; then 5 cycles of 94°C, 1 min; 60°C, 30 s; and 72°C, 2.5 min; followed by 35 cycles of 94°C, 30 s; 60°C, 30 s; and 72°C, 2.5 min.

4.4. Results

4.4.1. Gene splicing by SOE-PCR

Three separate expression constructs were isolated using *cdh* cDNA as a template: Tam-cdhHF, which encompassed the complete coding region of CDH (amino acids (aa) 1-749); Tam-cdhH, which comprised the heme domain of CDH (aa 1-201); and Tam-cdhF, which encoded the flavin domain of CDH (aa 202-749). The cDNA used for constructs Tam-cdhHF and Tam-cdhH shared a common 5' end, which corresponded to aa #1 of the mature CDH protein (Chapter 3). The *cdh* cDNA which was isolated by RT-PCR from *T. versicolor* mRNA is missing the DNA encoding the final four amino acids (AGGP) and stop codon (Chapter 3). Primers cdhHF-TaP (which was used to amplify cDNAs cdhHF and cdhF), and TaT-cdhHF (which was used to amplify amylase terminator DNA cohesive for cdhHF and cdhF cDNAs) were therefore designed to encode these amino acids. This altered the splice junction between the Taka-amylase terminator DNA and the cdhHF and cdhF cDNAs by giving only a 15 nt overlap between the two molecules; other SOE-PCRs had longer overlaps (Figure 4.3). Nevertheless, for the construct Tam-cdhF, the splicing reaction worked well with the shorter overlap (Figure 4.4). Thus, it was possible to repair the 3' end of the *cdh* cDNA in order to improve the chances of obtaining a functional construct.

The two laccase constructs, Tam-lccI and Tam-lccIV, were made using cDNAs isolated from the *T. versicolor* cDNA library (Appendix 1) by Ong et al. (230). Both of these cDNAs are full-length and include the 5' and 3' untranslated regions along with the signal peptide-encoding regions. The PCR primers that were used to amplify laccase cDNAs cohesive for *A. oryzae* Taka-amylase promoter and terminator DNAs were designed to yield a product starting at amino acid #1 of the mature protein (excluding the 5' UTR and laccase signal peptide) and ending at the final amino acid (excluding the 3'

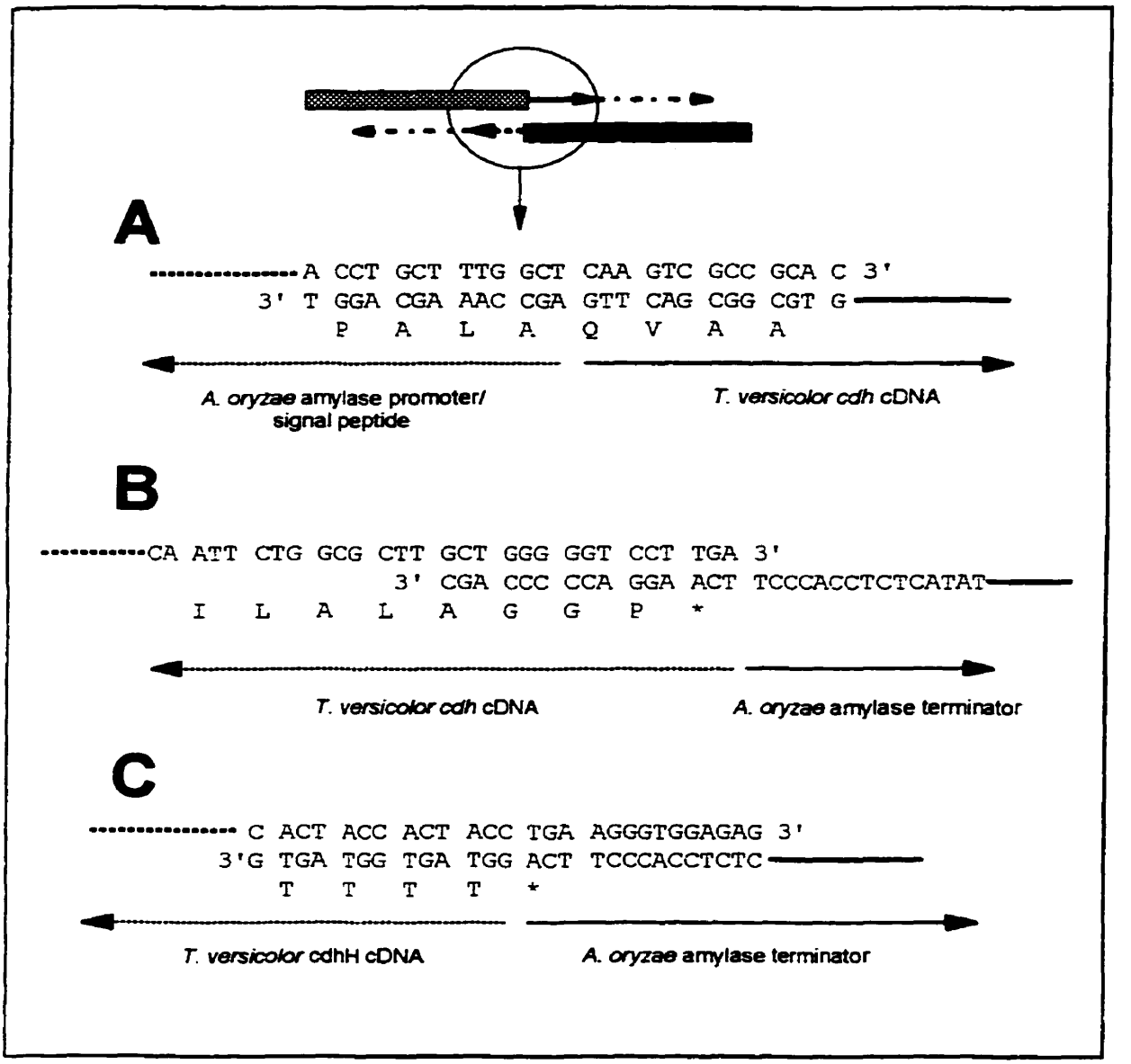


Figure 4.3. Detail of the splice junctions for the CDH constructs. A. Splice junction between the amylase promoter and the 5' end of *cdh* cDNA. B. Splice junction at the 3' end of the *cdh* cDNA depicting the repair of the 3' end of the *cdh* cDNA as it is spliced to the amylase terminator. C. Splice junction between the 3' end of the cdhH cDNA and the amylase terminator. The final amino acid of this construct (T) corresponds to amino acid 201 of the mature CDH protein (Chapter 3).

UTR and stop codon). These products were fused in-frame to PCR products encoding the *A. oryzae* Taka-amylase promoter and signal peptide (5' end) and stop codon/terminator DNA (3' end) in order to encourage proper heterologous mRNA and protein processing in *A. oryzae*. Both of these constructs were successfully isolated and cloned (Figure 4.5).

In the splicing reactions, there was an inverse correlation between cDNA length and the ease of the SOE-PCR. For the cdhH cDNA, which was 603 nt, a three-way splicing reaction including the amylase promoter, cDNA, and amylase terminator DNAs worked easily on the first attempt (Figure 4.4). However, three-way splicing reactions did not work at all for any of the other, longer cDNAs (not shown). For the cdhF, lccI, and lccIV cDNAs, all of which were around 1.5 kb in length, it was necessary to first fuse the amylase promoter and cDNA, purify the spliced product, then perform a second two-way splicing reaction with the amylase terminator DNA (Figures 4.4, 4.5). The cdhHF cDNA, which was 2.2 kb in length, did not work in splicing reactions despite repeated attempts (not shown). Since direct fusion by SOE-PCR of this construct was not feasible, we devised an alternative strategy (Figure 4.2). The 5' end of the *cdh* cDNA was successfully amplified using primer pair cdhHF-TaP and cdh1695 and the 1.7 kb product was fused to the amylase promoter (Figure 4.6). The fused product was cloned, digested with *Nru*I and *Hin*dIII, and ligated to the *Nru*I/*Hin*dIII-digested fragment of construct Tam-cdhF containing the 3' end of the *cdh* cDNA and the amylase terminator (Figure 4.6). The structure of all five cloned constructs were verified by re-amplification using primers L22 and L26, restriction mapping, and by sequencing the ends of the DNA (not shown).

4.4.2. Transformation of *A. oryzae* with expression constructs

Each of the five expression constructs was used in a co-transformation with the *amdS* gene from *Aspergillus nidulans* (p3SR2). Several acetamide-utilizing strains of *A. oryzae* were isolated on the original transformation plates (Table 4.4). However, there was much background growth on the minimal plates, including the "no DNA" control (not shown). The putative transformants that appeared the healthiest were chosen for further analysis.

4.4.3. Analysis of the transformants

Untransformed *A. oryzae* was unable to decolorize the $ABTS^{+}$ radical incorporated into minimal plates. However, four of the putative co-transformants, one

cdhF and three cdhHF, gave large zones of decolorization (not shown). The $ABTS^{+}$ plate assay appeared to be highly sensitive, since short term assays for CDH in the supernatant of liquid cultures of these transformants did not reveal any CDH activity (not shown). Furthermore, clearance zones on $ABTS^{+}$ plates without cellobiose were approximately 70% of the size of the zones on cellobiose-containing plates (not shown). Purified *T. versicolor* CDH was found to be capable of reducing and decolorizing the $ABTS^{+}$ radical using maltose as an electron source when incubation times were increased to overnight (not shown). This maltose oxidation was sufficiently slow compared to cellobiose oxidation that it was not observed in earlier work using shorter term colorimetric assays (Chapter 2). We also observed that the clearance zones appeared after the first overnight incubation of the *A. oryzae* strains, but disappeared thereafter. None of the *A. oryzae* strains co-transformed with constructs Tam-lccI or Tam-lccIV showed apparent laccase activity on minimal plates with ABTS.

Co-transformed construct	Number of strains growing on minimal plates with acetamide
Tam-cdhHF	13
Tam-cdhF	14
Tam-cdhH	11
Tam-lccI	8
Tam-lccIV	6

Table 4.4. Co-transformation of *A. oryzae* with expression constructs and p3SR2.

Total genomic DNA was isolated from several of the transformants. One of the strains, the cdhF co-transformant which produced clearance zones on the $ABTS^{+}$ plates, was shown to contain the cdhF expression construct (not shown). Curiously, the other three decolorizing strains did not give a band with primers specific for the construct Tam-cdhHF.

4.5. Discussion

Gene splicing by overlap extension (SOE-PCR) as described by Horton et al. (148) is an elegant application of the polymerase chain reaction which enables the precise fusion of DNA fragments without the need for conveniently located restriction sites. The power of this technique lies in the precise control of the splice junction it affords, which easily allows in-frame fusions to be made. We have applied this technique to the construction of vectors for the expression of *T. versicolor* proteins in the hyphomycete *A. oryzae*. Although the SOE-PCRs worked well, and enabled the fusion of *A. oryzae* Taka-amylase control regions to the cDNAs of *T. versicolor* CDH and laccase, we found an inverse correlation between the size of the cDNA and the facility of the SOE-PCR. A molar ratio very close to 1:1 of the products to be spliced appeared to be absolutely critical for this technique to work. In the present application, extreme size differences between the PCR products to be spliced may have exaggerated small errors in the determination of the concentration of the DNAs and made a direct 3-way splicing difficult. We found that the upper size limit for the cDNA for a two-way splicing reaction with the 0.7 kb promoter was approximately 1.7 kb. Thus, if the molar ratio is incorrect, SOE-PCR will not work well. For this reason, the use of a digital camera with software enabling the calculation of DNA concentration from fluorescence intensity was essential to success.

In addition, the choice of thermostable polymerase is important in SOE-PCR. In the present application, we chose *Pfu* polymerase for the amplification of the DNAs to be spliced. This polymerase was chosen due to its high fidelity (unlike *Taq*, it possesses a proofreading capability), and because it lacks the terminal A-transferase activity associated with *Taq* polymerase. The latter feature is important for SOE-PCR, because the template-independent addition of single A residues to the termini of the products may interfere with the subsequent splicing reaction or disrupt the reading frame of the spliced product. For the splicing reactions, we chose the ExPand Long Template enzyme, which is a mixture of *Taq* and *Pwo* polymerases. The *Pwo* polymerase component possesses a proofreading capacity, which makes the fidelity of the ExPand

mixture similar to that of the *Pfu*. The *Pfu* polymerase was tried in the SOE-PCRs and did not function well. The terminal A-transferase activity of the *Taq* component was not undesirable in the splicing reactions, since it facilitated cloning of the spliced products into a T-vector. In fact, the gel purified spliced products were incubated with dNTPs and *Taq* polymerase due precisely to this activity in order to prepare the products for cloning. Thus the judicious choice of thermostable polymerases allowed us to isolate and clone five expression constructs for *T. versicolor* proteins in *A. oryzae*.

CDH is a protein with several potential *in vivo* functions and industrial applications. However, the *T. versicolor* enzyme is difficult to purify in large quantities without extensive degradation (Chapter 2), which greatly hampers the study of the role of the protein in biological delignification. To facilitate such studies, we have constructed vectors for the expression of CDH and of its two domains in *A. oryzae*. We chose to express the domains separately because the catalytic function of the flavin domain is distinct from that of the complete protein (Chapter 2). These vectors should enable the purification of larger quantities of undegraded enzyme than have hitherto been possible from *T. versicolor*. Furthermore, these vectors provide an important tool for the study of structure/function relationships of amino acids within CDH by site-directed mutagenesis. For *T. versicolor* laccase, *ex vivo* delignification processes have been developed (48, 232) which may be of industrial significance, so that the purification of large quantities of enzyme may become necessary.

The hyphomycete *Aspergillus oryzae* is an industrially important fungus which is commonly used as a source of amylase, an enzyme of importance in the food industry. The fungus is a prodigious producer of extracellular amylase when grown on starch or maltose (289). For this reason, we chose the regulatory regions and signal peptide of *A. oryzae* Taka-amylase, which is a well-characterized gene (36, 120, 298, 309). Other groups have used a similar approach. Christensen et al. (69) used an amylase promoter to express an aspartic protease of *Rhizomucor meihei*. In addition, an MnP of *P. chrysosporium* was successfully expressed in *A. oryzae* (295), as was a laccase of

Myceliophthora themophila (32). Although other systems have also been successfully used to express ligninolytic enzymes, such as the yeasts *Pichia pastoris* (164) (M. Brown, unpublished) and *Saccharomyces cerevisiae* (186), and the filamentous ascomycete *Trichoderma reesei* (278), we felt that the *A. oryzae* system offered the most advantages. Transformation of aspergilli has been known for some time, and a number of optimized protocols are available (23, 62, 305, 332). Furthermore, the high level of expression of extracellular fungal proteins attainable by *A. oryzae* (128) made this the system of choice for expression of *T. versicolor* CDH and laccase.

In our initial attempts to transform *A. oryzae* with the expression constructs, we used a phleomycin selection system. These experiments were unsuccessful in that, although strains growing on phleomycin-containing plates were easily obtained, none of these were found to be CDH transformants. Such results have been noted before for *A. oryzae* (246), in spite of the fact that phleomycin resistance selection works well for the related fungi *A. nidulans* (19) and *A. niger* (246). For this reason, we used an acetamide selection system and plasmid p3SR2, which contains the *A. nidulans amdS* gene (151). This plasmid integrates stably into genomic DNA (305) and *amdS* confers the ability to grow on minimal plates containing acetamide as sole nitrogen source. This system has been successfully used for co-transformation in *A. oryzae*, which grows poorly with acetamide as sole nitrogen source (69, 295). However, some background growth on such media is commonly observed (305), which can make selection of $AmdS^+$ colonies difficult. This has been attributed to acetamide degradation by other enzymes produced by the fungus, as well as to nitrogenous impurities in the agar used to solidify the medium (305). In our work, CsCl was incorporated into the minimal medium to suppress this background growth, but a uniform lawn of slowly growing cells was obtained. Alternatively, the background growth by untransformed protoplasts that we observed could be due to leaky expression of an endogenous, functional *A. oryzae amdS* gene (127). We used a relatively uncharacterized strain of *A. oryzae* which had previously been subjected to several rounds of u.v. mutagenesis to maximize amylase production. Thus, the undesirable background growth that we observed may have been due to weak

expression of endogenous *amdS*. However, the presence of nitrogenous impurities in the agar is a more likely explanation, since the strain used grew very poorly on minimal plates solidified with electrophoresis-grade agarose instead of standard bacteriological agar (which is a relatively crude mixture of agarose and agaropectins). The high levels of background growth may have interfered with the identification of true transformants in certain cases.

Using similar amounts of each transforming plasmid, co-transformation frequencies ranging from 4% (62) to 100% (69) have been reported. We were able to unequivocally demonstrate *cdh* co-transformation (by PCR) for only 1 transformant out of 27 cdhF and cdhHF transformants analyzed (Table 4.4), which corresponds to a theoretical co-transformation frequency of 3.7%. However, since the chances of mis-identifying transformants were quite high due to the background growth (see above), the true co-transformation frequency cannot be calculated. The laccase transformants (none of which produced functional laccase), and the cdhH transformants (which would not be expected to produce an active protein) were not analyzed by PCR so the proportion of co-transformants is unknown. Furthermore, we attempted to detect the expression of nonfunctional CDH and laccase using polyclonal rabbit antibodies, but these experiments were inconclusive because the antibodies reacted with an unknown factor in the supernatants of liquid cultures. Supernatants from all strains, even untransformed *A. oryzae*, reacted with the antibodies used.

The acetamide selection system, as used in these experiments, was relatively unsuccessful, although one true co-transformant which may have secreted functional recombinant CDH was identified. Other selection systems have been used for aspergilli, such as auxotrophic complementation using *trp*C (332), *nia*D (62), and *pyr*G (23, 32). However, we were unable to use any of these selection systems due to the lack of an appropriate *A. oryzae* mutant. Improvements upon the acetamide system as used here will be necessary in order to obtain successful transformation with the expression constructs for laccase and CDH. For example, agarose should be used to solidify the selection medium, and a more characterized strain of *A. oryzae*, such as ATCC 11488

(295), should be used. Once successful high level heterologous expression is achieved, a powerful tool for the study of cellobiose dehydrogenase will have been acquired.

4.6. Conclusions

(1) Using the method of gene splicing by overlap extension (SOE-PCR), the *A. oryzae* Taka-amylase promoter and terminator DNAs were fused to cDNAs encoding *T. versicolor* CDH and laccase. These were designed as vectors for the heterologous expression of these proteins in *A. oryzae*.

(2) The molar ratio of DNA molecules to be spliced by SOE-PCR significantly affects the success of the reaction. A molar ratio close to 1:1 is important in order for the reaction to proceed.

(3) Several putative transformants of *A. oryzae* were obtained using growth on minimal medium with acetamide as sole nitrogen source for selection. However, the selection was plagued by high background growth, which may have interfered with the identification of true transformants.

(4) One true co-transformant was identified, which contained the cdhF expression construct. This strain may have produced low levels of functional recombinant CDH (flavin domain), as shown by decolorization in a plate assay with $ABTS^{+}$ radical. Three other strains were identified which produced clearance zones on such plates, but PCR analysis of the genomic DNA of these strains did not reveal the presence of the expression construct.

(5) A low co-transformation frequency for the two plasmids was observed. This frequency may be improved by switching to a more characterized strain of *A. oryzae* and by using agarose to solidify the selection medium to prevent the outgrowth of untransformed protoplasts using nitrogenous impurities present in regular agar.

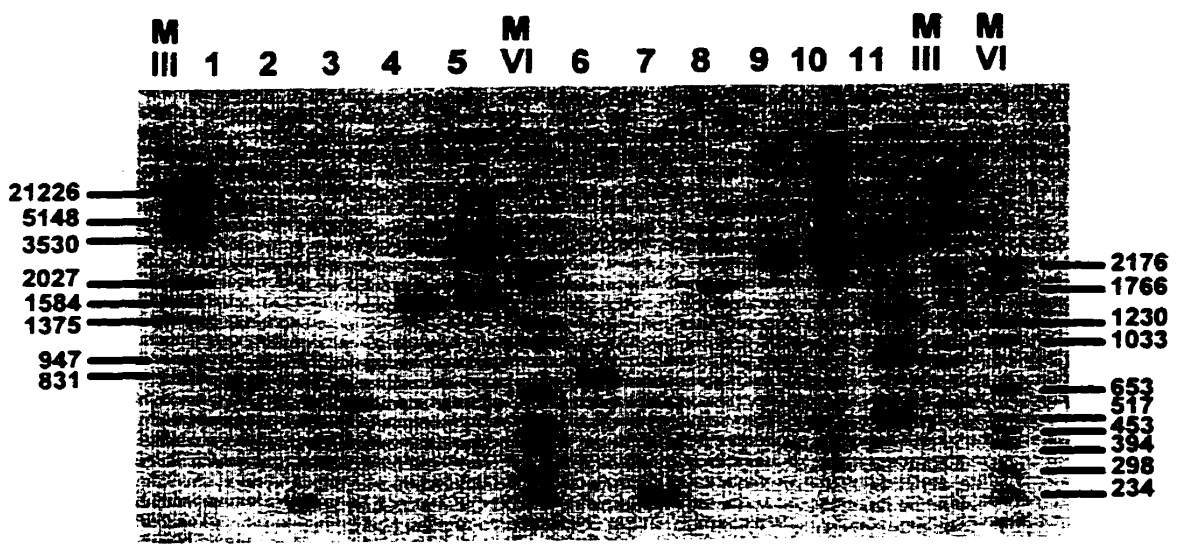


Figure 4.4. SOE-PCR of the CDH heme domain (cdhH) and flavin domain (cdhF) to the *A. oryzae* Taka-amylase promoter and terminator DNA and the cloning of the spliced products. Lanes: 1, cdhH-cohesive amylase promoter amplification; 2, cdhH-cohesive amylase terminator amplification; 3, amylase-cohesive cdhH cDNA amplification; 4, product of the three-way splicing reaction; 5, Cloned Tam-cdhH cut with *Bam*HI; 6, cdhF-cohesive amylase promoter amplification; 7, cdhF-cohesive amylase terminator amplification; 8, amylase-cohesive cdhF cDNA; 9, product of the two-way promoter/cdhF cDNA splicing reaction; 10, product of the promoter-cdhF/terminator splicing reaction; 11, Cloned Tam-cdhF cut with *Xba*I/*Pst*I. The sizes of the molecular weight markers (in bp) is shown.

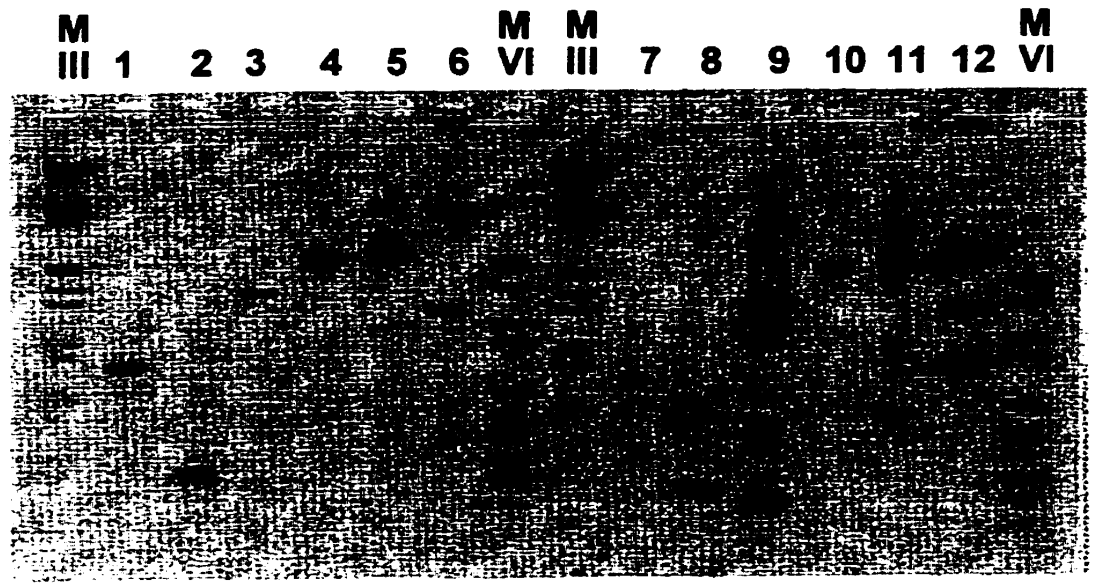


Figure 4.5. SOE-PCR of the laccase cDNAs to the *A. oryzae* Taka-amylase promoter and terminator DNA and the cloning of the spliced products. Lanes: 1, lccI-cohesive amylase promoter amplification; 2, lccI-cohesive amylase terminator amplification; 3, amylase-cohesive lccI cDNA amplification; 4, product of the two-way promoter/lccI cDNA splicing reaction; 5, product of the promoter-lccI/terminator splicing reaction; 6, Cloned Tam-lccI cut with *Bam*HI; 7, lccIV-cohesive amylase promoter amplification; 8, lccIV-cohesive amylase terminator amplification; 9, amylase-cohesive lccIV cDNA amplification; 10, product of the two-way promoter/lccIV cDNA splicing reaction; 11, product of the promoter-lccIV/terminator splicing reaction; 12, Cloned Tam-lccIV cut with *Bam*HI. Markers are the same as in Figure 4.4.

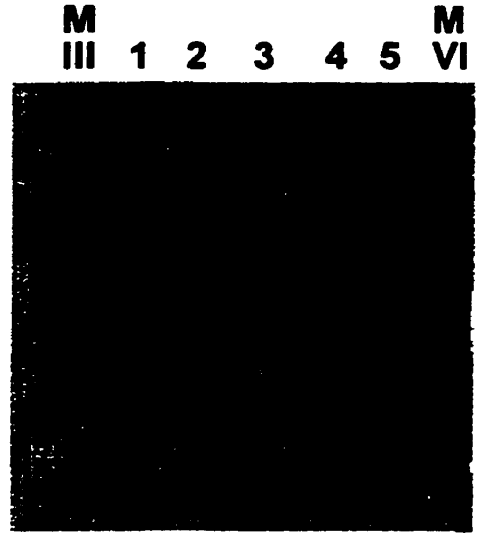


Figure 4.6. Construction of the Tam-cdhHF expression vector. Lanes: 1, cdhHF-cohesive amylase promoter amplification; 2, amplification of the 5' end of the *cdh* cDNA using primers cdhHF-TaP and cdh 1695 (cdh5'); 3, spliced product of amylase promoter and cdh5' cloned into pMOSBlue and cut with *Nru*I and *Hin*dIII; 4, NruI/*Hin*dIII Tam-cdhHF fragment (cdh3' plus terminator); 5, Ligation product of lanes 3 and 4 cut with *Xba*I and *Pst*I, corresponding to cloned Tam-cdhHF. Markers are the same as in Figure 4.4.

PREFACE TO CHAPTER 5

The high degree of similarity at the amino acid level between *T. versicolor* CDH and *P. chrysosporium* CDH (Chapter 3) may partially reflect the relatedness of the two fungi. CDH activity has been reported in a wide range of fungi, both white-rotters and others (6). The availability of a large number of CDH sequences from disparate organisms would be helpful in the determination of which amino acids are critical to CDH function. Furthermore, the screening of uncharacterized organisms for CDH activity can be a painstaking and difficult undertaking, since it is often produced in low amounts and since its activity can be masked by the activity of oxidative enzymes (268). A rapid method for the identification and cloning of *cdh*-like genes from any organism containing such genes was therefore developed. The experiments described in this chapter were performed under the guidance of S. Moukha, and the sequencing of the *P. cinnabarinus cdh* clone was performed commercially.

CHAPTER 5 A PCR-BASED SCREENING METHOD FOR ORGANISMS CONTAINING CELLOBIOSE DEHYDROGENASE *(CDH)*-LIKE GENES

5.1. Abstract

We have developed and tested a method for amplifying cellobiose dehydrogenase (*cdh*)-like genes from any organism having DNA homologous to the *cdh* genes of the basidiomycetes *T. versicolor* or *P. chrysosporium*. The method is based upon the polymerase chain reaction (PCR) using *cdh*-specific primers. Primers which may recognize any homolgous *cdh* were designed using regions of complete conservation of amino acid sequence between the CDHs of the white-rot fungi *T. versicolor* and *P. chrysosporium*. These are the only two CDHs for which complete amino acid sequences are available. Upstream primers hybridized to regions encoding the heme domain of CDH, including those amino acids thought to be involved in complexing the heme iron. Downstream primers were designed to recognize highly conserved regions within the flavin domain of CDH. To test the primers, we used genomic DNA from the white-rot fungus *P. cinnabarinus*, which is known to produce CDH but has a completely uncharacterized *cdh* locus. Eight different primer pairs yielded three PCR products close in size to the control amplification, which used cloned *T. versicolor cdh* as template. Reasons for the failure of certain PCRs included lack of complete conservation of amino acid sequences, differences in codon choice, and nonoptimal PCR conditions. The PCR products which were close to the control size were cloned and one of these, a 1.8-kb product, was completely sequenced. The PCR product was highly homologous to both *T. versicolor* and *P. chrysosporium cdh*, and contained eight putative introns. The cloned product encoded a predicted protein fragment with 88% homology to the corresponding fragment of *T. versicolor* CDH and 84% homology to the corresponding portion of *P. chrysosporium* CDH. We therefore conclude that one of the primer pairs successfully amplified a previously uncharacterized *cdh*, and that the primer sets may be tested with other, more distantly related fungi.

5.2. Introduction

Organisms which degrade lignin and cellulose do so by secreting a range of extracellular enzymes which together account for the complete degradation of these wood polymers. The degradation of cellulose appears to be mediated by a series of cellulase enzymes which hydrolytically cleave cellulose chains at the end of a chain (exo-1,4-β-glucanases, or cellobiohydrolases), or in the middle of a chain (endo-1,4-β-glucanases). In addition, β-glucosidases degrade cellobiose and cellodextrins, the products of the endo- and exo-cellulases, to glucose (29, 119). In contrast to the hydrolytic enzymes involved in cellulose degradation, lignin depolymerization occurs through the action of a series of extracellular oxidoreductases, such as manganese peroxidase (MnP), lignin peroxidase, (LiP), and laccase. These enzymes oxidize lignin subunits via 1-electron abstractions, probably using a low molecular weight mediator or manganese complex (49, 97), and this oxidation can lead to non-enzymatic fragmentation reactions (182).

Cellobiose dehydrogenase (CDH) is an extracellular oxidoreductase produced by a wide range of lignocellulolytic organisms (6). CDH contains both a protoporphyrin-IX-based heme and a flavin (FAD) prosthetic group (269, 325), and is organized into two domains: an amino terminal heme domain and a carboxy terminal flavin domain (196). The two domains are connected by a proteolytically sensitive hinge region (100, 196) which is rich in hydroxyamino acids (201, 249), similar to linkers found in cellulases (119). CDH also binds to cellulose, as do most cellulases, and appears to contain a structurally unique cellulose-binding domain (CBD) (144). The enzyme oxidizes cellobiose and cellodextrins while reducing a wide range of substrates, including quinones, metal ions, organic radicals, dyes, and other chromophores (142, 269). CDH is thought to be involved in the degradation of both cellulose and lignin. For example, by reducing Fe(III) to Fe(II), CDH can promote and sustain a Fenton's reaction in which the latter ion reacts with H_2O_2 to produce hydoxyl radicals (•OH), capable of abstracting an electron from and cleaving both lignin and cellulose (6, 188, 285, 300, 328). Furthermore, CDH is known to reduce phenoxy radicals generated by MnP and

laccase-mediated lignin oxidation; this reduction can decrease the tendency towards polymerization of these radicals and may result in a net depolymerization (12, 270).

CDH activity has been reported in a large number of white-rot fungi (10), in one brown-rot fungus (282), and in several other fungi which are cellulolytic but not ligninolytic (76, 89). Moreover, CDH has been purified and characterized from several fungi (27, 64, 149, 269, 275, 325). However, complete CDH amino acid sequences have been reported for only two fungi: *T. versicolor* (Chapter 3) and *P. chrysosporium* (201, 249). The two proteins are highly homologous to one another and display similar domain structures. However, given the relatively close taxonomic relationship between these two organisms, it is difficult to determine exactly which amino acids are critical for CDH function based on comparison of the two sequences. A rapid method for determining further CDH sequences from fungi known to produce CDH or from uncharacterized organisms would provide a useful tool with which to study structural differences between the various CDHs. Herein we describe a PCR-based method and show its utility by amplifying, cloning, and sequencing a fragment of a *cdh*-like gene from the white-rot fungus *P. cinnabarinus*. This method is similar to that described by D'Souza et al. (95) for the amplification of laccase-like sequences from white- and brown-rot fungi.

5.3. Materials and Methods

5.3.1. Strains and plasmids

The fungal strain used in these studies, *P. cinnabarinus* I-937 (CNCM, Institut Pasteur, Paris, France), is a wild strain originally isolated in Paris. Transformation-competent *Escherichia coli* JM109 was purchased from Promega (Charbonnières, France). Plasmid pBluescript was obtained from Stratagene (La Jolla, California). Restriction enzymes, *Taq* polymerase, and dNTPs were from Boeheringer-Mannheim (Meylan, France).

5.3.2. Isolation of genomic DNA

P. cinnabarinus I-937 total genomic DNA was isolated according to standard procedures (247). The DNA was diluted to a concentration of 0.1 mg/mL in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA) and stored at -20°C.

5.3.3. PCR primer design

The predicted amino acid sequences of *P. chrysosporium* (201, 249) and *T. versicolor* (Chapter 3) CDH were compared in order to determine short stretches of complete conservation of amino acid sequences between the two fungi. For primer design, stretches of 6 amino acids were chosen (corresponding to a primer length of 18 nucleotides). The upstream primers were based on conserved regions within the heme domain of CDH, and the downstream primers were based on the flavin domain. The codons used by both fungi to encode the amino acid sequences were then determined, and degenerate oligonucleotide primers were designed based on the respective codon choices. Possible primers which were degenerate in the last two (3') nucleotides were excluded. The primers ultimately designed, and the corresponding amino acid sequences, are shown in Table 5.1. Primers were synthesized by Eurogentec (Seraing, Belgium).

A. Heme domain — Upstream primers		
protein sequence	NSTHWK	ALGGAM
function	Heme binding (H residue)	Heme binding (M residue)
codons: *T. versicolor* *P. chrysosporium*	 AAC TCC ACG CAC TGG AAG AAC TCA ACC CAC TGG AAG	 GCT CTC GGA GGA GCC ATG GCC CTC GGT GGC GCC ATG
primer sequence (5'-3')	AAC TCM ACS CAC TGG AAG	GCY CTC GGW GGM GCC ATG
primer code	1-U	2-U

B. Flavin domain — Downstream primers		
protein sequence	VLLLER	VGAGPG
function	Flavin binding	Flavin binding (GXGXXG motif)
codons: *T. versicolor* *P. chrysosporium*	GTC CTA CTT CTC GAG CGT GTT CTC CTT CTC GAG CGC	GTC GGC GCT GGC CCT GGT GTC GGT GCT GGT CCT GGC
primer sequence (5'-3')	RCG CTC GAG AAG KAG RAC	RCC AGG RCC AGC RCC GAC
primer code	1-D	2-D
protein sequence	SEAGKK	NWADVW
function	Flavin binding?	Unknown
codons: *T. versicolor* *P. chrysosporium*	TCT GAA GCT GGC AAG AAA TCG GAG GCT GGC AAG AAG	AAC TGG GCT GAT GTG TGG AAC TGG GCT GAC GTC TGG
primer sequence (5'-3')	YTT CTT GCC AGC YTC MGA	CCA SAC RTC AGC CCA GTT
primer code	3-D	4-D

Table 5.1. Primers for amplification of *cdh*-like sequences from *P. cinnabarinus*. Codon choices for *P. chrysosporium cdh* are taken from the *cdh*-2 allele deposited in GenBank under accession number U65888 (200). Abbreviations: R = A or G; Y = C or T; M = A or C; K = G or T; S = G or C; W = A or T.

5.3.4. PCR

Thermocycling was performed using a Perkin Elmer Gene Amp PCR system 2400 (Perkin Elmer, Norwalk, CT, USA). To each 100 µL reaction mix was added 100 ng *P. cinnabarinus* genomic DNA; 0.2 mM each of dATP, dCTP, dTTP, and dGTP; 25 pmol each primer; 0.1 volume 10X *Taq* polymerase buffer; and 1 U *Taq* polymerase. The reaction mix minus *Taq* polymerase was heated at 94°C for 5 min before addition of *Taq*. Conditions for thermocycling were as follows: 5 cycles of 94°C, 5 minutes; 50°C, 30 seconds; and 72°C, 2 minutes; then 35 cycles of 94°C, 30 seconds; 50°C, 30 seconds, and 72°C, 2 minutes. A final 20 minute incubation at 72°C was then performed. As a positive control for each primer set, 10 ng of a plasmid containing cloned *T. versicolor cdh* was added instead of the *P. cinnabarinus* genomic DNA.

5.3.5. Cloning of PCR products

An aliquot of 10 μL of each PCR mixture was analyzed by agarose gel electrophoresis. Reactions which displayed a band close to the size of the *T. versicolor cdh* band were chosen for further analysis. These reaction mixtures were concentrated to a volume of 30 μL using QiaQuick affinity columns (Qiagen). The PCR products were again run on a 1% agarose gel, and the bands of interest were gel purified using a QiaQuick gel purification kit (Qiagen). The gel purified DNA was then incubated at 72°C for 2 hours with 0.2 mM each dNTP in a 100 μL reaction containing 2.5 U *Taq* polymerase and the buffer supplied by the manufacturer. The DNA was again concentrated and washed using a QiaQuick column (30 μL final volume). An aliquot of this solution was then ligated overnight at 16°C to a T-vector prepared from pBluescript (see below). The ligation reaction contained 7 μL of the PCR product, 1 μL of the T-vector, and 3 U of T4 DNA ligase in a 10 μL reaction volume. Ligation products were used to transform competent *E. coli* JM109, and recombinant (white) colonies were selected from LB plates containing 50 μg/mL ampicillin, 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal), and 40 μg/mL isopropylthio-β-D-galactoside (IPTG). Plasmid DNA from such colonies was prepared by the alkaline lysis miniprep protocol (20) and the presence of the desired PCR product verified by restriction enzyme digestion and agarose gel electrophoresis.

The T-overhang vector was prepared according to the procedure of Hadjeb and Berkowitz (134). Briefly, 10 μg of pBluescript was digested with *Eco*RV at 37°C for 60 min, and the digested plasmid was washed free of enzyme and buffer using a QiaQuick column. The DNA was incubated with 2 mM dTTP and 5 U *Taq* polymerase at 72°C for 120 min. After phenol/chloroform extraction and precipitation with ethanol, the vector was self-ligated overnight at 16°C using 3 U T4 DNA ligase (Promega). Following this incubation, vectors which received the T-overhang were resolved from the blunt-ended, concatemerized molecules by electrophoresis in a 1% agarose gel in Tris-acetate-EDTA buffer. The sharp band corresponding to linear plasmid (the T-vector) was excised from the gel, purified using GeneClean (Bio 101), and resuspended in a final volume of 10 μL

of sterile distilled water. An aliquot of the gel-purified T-vector was analyzed by agarose gel electorphoresis in order to approximate its concentration. The T-vector was immediately stored at -70°C until use.

5.3.6. DNA sequencing

Cloned PCR products were sequenced by the primer walking method using an automated DNA sequencer (Genome Express, Paris). The sequence was compiled and analyzed using MacVector version 6.0.1.

5.4. Results

5.4.1. Amplification and cloning of *cdh*-like sequences from *P. cinnabarinus*

Both upstream primers were tested against each of the four downstream primers using *P. cinnabarinus* genomic DNA as template. As a control, each primer set was also used in a PCR with cloned *T. versicolor cdh* as template. The results of these amplifications are shown in Figure 5.1. All primer pairs produced bands of the expected size in the control reactions. With *P. cinnabarinus* genomic DNA as template, upstream primer 1U produced weak or no bands when paired with downstream primers 3D and 4D, and amplified a large number of bands with downstream primer 2D. A single, clear band was observed using primer set 1U/1D which was reasonably close to the size of the control band. Upstream primer 2U gave better results; in three of the four reactions, a band close to the size of the control was observed, although primer sets 2U/1D and 2U/2D also contained a number of nonspecific bands. Primer set 2U/3D did not produce any products at all. Encouraging results were observed using primer set 2U/4D, which resulted in the amplification of a single product identical in size (1.8 kb) to the control DNA.

Bands close to the size of the control band in each of reactions 1U/1D, 1U/2D, 2U/1D, 2U/2D, and 2U/4D were chosen for gel purification and cloning. The average PCR product cloning efficiency using the pBluescript-based T-vector was approximately 95%. Five white colonies from each transformation were chosen for miniprep analysis

and were screened using restriction enzymes *Eco*RI and *Xho*I. All plasmids analyzed contained an insert of the expected size (data not shown).

5.4.2. Sequencing the cloned PCR products

The PCR product produced by primer set 2U/4D was the longest product generated and therefore potentially contained the largest proportion of *P. cinnabarinus cdh*. For this reason, it was chosen for DNA sequence analysis. The complete sequence of PCR product 2U/4D is shown in Figure 5.2. The sequence was 1864 nucleotides in length, and contained 55.8% G+C. It displayed very high homology both to *T. versicolor cdh* (between nucleotides 535 and 2401), and to *P. chrysosporium cdh* (between nucleotides 765 and 2669 of the *cdh*-2 allele (200)). A total of 8 putative introns were found in the PCR product, which were identified by alignment to the known *cdh* genes as well as by translation of the gene with the putative introns removed. A comparison of the known intron locations of *T. versicolor* and *P. chrysosporium cdh* to the putative introns of PCR product 2U/4D is shown in Figure 5.3.

The predicted protein fragment encoded by PCR product 2U/4D contained 479 amino acids. The protein fragment displayed very high homology to the corresponding amino acid sequences of both *T. versicolor* CDH (88% homology between amino acids 56-533) and of *P. chrysosporium* CDH (84% homology between amino acids 60-539) (Figure 5.4). Moreover, there appeared to be a strict conservation of amino acid sequence among the three proteins at areas of known functionality, such as the heme- and FAD-complexing amino acids, and the cellulose-binding domains (Figure 5.4). The high homology of PCR product 2U/4D to *T. versicolor* and *P. chrysosporium cdh* at both the nucleotide and predicted amino acid levels and the relatively close conservation of intron positions led us to conclude that this PCR product corresponded to a fragment of *P. cinnabarinus cdh*.

5.5 Discussion

D'Souza et al. (95) have described a PCR-based screening method for amplifying laccase-like sequences from various organisms. Their method involves the

use of primers specific for regions encoding copper-complexing amino acids. These amino acid sequences are known to be highly conserved in laccases from a wide variety of organisms, since a very large number of laccase gene sequences is available (95). At the present time, DNA sequences are available only for the *cdh* genes of two organisms: *T. versicolor* (Chapter 3) and *P. chrysosporium* (200). However, CDH is known to be produced by a large number of white-rot fungi (6, 9), by one species of brown-rot fungus (282), and by several species of soft-rot fungi (76, 89, 276). The full range of organisms which produce CDH is currently unknown. A series of primer sets were therefore developed with the intention of enabling the amplification of *cdh*-like sequences from organisms known or suspected to contain such genes. Given the paucity of amino acids within the two proteins with a known functionality, the primers were designed to amplify genes encoding short stretches of amino acids that are completely conserved between the *T. versicolor* and *P. chrysosporium* CDH proteins. Moreover, the choice of upstream primers encoding amino acids thought to be involved in complexing the heme iron and downstream primers from the flavin domain increased the likelihood of amplifying heme/flavin proteins. As a test of the primer sets so designed, we chose the fungus *P. cinnabarinus* I-937. This fungus is known to produce small quantities of a CDH which binds to anti-*P. chrysosporium* CDH polyclonal antibodies (C. Laugero, unpublished observation), but has never been analyzed for the presence of *cdh*-like genes.

In a preliminary trial without optimization of the PCR conditions, several of the primer sets produced products that were close in size to those generated using cloned *T. versicolor cdh* as a template. Upstream primer 1U, which encoded the sequence NSTHWK, appeared to work less efficiently than upstream primer 2U, which encoded ALGGAM (Figure 5.1). The general failure of upstream primer 1U was likely due to a difference in codon choices at the critical 3' end; in *P. cinnabarinus*, the K residue is encoded by AAA, while in *T. versicolor* and *P. chrysosporium* (and therefore in the primer sequence), this codon is AAG (Table 5.1; Figure 5.2). A similar explanation is likely for downstream primer 1D, which encodes the sequence VLLLER, except that the

wide redundancy of leucine codons and the conservative substitution of an isoleucine residue in the *P. cinnabarinus* sequence probably accounts for its failure. Thus, the three PCR products that were cloned using upstream primer 1U and downstream primer 1D were almost certainly nonspecific amplification products: Although their size was close to the control, the differences that were observed were more than would be expected given the sequence of the specific product amplified using primer set 2U/4D (Figure 5.2). The failure of the PCR using primer set 2U/3D was likely due to nonoptimal reaction conditions rather than to the lack of a corresponding sequence or to differences in codon choices, as the *P. cinnabarinus* CDH sequence ultimately showed (Figure 5.4).

The reaction conditions for primer set 2U/3D should be optimized before using these primers to amplify *cdh*-like sequences from unknown organisms, since it would be advantageous to probe an unknown organism with more than one set. This is because a previously uncharacterized organism which possesses a *cdh*-like gene may contain an inconveniently located intron which would prevent hybridization of one or both primers, may differ in its codon choices, or may simply lack homology to other CDHs in the amino acids encoded by the primers. However, the latter possibility should diminish as other CDH sequences accumulate and amino acids critical to CDH function become known. In the present case, with only two CDH sequences available, we chose upstream primers hybridizing to the heme domain and downstream primers hybridizing to the flavin domain, in order to maximize the chances that a heme/flavin protein-encoding gene was amplified. In this context it is interesting to note that downstream primer 2D, encoding the general flavin-binding motif GXGXXG, produced a number of bands with both upstream primers; it is possible that many of the bands amplifed using these primer sets corresponded to other heme/flavin proteins, but this possibility was not tested.

PCR products produced by the different primer sets which were close in size to the control were selected for cloning. One of these, a 1.8-kb product produced by primer set 2U/4D, was fully sequenced (Figure 5.2). That this PCR product corresponded to a fragment of *P. cinnabarinus cdh* was shown by its strong homology to *T. versicolor cdh*

and *P. chrysosporium cdh*, by its conservation of intron positions (Figure 5.3), and by its very high homology at the amino acid level to the known CDHs (Figure 5.4). The *P. cinnabarinus cdh* fragment contained eight putative introns, all of which were similar in length and structure to introns found in other basidiomycetes (286). The intron/exon structure of *P. cinnabarinus cdh* was more similar to *T. versicolor cdh* than to *P. chrysosporium cdh* (Figure 5.3); consistent with this, the predicted amino acid sequence encoded by this fragment of *P. cinnabarinus cdh* is more highly homologous to *T. versicolor* CDH (88% homology to *T. versicolor* CDH vs. 84% homology to *P. chrysosporium* CDH). The *T. versicolor* and *P. chrysosporium* CDH proteins are 83% homologous (Chapter 3). Thus, the *P. cinnabarinus* and *T. versicolor* CDHs are more similar to one another than to *P. chrysosporium* CDH. These observations are consistent with the taxonomic classifications of the three fungi; *P. chrysosporium* is in Order Stereales, while both *T. versicolor* and *P. cinnabarinus* are in Order Apyllophorales. Only at the level of Family do the latter two fungi diverge (*T. versicolor* is a member of the Polyporaceae, while *P. cinnabarinus* is a member of the Schizophyllaceae). Nevertheless, the predicted amino acid sequences of all three proteins are highly similar (Figure 5.4). Furthermore, regions of CDH hypothesized to be involved in complexing the heme and flavin cofactors, and the putative CBDs (144) are strongly conserved in all three proteins.

The method described in this paper could be used to amplify *cdh*-like genes from a variety of organisms. However, it is unknown whether all CDH proteins are as highly conserved as the three that are known to date; thus, at least two primer sets would likely be required to screen an uncharacterized organism. We suggest that primer set 2U/4D be used in conjunction with optimized reactions with primer sets 2U/2D or 2U/3D. In testing the primer pairs, we have chosen an organism, *P. cinnabarinus*, that was previously known to produce a CDH that was similar to the *T. versicolor* and *P. chrysosporium* proteins. Testing the recommended primer sets with a series of CDH-producing organisms from different taxonomic groups is the next logical step. It is probable that, as more sequences become known, more rational primer sets can be

designed. These experiments should provide valuable insights into the structure and function of CDH.

5.6. Conclusions

(1) PCR primers designed to amplify *cdh*-like genes from a variety of organisms were developed and used successfully to amplify most of a CDH-encoding gene from *P. cinnabarinus*, an organism which produces a previously uncharacterized CDH.

(2) All three known CDH proteins are highly homologous, although the CDHs from *P. cinnabarinus* and *T. versicolor* are more similar to one another than to *P. chrysosporium* CDH.

(3) Amino acids within CDH that were previously hypothesized to be involved in the complexation of the heme and flavin prosthetic groups, and in the cellulose binding domain, are conserved in *P. cinnabarinus* CDH.

(4) Due to potential differences in codon choices, intron positions, and amino acid sequences among the CDH-encoding genes of various organisms, at least two primer pairs should be used to screen an uncharacterized organism for *cdh*-like genes.

(5) Primer set 2U/4D could be used in conjunction with optimized reactions using primer sets 2U/2D or 2U/3D to amplify *cdh*-like genes from other organisms.

Figure 5.1. PCR amplification of *cdh*-like gene fragments from *P. cinnabarinus*. **A.** Reactions using upstream primer 1U. **B.** Reactions using upstream primer 2U. Primers are coded as shown in Table 5.1. Arrows indicate bands that were chosen for gel purification and cloning.

A
1U/1D
1U/2D
1U/3D
1U/4D
MW T. vers. P. clnn. T. vers. P. clnn. T. vers. P. clnn. T. vers. P. clnn.
2176
1766
1230
1033
653
517
453
394
298
234
B
2U/1D
2U/2D
2U/3D
2U/4D
MW T. vers. P. clnn. T. vers. P. clnn. T. vers. P. clnn. T. vers. P. clnn.
2176
1766
1230
1033
653
517

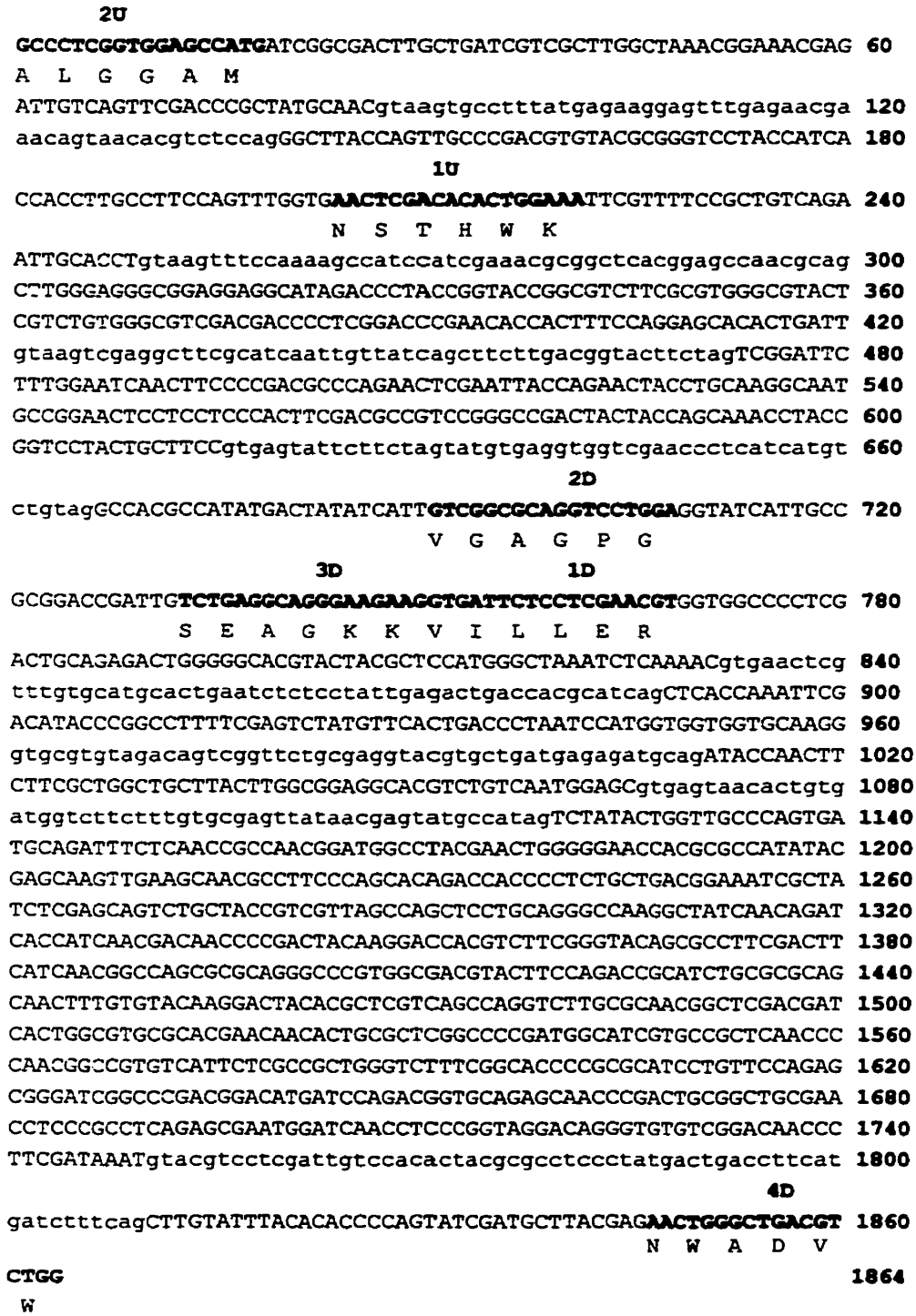


Figure 5.2. The nucleotide sequence of PCR product 2U/4D amplified from *P. cinnabarinus*. The regions corresponding to the primers shown in Table 5.1 are shown in bold, and the corresponding predicted amino acids sequences are indicated. Predicted introns are shown in lower-case lettering.

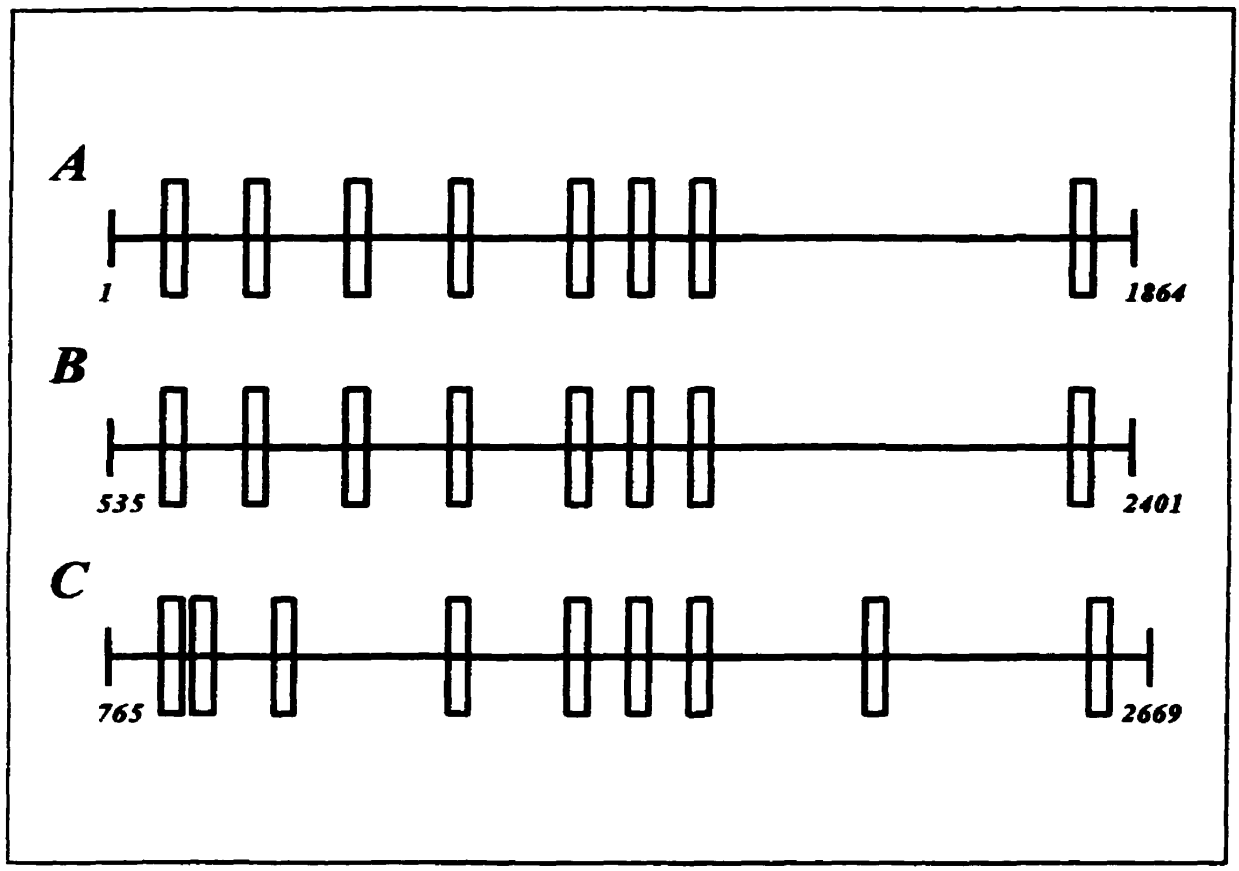


Figure 5.3. Alignment of the predicted introns of PCR product 2U/4D from *P. cinnabarinus* (**A**) to the known intron locations of the corresponding regions of **B.** *T. versicolor cdh* (Chapter 3), and **C.** *P. chrysosporium cdh* (200). Introns diagrammed above correspond to introns II-IX of *T. versicolor cdh* and II-X of *P. chrysosporium cdh*.

Figure 5.4. Optimized global alignment of the predicted amino acid sequences of *T. versicolor* CDH, *P. chrysosporium* CDH, and PCR product 2U/4D from *P. cinnabarinus*. Conserved amino acids are shown in dark grey, while conservative changes (amino acids with similar functional groups) are shaded light grey. Dissimilar amino acids are not shaded. Alignment was performed using the CLUSTALW algorithm (MacVector 6.0.1). Amino acids thought to be involved in CDH function are as follows: M (heme binding), aa 65; H (heme binding), aa 114; S/T/P-enriched linker, aa 191-216; GXGXXG (flavin binding), aa 223-228; cellulose-binding domain, aa 252-300.

ClustalW Formatted Alignments

PREFACE TO CHAPTER 6

The biochemical and molecular characterization of CDH reported in the previous chapters provided interesting new information regarding this enzyme. However, these experiments did not directly address the fundamental question of the role of CDH in delignification, cellulose degradation, and pulp biobleaching by *T. versicolor*. A CDH-deficient mutant was sought in order to provide a means of addressing this question. The availability of the genomic clone encoding CDH (Chapter 3) allowed the possibility of using targeted deletion as a means of accomplishing this goal. Gene targeting by disrupting a cloned version of a gene before transformation has been used for many years in yeast systems and has also been reported in filamentous fungi. The following chapter describes efforts to isolate such a mutant.

K. Bartholomew was responsible for the optimization of the protoplasting and transformation protocol for *T. versicolor*, and performed all of the transformations with the mutagenic vector. In addition, L. Valeanu screened the transformants for CDH activity and performed the biobleaching and delignification analyses. I performed all of the other experiments described in this chapter.

CHAPTER 6 ISOLATION OF A CELLOBIOSE DEHYDROGENASE-DEFICIENT MUTANT OF *TRAMETES VERSICOLOR*

6.1. Abstract

Although cellobiose dehydrogenase (CDH) is capable of reducing a wide range of substrates, including transition metal ions, quinones, and phenoxy radicals, its true *in vivo* role has remained mysterious. In order to study the potential importance of CDH to lignocellulose degradation and pulp biobleaching, we constructed a CDH-deficient strain of the polypore basidiomycete *T. versicolor*. This strain was developed by transforming protoplasts of *T. versicolor* strain 52J with a plasmid consisting of a disrupted version of cloned *T. versicolor cdh* containing a phleomycin resistance cassette. Of 143 phleomycin-resistant colonies analyzed, 3 (2.1%) did not produce measureable CDH over two successive two-week culture periods. Of these three, two yielded normal amplification products with *cdh*-specific primers. The third, cdhm25C, consistently failed to give an amplification product with *cdh*-specific primers and had lost its ability to grow on phleomycin-containing plates. We conclude that strain cdhm25C is a true *cdh* mutant which has undergone a targeted insertion followed by a genetic rearrangement which resulted in the excision of the phleomycin resistance cassette and all or part of the CDH coding region. Cultures of two of the *cdh* mutants, cdhm25C and cdhm4D, biobleached and delignified industrial unbleached kraft pulp as efficiently as wild-type *T. versicolor*, which suggests that CDH does not play an important role in these processes.

6.2. Introduction

As discussed previously (Chapter 1), *T. versicolor* is able to degrade both lignin and cellulose, and its unique abilities have led us to investigate its potential application in a pulp "biobleaching" system. In this system, washed, unbleached kraft pulp is incubated with the fungus in a growth medium for several days, resulting in a substantial increase in pulp brightness and a concomitant decrease in the lignin content (18). Although biobleaching with *T. versicolor* offers potentially great chemical savings and

reduced organochlorine emissions from pulp mills, using the complete fungal system to biobleach and delignify kraft pulp is impractical for mill application because it takes too long (18). We have therefore investigated the enzymatic system employed by the fungus to delignify with a view to applying the isolated enzymes. Enzymes from *T. versicolor* which have been shown to drive systems delignifying kraft pulp are manganese peroxidase (MnP) (234) and laccase (48). However, neither of these enzymes have been able to match the rate and extent of delignification and biobleaching afforded by the complete fungal system (232). This has led to the search for other *T. versicolor* enzymes that may be important in biobleaching.

Cellobiose dehydrogenase (CDH) is one potential candidate. CDH is an inducible extracellular hemoflavoenzyme which oxidizes cellobiose, short cellodextrins, and the reducing ends of cellulose molecules to acids (6). The enzyme reduces a very wide range of substrates, including quinones, metal ions, and phenoxy radicals (6, 27, 142, 269). A more complete review is given in Chapter 2. This lack of specificity has led to a number of different hypotheses regarding the true *in vivo* role of CDH. Many workers see CDH as primarily a cellulolytic enzyme (26, 107, 260), while others postulate that it is involved in lignin degradation through various interactions with the lignin oxidizing enzymes (12, 270) and reduction of oxidized lignin quinones and phenoxy radicals to phenolics. CDH may well be involved in both lignin and cellulose degradation through its ability to promote the production of hydroxyl radicals, which are powerful oxidizing agents that can cleave both lignin and cellulose (141, 328) (Chapter 2). Each of these putative functions of CDH are based upon the demonstrated *in vitro* biochemical capabilities of the enzyme, but none have been proven unequivocally to be important *in vivo*. One effective means of determining the real role of CDH is to generate mutants that are deficient only in the production of CDH. To date, no such mutants have been reported for any of the CDH-producing fungi.

Traditionally, the isolation of a desired fungal mutant has been achieved by randomly mutagenizing a conidial or protoplast preparation, usually by ultraviolet light,

and screening the surviving cells for the desired phenotype. Although such an approach has been successfully used to study the roles of laccase (8) and MnP (3), the method is limited by the necessity of screening large numbers of mutants and by the requirement for an effective screen for the desired phenotype. Moreover, it is never certain that only the desired gene has been mutated. In the case of CDH, no efficient screening method has been developed since secreted oxidative enzymes tend to interfere by re-oxidizing CDH-reduced chromophores, which has hindered the search for CDH-less mutants.

An alternative approach to generating a desired mutant involves the *in vitro* disruption of a cloned version of the gene under study and the subsequent introduction of the disrupted version of the gene into the host organism (281). In a certain percentage of such transformants, the nonfunctional version of the gene will recombine into the host genome directly at the targeted locus, displacing the endogenous gene and resulting in a "knockout" mutant. This approach has been used for many years to disrupt genes in yeast (264), and has more recently been successfully applied to filamentous fungi (4, 37, 113). CDH is an ideal target for such studies since it is encoded by a single structural gene (Chapter 3), and therefore the phenotype of a knockout mutant is unlikely to be masked by the activity of other isozymes, as has been observed in other systems (179), and as would be the case for *T. versicolor* laccase or MnP (72, 218).

Here we describe the application of this method to the development of a CDH-deficient strain of *T. vesicolor*. Using a phleomycin-based transformation vector for basidiomycetes developed by Schuren and Wessels (287), we have constructed a vector designed to integrate into the *cdh* locus of *T. versicolor*. A strain which does not produce CDH and which possesses an altered *cdh* locus has thereby been developed.

6.3. Materials and Methods

6.3.1. Organism

The fungal strain employed in this study was *T. versicolor* 52J (ATCC 20869). All plasmid manipulations were done using the *E. coli* host strain DH5α (137).

6.3.2. Construction of transformation vectors

Plasmid pBScdhgen1-1E, which contains a 5 kb *Eco*RI fragment of *T. versicolor* genomic DNA including the complete *cdh* gene (Chapter 3), was used as the basis for the construction of a *cdh* knockout vector (Figure 6.1). The 5 kb insert was subcloned into pUC7, a vector which lacks a *Sac*I site, to form plasmid pUC7-cdh. This plasmid was digested with *Sac*I, thereby removing an internal 2 kb *Sac*I fragment from the insert which contains most of the coding region of *cdh*, and re-ligated. A plasmid lacking the 2 kb *Sac*I fragment of *cdh* was retrieved and designated pUC7-cdhΔS. Plasmid pGPhT (287) was digested with *Pvu*II to release a blunt-ended fragment containing the entire phleomycin-resistance cassette. This fragment was ligated to *Sac*I-digested and filled-in (T4 DNA polymerase) pUC7-cdhΔS to form plasmid pcdhΔS-GPht, and was also ligated to *Sma*I-digested pBluescript KS^+ (Stratagene) to form plasmid pBS-GPhT.

6.3.3. Transformation of *T. versicolor*

Cultures of *T. versicolor* 52J were prepared for protoplasting as follows: Four colonies of 2-3 cm diameter grown on YM agar plates (3 g malt extract, 3 g yeast extract, 5 g peptone, and 10 g glucose per liter with 1.5% agar) were transferred into a 100 mL Eberbach semimicro blender cup and blended at high speed in 100 mL YM broth for 40 s. The mycelial macerate was transferred to a 500 mL flask and incubated at 30°C with shaking at 200 rpm until the culture appeared saturated (2-3 days). For protoplast preparation, 5-10 mL of this liquid culture was transferred to 100 mL fresh YM broth, blended for 40 s as above, and incubated in a fresh flask at 30°C with shaking (200 rpm) for 12-18 h.

Mycelia from these overnight cultures were harvested by centrifugation at 2500 g for 5 min. The mycelia were washed twice with 0.5 M Mg osmoticum (0.5 M $MgSO_4$, 10 mM MES, pH 6.3). Washed mycelia were suspended in an equal volume of 1 M Mg osmoticum (1 M $MgSO_4$, 20 mM MES, pH 6.3), and Novozyme 234 (InterSpex

Products, Inc.) was added to a final concentration of 500 μg/mL. The mycelia were incubated at 30°C for 4 h with gentle mixing, then 1.0 vol of sterile H_2O was added and the cultures were centrifuged at 2500 g for 5 min to collect the digested mycelia and protoplasts. The pellet was resuspended in 25 mL 0.5 M Mg osmoticum, mixed, and recentrifuged. The protoplast-containing supernatant was saved, and the pellet was again resuspended in 0.5 M Mg osmoticum. This step was repeated once more, and the resulting supernatants were pooled. Protoplasts were pelleted by adding an equal volume of 1 M sorbitol osmoticum (1 M D-sorbitol, 10 mM MES, pH 6.3), and centrifuging at 2500 g for 10 min. The collected protoplasts were resuspended in 2-3 mL 1 M sorbitol osmoticum, counted using a hemocytometer, and their concentration adjusted to 10^8/mL. The protoplasts were stored on ice and used within 1 week of preparation.

Plasmid DNA to be used for transformations was purified on a CsCl gradient using standard procedures (20). DNA (5μg) was diluted with water and $CaCl_2$ was added to a final concentration of 50 mM in a total volume of 120 μL. An aliquot of 200 μL of the protoplasts (2×10^7) was transferred to a 15 mL sterile plastic centrifuge tube and incubated for 20 min on ice. The protoplasts were then mixed with the DNA solution and incubated for another 20 min on ice. Following this incubation, 320 μL of 50% PEG 3350 was gently added, the suspension was incubated again on ice for 5 min, and 3.5 mL of recovery media (YM/0.5 M $MgSO_4$/100 μg/mL phleomycin/40 mM MOPS, pH 7.0) was added. The transformation mixture was incubated overnight at 4°C, then added to several soft agar overlays (recovery media with 0.7% agar) and spread evenly on the surface of YM-phleomycin 100 plates (100 μg/mL phleomycin/50 mM MOPS pH 7.0). Plates were incubated at 30°C until phleomycin-resistant colonies were observed.

6.3.4. Phenotypic screening of transformants for CDH production

Phleomycin-resistant transformants were transferred from the initial transformation plates onto fresh YM-phleomycin 50 plates (50 μg/mL phleomycin/40 mM MOPS pH 7.0). Colonies which grew on these secondary plates were subcultured onto YM plates without phleomycin and portions of these colonies were used to

inoculate 50 mL Erlenmeyer flasks containing 20 mL each of CDH production medium (Chapter 2) with 5 g/L cellulose as the carbon source. These flasks were incubated at 25°C with shaking. Starting on day 4, aliquots of the culture supernatants were removed and assayed for CDH using the chlorpromazine radical reduction assay (268). Cultures were tested every two days for a two-week period, and any strains exhibiting measurable CDH activity were discarded. Transformants which did not produce detectable CDH during the primary assay were re-inoculated in duplicate into fresh CDH production medium as above and analyzed for another two-week period. Strains which still did not produce CDH in either flask were considered CDH(-) and were subjected to genetic analysis.

6.3.5. Genotypic analysis of transformants

Genomic DNA was isolated from each transformant as previously described (322). The *cdh* locus was amplified from total genomic DNA using an upstream primer, 5'-CAT TGG TCA TCC CTC GAG TCC-3', which hybridized to *T. versicolor* genomic DNA 150 nucleotides upstream of the *Eco*RI site corresponding to the 5' end of *cdh* on plasmid pBS-cdhgen1-1E. This primer was chosen so that only the endogenous *cdh* locus, and not the *cdh* fragment from ectopic integration events, was amplified. The downstream primer, 5'-ACA AGT ACA GCA CAC GAC GG-3', hybridized downstream of a *Pst*I site within the endogenous *cdh* gene. These *cdh* primers were designed to yield a *Pst*I-digestible product of 3.55 kb from wild-type *T. versicolor* 52J genomic DNA. The primers (25 pmol each) were used in a PCR containing 0.2 mM dNTPs, 1 U *Taq* polymerase (Pharmacia), and 1 U *Taq* extender (Stratagene), in a 100 μL reaction volume. Amplification conditions: 94°C, 5 min followed by 5 cycles of 94°C, 1 min; 52°C, 30 s; 72°C, 2.5 min, then 35 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 2.5 min. Amplification of the laccase III gene was effected using primers 5'-TAC TGT GAT GGT CTG AGG-3' (upstream) and 5'-GTC TTG CGC GTT CTG CGC-3' (downstream). The amplification was done in a 50 μL reaction mixture containing 10% DMSO, 0.2 mM dNTPs, 20 pmol each primer, and 1 U *Taq* DNA polymerase

(Pharmacia). Cycling conditions: 94°C 5 min, then 35 cycles of 94°C, 2 min; 60°C, 2 min; 72°C, 2 min, followed by 10 min at 72°C. An aliquot of 10 μL of the amplifications was analyzed by agarose gel electrophoresis.

6.3.6. Biobleaching

Flasks containing 1% (w/v) unbleached hardwood kraft pulp from a Quebec mill in 200 mL of 83 mM glucose, 5 mM glutamine, 5 mM NaCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM dimethylsuccinate, 0.8 mg/L thiamine, and a trace metal solution (267) were inoculated with 10% (v/v) of a liquid fungal culture prepared in the same medium. All flasks were supplemented with 75 μM $MnSO_4$ to facilitate bleaching. Flasks were incubated at ambient temperature for nine days with shaking at 200 rpm. Culture supernatants were analyzed periodically for laccase, MnP, and CDH activity. At the end of the incubation, handsheets were prepared from the pulp/mycelium mixture and the brightness measured using standard methods (CPPA Standard Method E.1, 1990). Residual lignin was measured using the micro-kappa method (CPPA Standard Method G.18, 1997).

6.4. Results and Discussion

6.4.1. Construction of a mutagenic transformation vector for *T. versicolor cdh*

Plasmid pGPhT was developed by Schuren and Wessels (287) as a transformation vector for basidiomycetes. This plasmid contains the *ble* gene of *S. hindustanus*, which encodes a phleomycin binding protein, under the control of regulatory sequences from the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) gene of the basidiomycete *S. commune*. Previously we have determined that this plasmid confers upon *T. versicolor* the ability to grow in the presence of phleomycin, and that the plasmid integrates stably and ectopically into genomic DNA (G. Dos Santos *et al.*, manuscript in preparation). Plasmid pGPhT was reported to confer phleomycin resistance to *S. commune* when the *GPD* promoter was deleted up to position -130 (287),

which suggested that a 1.5 kb *Pvu*II fragment of pGPhT should contain the phleomycin resistance cassette. To determine whether this was in fact the case, plasmid pBS-GPhT was constructed. Protoplasts of *T. versicolor* 52J transformed with this plasmid yielded phleomycin-resistant colonies, while control transformations without plasmid DNA did not yield any colonies (data not shown). A typical transformation gave 5-10 transformants per μg of DNA. The plasmid probably integrated into genomic DNA ectopically, as did plasmid pGPhT, but the transformants were not analyzed further. To construct a *cdh*-deficient mutant of *T. versicolor*, plasmid pcdhΔS-GPht was constructed with the aim of targeting the insertion of the phleomycin resistance cassette to the chromosomal region containing *cdh*. The mutagenic plasmid was constructed as diagrammed in Figure 6.1 and its structure was verified by restriction mapping (not shown). This plasmid was used to transform protoplasts of *T. versicolor*.

6.4.2. Phenotypic analysis of phleomycin resistant transformants

A total of 142 phleomycin-resistant transformants of *T. versicolor* 52J were isolated. These strains were analyzed for their ability to produce CDH, and of these, 9 (6.3%) did not produce measurable CDH over the two week assay period. These 9 strains were then re-inoculated into duplicate flasks of CDH production medium and analyzed for a second two-week period. Of the 9 strains, 6 produced detectable CDH in at least one flask during the second assay period, although all of these produced CDH substantially later than untransformed *T. versicolor* (day 4 for the untransformed vs. day 6 or later for the transformants). The reason for this delay in CDH production is unknown, but may be due to differences in growth rates. Three strains of the 142 originally tested (2.1%) produced no measurable CDH in either flask over the second two-week assay period. These strains were considered to have a CDH(-) phenotype.

6.4.3. Genetic analysis of CDH(-), phleomycin resistant *T. versicolor* strains

The three CDH(-) strains, cdhm4D, cdhm9D, and cdhm25C were subjected to genetic analysis. In theory, a targeted double recombination event would result in the

complete replacement of the endogenous *cdh* with the vector DNA, which contains a large deletion in the coding region of the gene (the 2 kb *Sac*I fragment is replaced with the 1.5 kb phleomycin resistance cassette). Such a gene replacement is expected to occur in a small but detectable proportion of the transformants (4, 112) and should result in a strain which cannot produce functional CDH. Total genomic DNA was isolated from each CDH(-) strain and used in a PCR with *cdh*-specific primers. A targeted double recombination event would have resulted in an amplification product of 3.05 kb which contains a *Bam*HI site but does not contain a *Pst*I site. This is in contrast to an unaltered *cdh* locus, which gives a 3.55 kb product which contains a *PstI* site but no *Bam*HI site. Of these three strains, two (cdhm4D and cdhm9D) possessed an apparently normal *cdh* locus. The other strain, cdhm25C, did not give an amplification product with *cdh* primers (Figure 6.2), even though the experiment was repeated three times. However, the same strain gave a normal amplification product using laccase-specific primers (Figure 6.2). Moreover, strain cdhm25C, in contrast to the other two CDH(-) strains, appeared to have lost the ability to grow on YM-phleomycin plates (Figure 6.3). These results lead to the conclusion that the mutagenic plasmid initially inserted at the *cdh* locus and conferred the ability to grow in the presence of phleomycin for some time, but that a genetic rearrangement later occured at the *cdh* locus which resulted in the excision of the phleomycin resistance cassette along with some or all of the endogenous CDH-encoding gene. Thus strain cdhm25C can probably be considered a true *cdh* mutant.

The proportion of CDH(-) strains that were isolated was small (2.1%). Although gene targeting is known to occur with a high frequency in yeast systems (112, 264), the major mode of integrative transformation in filamentous fungi is ectopic (112). Alic et al. (4) studied the targeted integration into *P. chrysosporium* genomic DNA of a plasmid containing an *S. commune* gene conferring adenine prototrophy cloned into a disrupted version of the *P. chrysosporium ura*3 gene. In this system, a similarly small proportion of transformants were shown to be due to homologous transformation (these were selected on 5-fluoroorotic acid), and all were shown to be due to a double recombination event leading to a knockout of the *ura*3 gene (4). In the present case, 97.9% of the

phleomycin-resistant transformants were presumably due to ectopic integration, since their CDH phenotype was unaffected. Alternatively, some of these strains with an unaffected CDH phenotype may have been due to homologous integration near the *cdh* locus by a single crossover event occuring in a region outside of that required for proper expression of the gene (for example, within the 2 kb immediately downstream of the gene). Nevertheless, we were able to identify three CDH(-) strains by direct enzymatic assays of a relatively small sampling of phleomycin-resistant strains. In none of the CDH(-) strains identified did a double recombination (knockout) event occur; however, the mutagenic plasmid probably integrated at the targeted locus in at least one case and resulted in a strain with the desired phenotype. Thus this method may be of general utility in creating mutants of any gene of *T. versicolor* for which a clone is available.

6.4.4. Nature of the CDH(-) phenotype

The three CDH(-) strains which were isolated were used to evaluate the potential role of CDH in pulp biobleaching. In all three strains, levels of pulp bleaching and delignification close to that of the wild-type *T. versicolor* 52J were observed (Figure 6.4). In addition, CDH was undetectable in the wild type strain at the end of the bleaching assay (not shown). These results indicate that CDH is probably not important in the pulp biobleaching effect of *T. versicolor*. However, the levels of the known delignifying enzymes, MnP and laccase, varied widely among the various strains (not shown). Further studies are underway to determine whether or not CDH is important in delignification and to elucidate its true role.

6.5. Conclusions

(1) A 1.5 kb *Pvu*II fragment of pGPhT, a transformation vector developed for basidiomycetes (287), contains the phleomycin resistance cassette. This fragment confers phleomycin resistance to *T. versicolor*.

(2) A mutagenic transformation vector, pcdhΔS-GPhT, designed to target its insertion to the endogenous CDH-encoding gene of *T. versicolor*, was constructed.

(3) Of 142 phleomycin-resistant strains isolated using this vector, 3 (2.1%) were classified as possessing a CDH(-) phenotype. Of these three, two possessed an ostensibly normal *cdh* locus, as determined by PCR analysis. The other, cdhm25C, appeared to contain an altered *cdh* locus and had lost the ability to grow on phleomycin-containing plates. This strain is likely a true *cdh* mutant which occured by targeted insertion followed by genetic rearrangement which deleted all or part of the endogenous *cdh*.

(4) Analysis of the CDH(-) phenotype indicates that CDH is probably not involved in pulp biobleaching or delignification by *T. versicolor*.

(5) *T. versicolor* 52J mutant 25C is a uniquely effective tool for elucidating the true biological role of CDH.

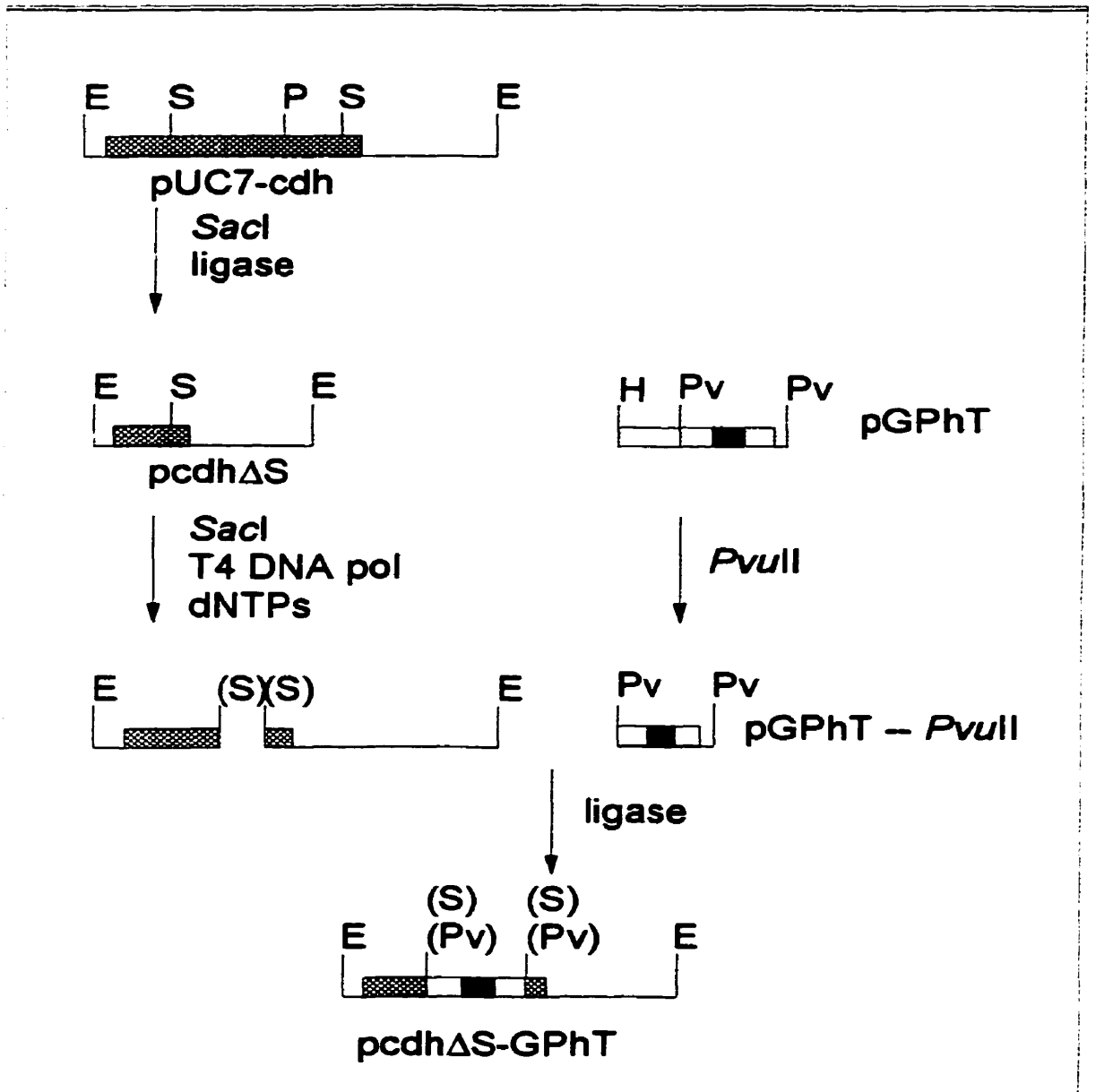


Figure 6.1. Construction of knockout vector pcdhΔSGPhT. The hatched box corresponds to the *T. versicolor cdh* coding sequence, the filled box to the *Streptoalloteichus hindustanus ble* gene encoding phleomycin resistance, and the open box to *Schizophyllum commune GPD* regulatory sequences from plasmid pGPhT (287). Symbols: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sac*I. Symbols in parentheses indicate sites destroyed by blunting and re-ligation.

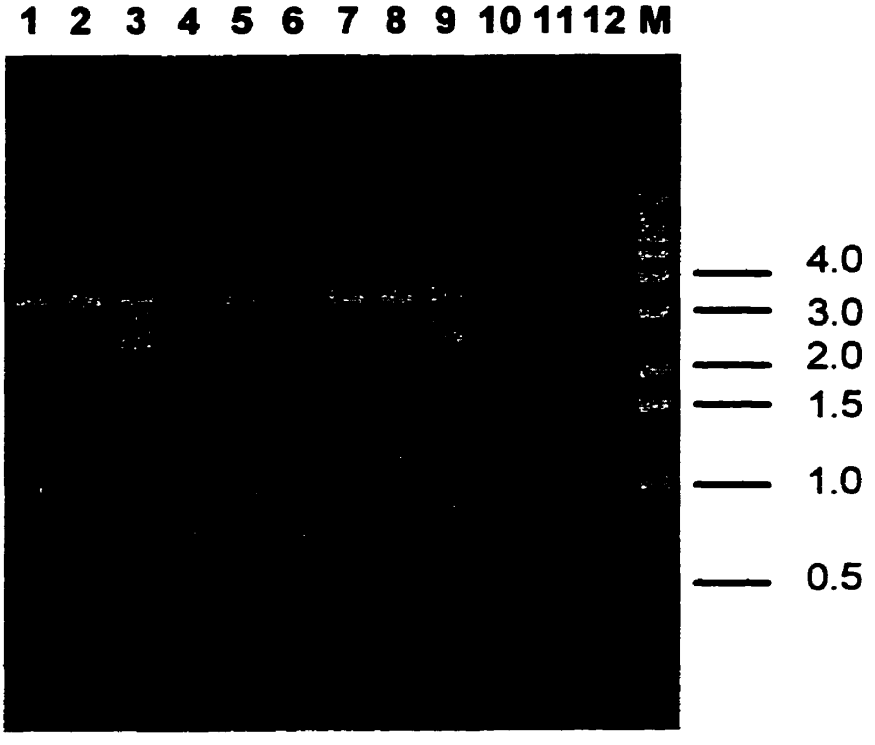


Figure 6.2. PCR analysis of the CDH(-) *T. versicolor* strains transformed with knockout vector pcdhΔS-GPhT. Lanes 1-10 correspond to amplifications using *cdh*-specific primers on genomic DNA from untransformed *T. versicolor* 52J (lanes 1-3), cdhm4D (lanes 4-6), cdhm9D (lanes 7-9), and cdhm25C (lane 10). U, uncut amplification product; B, *Bam*HI-cut amplification product; P, *Pst*I-cut amplification product; C, uncut *cdh* amplification product of cdhm25C. Lanes 11-12 correspond to amplifications using *lcc*-specific primers on genomic DNA from cdhm25C (lane 11) and untransformed *T. versicolor* 52J (lane 12). M, molecular weight markers (1 kb ladder from Boehringer-Mannheim; sizes (in kb) are indicated).

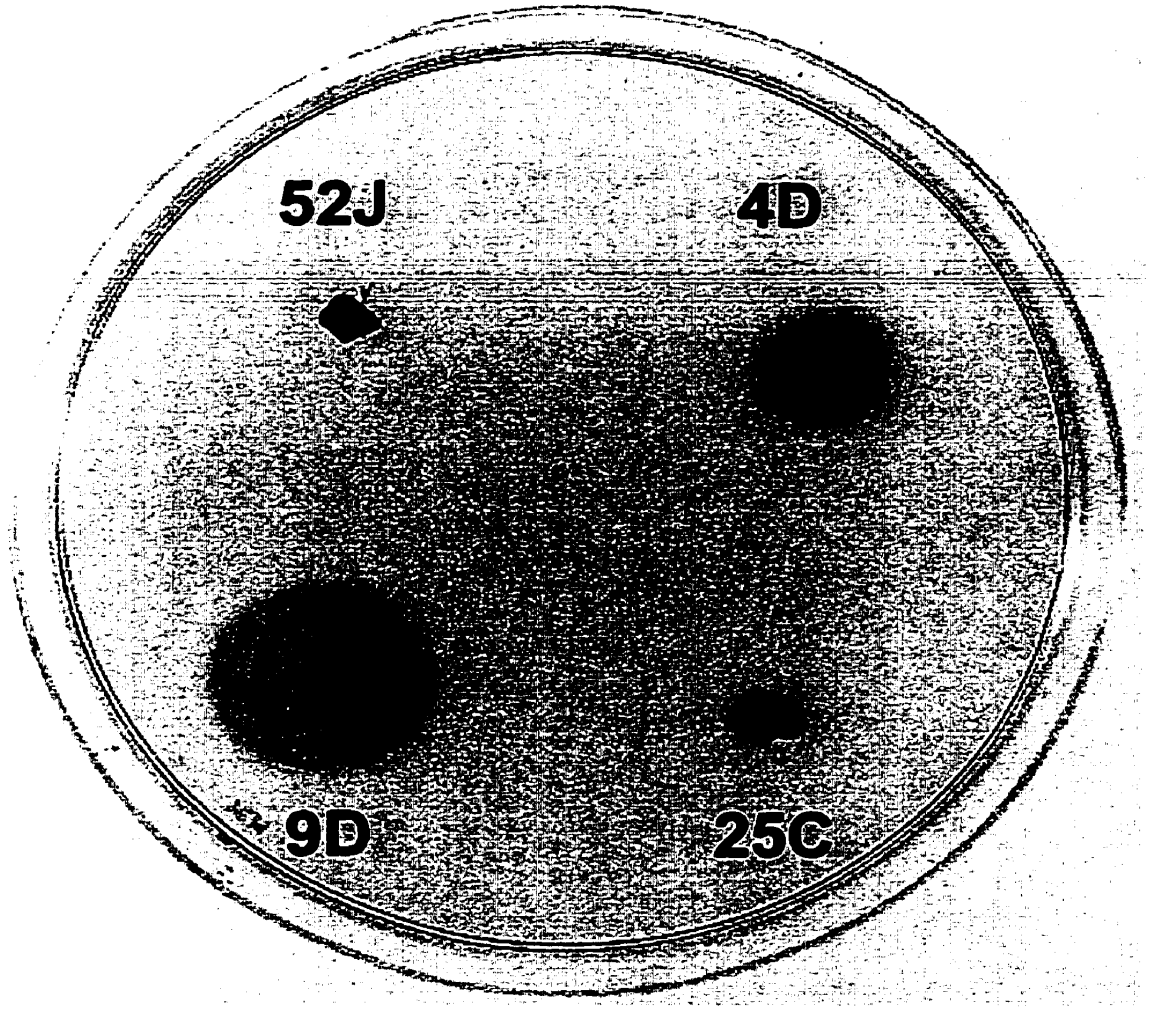
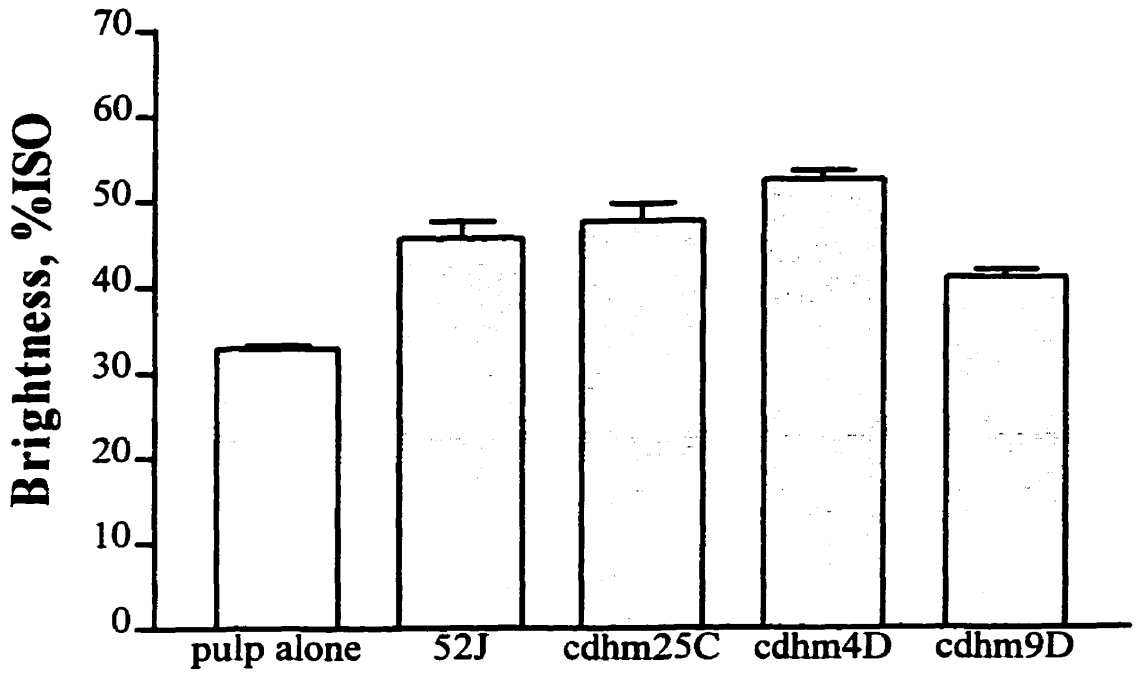


Figure 6.3. Growth of CDH(-) transformants on YM-phleomycin 100 plates. The three mutants and untransformed *T. versicolor* 52J were subcultured to these plates from YM plates without phleomycin and incubated for three days at 30°C.



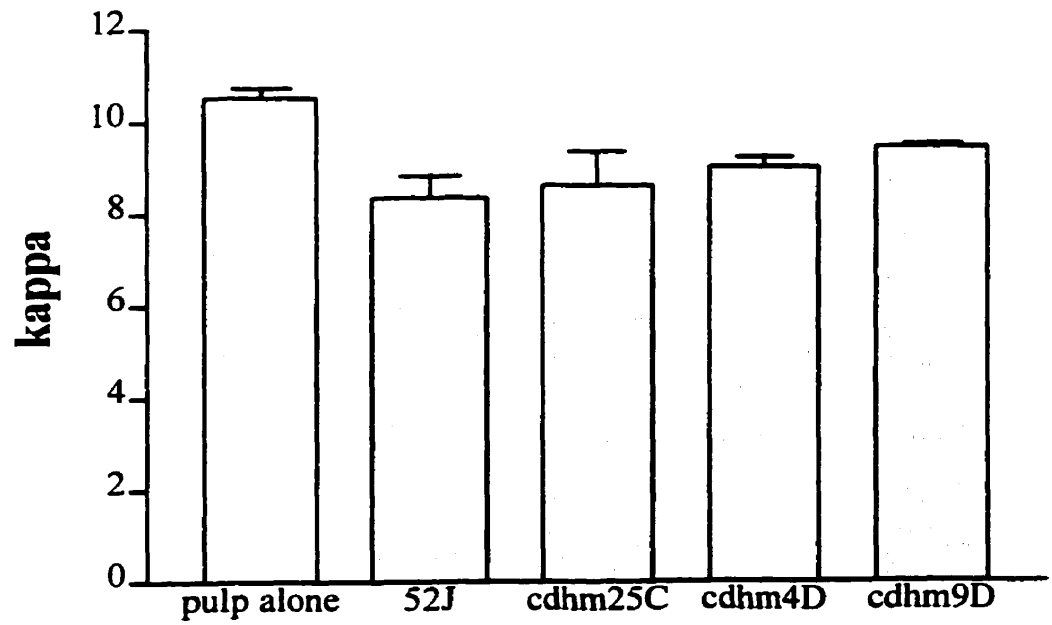


Figure 6.4. Biobleaching and delignification by the CDH(-) *T. versicolor* strains. A, biobleaching; B, delignification. Results are expressed as the mean of three determinations ± standard deviation. For each mutant strain compared to *T. versicolor* 52J, $p > 0.02$, with the exception of the biobleaching by mutant cdhm4D compared to the parent.

CHAPTER 7 GENERAL DISCUSSION

The objectives of this work were essentially twofold: To develop general tools useful in the analysis of the phenomenon of kraft pulp biobleaching and delignification by *T. versicolor*; and to apply these tools to a molecular analysis of the CDH produced by this fungus. Ultimately, the objective was to apply the molecular tools to an analysis of the role of this enigmatic enzyme in the degradation of lignin and/or cellulose and in pulp biobleaching. The work described in this thesis has led to an evolution in our understanding of the characteristics and role of *T. versicolor* CDH.

Molecular tools

Prior to the present studies, few studies of the molecular genetics of pulp biobleaching by *T. versicolor* 52J had been undertaken. The tools required for such an analysis had therefore to be developed. Accordingly, genomic and cDNA libraries were produced (Appendix 1, Appendix 2) which should enable the isolation of further important clones. Furthermore, the constructs for the expression of *T. versicolor* CDH and laccase in *A. oryzae* (Chapter 4) may prove to be very useful for isolating larger quantities of these enzymes if better expression levels can be achieved. These vectors would also enable the study of structure/function relationships within CDH and laccase by site-directed mutagenesis, again assuming that appropriate levels of properly expressed protein can be attained. In addition, the *cdh*-specific primers (Chapter 5) should help in the rapid isolation of further clones encoding CDH from diverse organisms, which will aid in the identification of amino acids critical to CDH function.

The application of these molecular tools to the study of *T. versicolor* CDH has contributed to a new level of understanding of this enzyme. The genomic and cDNA clones (Chapter 3) were the first CDH-encoding clones outside of *P. chrysosporium*. The predicted amino acid sequence of *T. versicolor* CDH corroborated interesting hypothesized features of the analogous enzyme from *P. chrysosporium*, including the potentially novel cellulose binding domain. In addition, the heme-complexing amino acid residues were identified, although the identification of H109 of *T. versicolor* CDH

as the heme chelating residue required comparison to the sequences of *P. cinnabarinus* and *T. heterothallica* CDH (Chapter 3). Most importantly, however, the cloning of *T. versicolor cdh* allowed us to construct a knockout vector, which enabled the isolation of three strains of *T. versicolor* deficient in CDH production. The fact that these strains are capable of carrying out substantial levels of kraft pulp delignification and biobleaching (Chapter 6) has modified our thinking about the role of CDH in this system. This will be considered in more detail below.

Role of CDH in kraft and native lignin degradation

When the present work was started, a series of hypothesized roles in lignin degradation and pulp biobleaching had been put forward for *T. versicolor* CDH based upon the *in vitro* catalytic activity of the enzyme (266, 270). For example, the ability of CDH to reduce phenoxy radicals generated by the action of the oxidative enzymes laccase and MnP produced by *T. versicolor* were proposed to result in a dampening of the tendency of these radicals to polymerize, shifting the balance of lignin polymerization/depolymerization in favour of the latter. This re-reduction of phenoxy radicals was hypothesized to enhance lignin degradation and removal from wood or wood pulp. Furthermore, CDH has been shown to be capable of reducing insoluble MnO_2 to more bioavailable forms, and the cellobionic acid formed from cellobiose oxidation was shown to be an effective Mn(III) chelator (270). These observations were thought to at least partially explain why the complete *T. versicolor* system is so much more effective in bleaching kraft pulp than any of its isolated enzymes, alone or in combination, have hitherto been shown to be. Thus CDH was seen to act synergistically with MnP, the one *T. versicolor* enzyme which is known to biobleach and delignify.

However, the present work has cast doubt on the importance of these hypothesized interactions in lignin degradation. Although purification of the enzyme confirmed many of its previously known catalytic capabilities (Chapter 2), the preliminary results described in Chapter 6 seem to argue against the involvement of CDH in the degradation of lignin. More recent results with the CDH(-) mutant strains

have also shown little or no decrease in the ability to degrade DHP lignin, again arguing that CDH does not likely play a major role in lignin degradation in this system. It should of course be noted that the biobleaching and DHP degradation assays are not necessarily reflective of the degradation of wood by the fungus in nature, since the cultures are not static but are vigourously shaken: A role for CDH in lignin degradation in static cultures has not been ruled out. Previous *in vitro* assays with purified CDH and MnP showed a very slight delignification of kraft pulp, and it was hypothesized that optimization of the reaction conditions would lead to further improvements in delignification (266). However, despite numerous subsequent attempts in this laboratory, no substantial *in vitro* synergy between CDH and either laccase or MnP in ligninolysis or kraft pulp biobleaching has been demonstrated (F. Archibald and L. Valeanu, unpublished results). It therefore seems unlikely that CDH plays a major role in kraft pulp delignification in *T. versicolor*.

Biobleaching

The biobleaching results with the CDH(-) strains (Chapter 6) seem to constitute strong evidence that CDH is not important in pulp biobleaching by *T. versicolor*. However, it remains possible that some other reductive activity is masking the effect of the lack of CDH by performing some its hypothesized reductive reactions. One potential candidate is pyranose oxidase, which oxidizes glucose and reduces some of the same substrates as CDH. This possibility should be tested using a co-substrate other than glucose in a biobleaching assay. Very recent results indicate that the CDH(-) strains can in fact biobleach effectively using xylose, which is not a substrate for pyranose oxidase, as a co-substrate. This is not consistent with the above hypothesis, and argues that the reductive activity of CDH does not contribute substantially to the pulp biobleaching effect.

Fenton's chemistry and cellulose degradation

CDH may promote the production of hydroxyl radicals through its ability to reduce Fe(III) and Cu(II), although the *T. versicolor* enzyme does not appear to be

capable of directly producing H_2O_2 by the reduction of oxygen (Chapter 2). In this characteristic, *T. versicolor* CDH seems to be distinct from the *P. chrysosporium* enzyme, but is similar to the CDH produced by *C. puteana*, a brown rot fungus (282). One possible source of H_2O_2 is the auto-oxidation of Fe(II) complexes to Fe(III) and $O_2^{\cdot-}$; the superoxide thereby formed could react with Fe(II) to form Fe(III) and H_2O_2 (328). Wood (328) suggested that Fe(II)-oxalate complexes, which readily auto-oxidize, could be an important source of H_2O_2 *in situ*, and oxalate is in fact produced by cultures of *T. versicolor* (267). Moreover, extracellular peroxide is necessarily produced by *T. versicolor* in order to support the action of its peroxidases. The hydroxyl radicals produced by the Fenton reaction are capable of chemically cleaving both lignin and cellulose. The presence of a putative cellulose binding domain on CDH (albeit a structurally novel one) suggests that these radicals, which have a very short half-life due to their reactivity, may preferentially act on cellulose when the enzyme is bound. In this context, it is interesting to note the observation that CDH 6.4, the flavin-containing isoform of CDH, is unlikely to generate hydroxyl radicals under physiological conditions due to its low reactivity with Fe(III) and Cu(II) (Chapter 2). The observation in the *P. chrysosporium* system that the heme/flavin (CDH) isoform is only cleaved to the flavin isoform (CBQ) when the enzyme is bound to cellulose (132) suggests that the fungus has an interest in limiting hydroxyl radical production, perhaps in order to protect the nearby fungal mycelium from oxidative damage. *In situ*, there may be a delay between the binding of CDH to cellulose and the cleavage of the heme domain by the fungal proteases which allows the enzyme time to act. The oxidative cleavage of cellulose chains by hydroxyl radicals may be especially important in opening up the cellulose structure within the crystalline regions by decreasing the hydrogen bonding between adjacent chains, allowing exo- and endo-cellulases to enter and degrade the cellulose polymer. The flavin domain of CDH which is produced by proteolysis would no longer produce hydroxyl radicals, but could still function in oxidizing cellobiose if an appropriate electron acceptor (such as phenoxy radicals produced by laccase or MnP activity) was present. Since in the wood substrate lignin and cellulose are closely

associated, a dual role for the enzyme in ligninolysis (by the interactions previously hypothesized) and cellulolysis is possible. However, CDH binds to cellulose, is induced by cellulose, and specifically oxidizes the monomeric unit of cellulose. Its reductive half reaction can sustain the production of hydroxyl radicals, which can attack cellulose. Furthermore, a structurally and catalytically similar CDH is produced by a non-ligninolytic fungus (282). In our system, CDH(-) mutants are capable of carrying out delignification and pulp biobleaching to the same extent as wild type *T. versicolor* (Chapter 6). Subsequent experiments have shown that these CDH(-) strains have a greatly decreased ability to degrade fresh, dry birch wood blocks and grow very poorly on highly crystalline purified cellulose as a carbon source compared to the CDH(+) parent. The weight of the evidence so far attained therefore indicates that CDH can most properly be considered a cellulase that may also play a role in the degradation of lignin through its reductive capacity, but is not an important delignifying enzyme in *T. versicolor*.

APPENDIX 1 CONSTRUCTION OF A cDNA LIBRARY FROM *TRAMETES VERSICOLOR* 52J

A1.1. Preface

One of the goals of this project was to isolate DNA clones from *T. versicolor* 52J encoding proteins of relevance to its kraft lignin biobleaching abilities. In the absence of appropriate clone banks, it was necessary to construct a cDNA library from mRNA isolated under biobleaching conditions. Our intention was to create a library that was likely to contain clones encoding proteins that are important in the biobleaching process, possibly including previously unknown proteins. The following pages document the efforts to create such a library.

The clonal composition of a cDNA library is dependent on the pattern of gene expression as mRNA at the time of metabolic shutdown (harvest); for this reason, the selection of growth conditions is critical. We therefore carefully evaluated the conditions of growth of *T. versicolor* 52J conducive to biobleaching. The following experiments are an extension of earlier observations (147, 183). Kirkpatrick et al. (183) demonstrated that low levels of soytone used as sole nitrogen source in a semi-defined medium results in a substantial brightening effect on HWKP in biobleaching cultures that is due to a chemical effect of the fungus on the pulp. In addition, the work of Ho et al. (147) showed that the inclusion of a small amount of HWKP in inoculum cultures improves the brightness ultimately obtained, and that the brightness increase observed is due primarily to delignification of the pulp.

A1.2. Materials and Methods

A1.2.1. Organism

The fungus chosen for these experiments was *T. versicolor* 52J (ATCC 20869), a white-rot basidiomycete known to produce a marked biobleaching effect on hardwood kraft pulp (2, 233).

A1.2.2. Growth conditions

To evaluate the effect of various growth conditions on biobleaching activity, a two-stage growth protocol was utilized. The "prebleaching" stage was inoculated with three 1 cm diameter punchouts from a colony of *T. versicolor* 52J on an MB agar plate (blended in 10 ml medium) in a total of 200 ml. A glass marble was added to the flasks, which were subsequently incubated at 25°C for 5 days with shaking at 200 rpm. Following this, 10 ml of the prebleaching stage cultures were used to inoculate triplicate "biobleaching" stage cultures containing growth medium plus 2% HWKP. The growth medium common to all biobleaching flasks contained 0.2% Bacto-Soytone, 83 mM glucose, 5 mM NaCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 mM thiamine, and a trace metal solution consisting of 3 μM $FeSO_4$, 1 μM $CuSO_4$, 5 μM $ZnCl_2$, 1 μM $MnSO_4$, 0.1 μM $NiCl_2$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$. The additions to this medium in both growth stages which were used to evaluate effects on biobleaching are detailed in Table A1.1. A control biobleaching flask with no fungal inoculum was also included. The pulp used in both prebleaching and bleaching flasks, as well as the flasks containing the cultures, had been acid washed prior to the experiment.

Medium Group number	Prebleaching flasks	Bleaching flasks[1]
1	30 μM $MnSO_4$	30 μM $MnSO_4$
2	30 μM $MnSO_4$, 0.25% HWKP	30 μM $MnSO_4$
3	----	30 μM $MnSO_4$
4	0.25% HWKP	30 μM $MnSO_4$
5	30 μM $MnSO_4$	----
6	30 μM $MnSO_4$, 0.25% HWKP	----
7	----	----
8	0.25% HWKP	----

[1]All bleaching flasks contained 2% HWKP.

Table A1.1. Additions to growth medium to create the differential growth conditions used to evaluate biobleaching induction.

A1.2.3. Enzyme assays and brightness monitoring

Aliquots of 25 ml were removed daily from each biobleaching culture, and the supernatants were used to monitor laccase and MnP activities. The solids (pulp and fungal hyphae) of these samplings were used to make mini-handsheets which were then used for brightness measurements. MnP activity was monitored using the Mn(II)-malonate assay, and laccase was measured using the ABTS oxidation assay.

A1.2.4. RNA extraction

T. versicolor 52J was cultured in the medium described above containing 30 μM $MnSO_4$, 0.25% HWKP, and 2 mM cellobiose for three days at 25°C with shaking at 200 rpm. Biomass and pulp were separated from the growth medium by filtration, then added to a mortar containing liquid nitrogen and ground to a fine powder using a pestle. The extraction of RNA from the biomass proceeded essentially according to previously published protocols (139). Total RNA was loaded onto an oligo dT spin column (Clontech) and poly A^+ RNA was isolated by following the protocol recommended by the manufacturer.

A1.2.5. cDNA synthesis and library construction

An aliquot of the poly A^+ RNA (5 μg) was used as a substrate for cDNA synthesis using StrataScript™ RNaseH$^-$ reverse transcriptase (Stratagene) and following manufacturer's protocols. Briefly, cDNA synthesis was initiated using a 50-nt linker-primer with an 18-nt poly (dT) sequence and an internal *Xho*I site. First strand synthesis proceeded with 5-methyl dCTP in the reaction mix to protect any internal restriction sites; second strand synthesis was catalyzed by RNase H and DNA polymerase I and proceeded in the absence of 5-methyl dCTP. The resultant ds cDNA termini were filled in with the Klenow fragment of DNA polymerase I, and *Eco*RI adaptors were ligated to the blunt ends. Following this, *Xho*I digestion released the *Eco*RI adaptor from one end, resulting in a cDNA molecule that was cohesive for *Eco*RI on the 5' end and for *Xho*I on the 3' end. Following this procedure assured that all cDNA molecules were ligated directionally into the λZAP™ vector (Stratagene) arms with their 5' ends adjacent to the

lacZ promoter, thereby facilitating antibody-based screens by ensuring that cDNA molecules would be transcribed from the sense strand. The cDNA was fractionated using a Sephacryl S-400 spin column (Stratagene) to remove the released adaptor and leftover linker-primer. Appropriate fractions were pooled, extracted with phenol/chloroform, precipitated, washed, and dried. Pooled cDNA fractions were redissolved in 10 µl sterile H_2O. A 2 µl aliquot of this cDNA (about 100 ng) was ligated to the arms of the λZAP™ vector according to the manufacturer's instructions. A 1 µl aliquot of this ligation was packaged in 5 separate reactions using GigaPack® Gold packaging extracts (Stratagene) and following the recommended protocol. The packaged phage was plated on *E. coli* XL-1 Blue (Stratagene) to determine its titre. The cDNA library was amplified once on *E. coli* XL-1 Blue; the amplified library was collected by layering 10 ml of suspension medium (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl, pH 7.5, 0.01% (w/v) gelatin) onto each plate and incubating overnight at 4°C. The pooled, amplified cDNA library was titered once again on *E. coli* XL-1 Blue and stored at -70°C in 7% DMSO. The percentage of recombinant phage in the library was evaluated by plating aliquots of appropriate dilutions on *E. coli* XL-1 Blue on plates containing 4 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal) and 2.5 mM isopropyl thio-β-D-galactoside (IPTG); blue plaques contained no inserts.

A1.3. Results

A1.3.1. Determination of appropriate growth conditions

Since the clonal composition of a cDNA library is determined by the gene expression pattern at the time of biomass harvest and RNA isolation, we carefully evaluated the growth conditions that led to the production of proteins that may be involved in the biobleaching phenomenon. The results of these experiments are summarized in Figure A1.1. The different growth conditions gave rise to large differences in the rate of brightness increase and in the final brightness obtained. Furthermore, consistent with previous observations (147), the fungus seemed to respond to 0.25% HWKP in the prebleaching medium to give higher brightness levels in the bleaching cultures than are observed when no HWKP is included. In addition, the

inclusion in the medium of 30 μM $MnSO_4$, which is known to be an inducer of MnP, appears to be required for this effect. However, the biobleaching differences between cultures containing HWKP and those lacking it are not completely explained by the production of either MnP or laccase, as similar levels of these enzymes were observed in both cultures (data not shown). This suggests that some other factor or factors, possibly including CDH, is induced by HWKP and contributes to the biobleaching effect shown by *T. versicolor*.

A1.3.2. cDNA library synthesis

The growth medium that was determined to be optimal for the production of biobleaching-related proteins (Medium Group 2) was used to culture *T. versicolor* 52J. Total RNA was extracted at day 3 post-inoculation, yielding approximately 1 mg of non-degraded RNA. A total of 700 μg of total RNA was loaded onto an oligo (dT)-cellulose column, and 25 μg of poly(A)$^+$ RNA was recovered (this corresponds to a yield of 3.5%). The poly(A)$^+$ RNA was analyzed by agarose gel electrophoresis and observed to be free from degradation (data not shown). A 5 μg aliquot of this preparation was used as a template in a cDNA synthesis reaction, yielding approximately 500 ng of cDNA. The size range of the cDNA was tested by running 100 ng on a 1.5% agarose gel with molecular weight markers; the top of the smear indicated a maximum cDNA length of approximately 10 kb, and a minimum length of <1kb (data not shown). This cDNA was ligated into dephosphorylated, *Eco*RI/*Xho*I-digested λZAP vector arms, packaged in five separate reactions, and plated on *E. coli* XL-1 Blue. The titre of the packaged phage was determined (Table A1.2.) and the titres of all five packaging reactions were added together to give the pre-amplification base of the library (Table A1.2.). All of the recombinant phage so generated were combined and amplified once on *E. coli* XL-1 Blue, and the resultant amplified library was again titred, giving a final phage concentration of 1.6 x 10^8 pfu/ml. Aliquots of this library were stored at -70°C in 7% DMSO. To evaluate the percentage of recombinant phage in the library, phage were plated on *E. coli* XL-1 Blue cells on plates containing XGal and IPTG. The results showed that 98.3% of the phage population was recombinant.

A1.4. Discussion

To produce a cDNA library from *T. versicolor* 52J which is likely to contain clones encoding biobleaching-related proteins, we evaluated the growth conditions which produce the biobleaching effect. Conditions which produced the maximum brightness were used in a subsequent experiment to extract RNA which was used to produce the cDNA library. Note that a single time point was chosen for RNA harvesting (day 3), which was essentially arbitrary and represented the middle of the growth cycle when enough biomass was present to extract an acceptable amount of RNA. Short of producing multiple libraries, no consideration could have been taken of the temporal expression of various genes; thus the library only contains clones that were being produced at the time of RNA isolation and may not contain cDNA molecules representing all proteins of importance to the effect.

The pre-amplification base of the library corresponds to the number of cDNA molecules that were originally packaged, and can be used to determine whether the library is truly representative of all of the genes that were being expressed when the RNA was harvested. The probability, P, that the rarest mRNA will be represented at least once in the library may be calculated by the following formula (185):

$$P = 1-[1-(n/T)]^N$$

where n = number of molecules of the rarest mRNA

T = total number of mRNA molecules in the cell

N = number of clones in the library (pre-amplification base)

P may be calculated for a chosen value of n when T is known; an estimate of T (for mammalian cells) has been made at 5.6×10^5 (185). Using this value, for n = 10, P = 0.94, meaning that there is a strong probability that all clones represented by 10 or more molecules are represented in the library. The probability is even greater if T is lower in *T. versicolor* than in a mammalian cell. Combined with the observation that

over 98% of the clones are recombinant, we are quite confident that the library is representative of the pattern of gene expression at the time of RNA harvesting. Indeed, two full-length and one truncated laccase-encoding cDNAs have been isolated from this library by another group (230), and one of the laccase-encoding clones isolated from this library has been successfully used to express functional recombinant *T. versicolor* 52J laccase in the yeast *Pichia pastoris* (M. Brown, unpublished results).

Packaging reaction	Dilution	Number of plaques	Pfu/ml[1]	Average	Pfu plated[2]
1	undiluted	53	$5.3X10^4$	$5.2X10^4$	$2.61X10^4$
	10^{-1}	5	$5X10^4$		
	10^{-2}	0	----		
2	undiluted	40	$4.0X10^4$	$4X10^4$	$2.0X10^4$
	10^{-1}	4	$4X10^4$		
	10^{-2}	0	----		
3	undiluted	55	$5.5X10^4$	$4.3X10^4$	$2.2X10^4$
	10^{-1}	3	$3X10^4$		
	10^{-2}	0	----		
4	undiluted	45	$4.5X10^4$	$4.3X10^4$	$2.2X10^4$
	10^{-1}	4	$4X10^4$		
	10^{-2}	0	----		
5	undiluted	35	$3.5X10^4$	$1.3X10^5$	$6.5X10^4$
	10^{-1}	5	$5X10^4$		
	10^{-2}	3	$3X10^5$		
				TOTAL:	**$1.55X10^5$**

[1]The calculation of pfu/ml was as follows: # plaques X 1000 X dilution factor, since 1 µl of the ligation reaction was packaged.

[2]The pre-amplification base was calculated by multiplying the titre of the packaging reaction by the total volume plated (167 µl on each of three plates) and adding the totals obtained for all five reactions.

Table A1.2. Titre of unamplified packaging reactions.

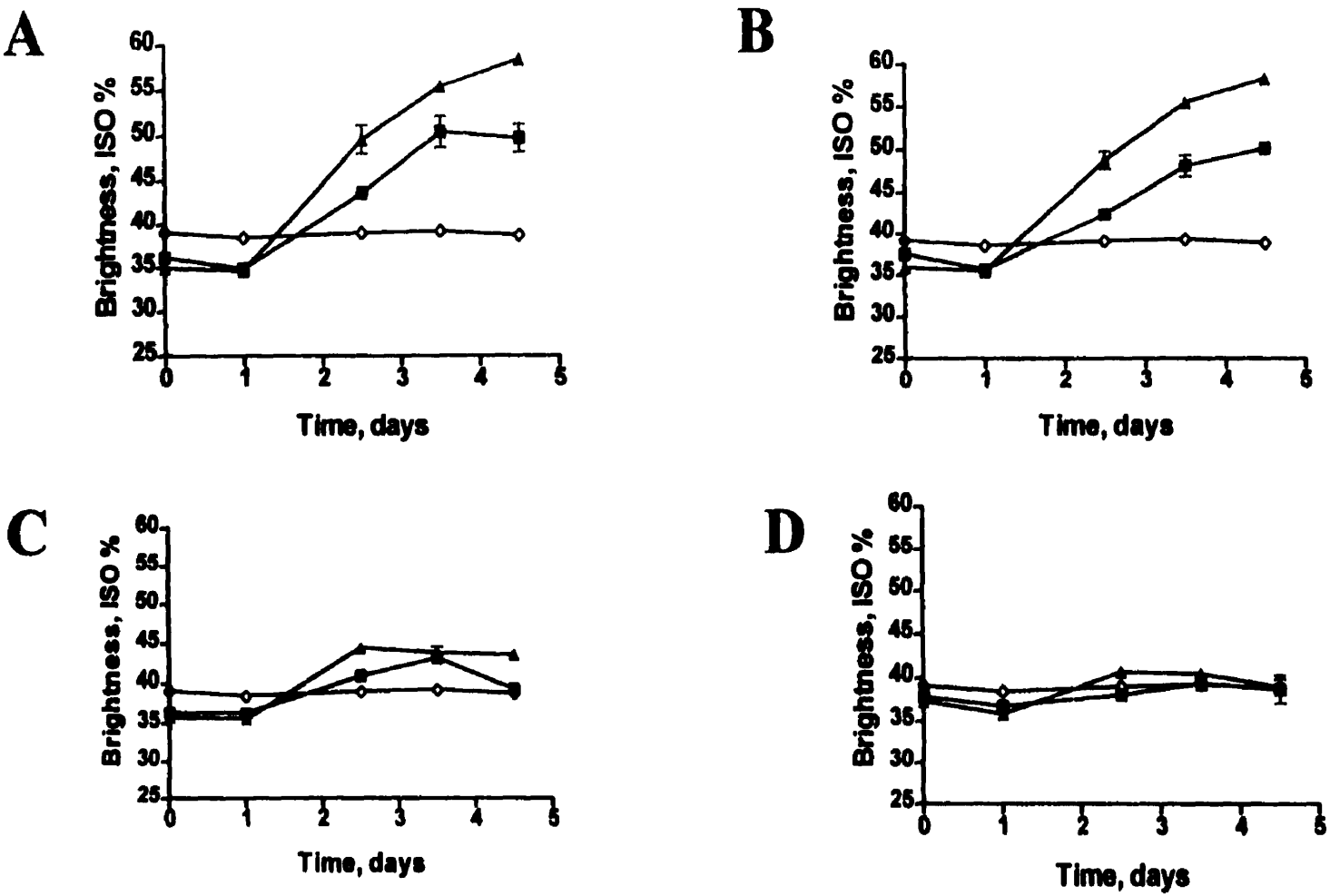


Figure A1.1. Brightness of HWKP in biobleaching cultures of *T. versicolor* 52J grown under various conditions. **A.** Medium group 1 (■); Medium group 2 (▲); no fungus (◊). **B.** Medium group 3 (■); Medium group 4 (▲); no fungus (◊). **C.** Medium group 5 (■); Medium group 6 (▲); no fungus (◊). **D.** Medium group 7 (■); Medium group 8 (▲); no fungus (◊). Medium group numbers correspond to those described in Table A1.1.

APPENDIX 2 CONSTRUCTION OF A GENOMIC LIBRARY FROM *TRAMETES VERSICOLOR* 52J

A2.1. Preface

The cDNA library that was constructed in this project provided an important tool for use in the molecular biological analysis of delignification/ pulp biobleaching by *T. versicolor* 52J. However, as described, the library did not necessarily contain clones representing all proteins of importance to biobleaching, since only the mRNA synthesized during a single time point in the fungal growth cycle was chosen for analysis. Therefore, a representative genomic library which contains clones representing all genes in the entire genome of the fungus was sought. In this case, the conditions under which the fungus is grown do not affect the clonal composition of the library; the only consideration is to generate enough biomass to enable the extraction of an adequate amount of undegraded DNA.

A2.2. Materials and Methods

A2.2.1. Organism

The fungus used in these experiments was *T. versicolor* 52J (ATCC 20869).

A2.2.2. Growth of fungus and extraction of DNA

T. versicolor 52J was grown in YM broth (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, and 20 g/L glucose) until sufficient biomass was present. Extraction of DNA was according to previously published protocols (247).

A2.2.3. Partial digestion of DNA and size selection

High molecular weight genomic DNA from *T. versicolor* 52J was digested with several serial dilutions of *Sau*3A for exactly 30 min at 37°C. Aliquots of these reactions were analyzed on a 0.7% agarose gel prepared in TAE buffer and the amount of restriction enzyme which generated an optimum amount of DNA fragments in the desired size range (12-18 kb) was chosen for a subsequent preparative digestion. The partially *Sau*3A digested DNA was fractionated on a 10%-40% sucrose gradient prepared as described (20) by centrifuging at 30000 rpm for 24 hours (Beckman L8-70

centrifuge, SW41 rotor, 1.54 x 10^5 g at r_{max}; 6.8 x 10^4 g at r_{min}). Fractions of 700 µL were removed from the top of the gradient and aliquots were analyzed by agarose gel electrophoresis (0.7% gel prepared in TAE buffer). Fractions containing DNA of the desired size range were concentrated using Microcon-30 concentrators (Amicon) and washed with TE buffer to remove the sucrose.

A2.2.4. Preparation of the genomic library

An aliquot of 75 µg of DNA from the λGEM-12 cloning vector (Promega) was digested with *Bam*HI (5U/µg) in a 500 µl reaction volume for 60 min at 37°C. The digested DNA was concentrated to 100 µl using a Microcon-30 concentrator, layered on top of a 10%-40% sucrose gradient and fractionated as described above. Fractions of 500 µl taken from the top of the gradient were analyzed by electrophoresis on a 1% agarose gel (TAE buffer). Fractions which contained *Bam*H1-digested arms were pooled, concentrated using Microcon-30 concentrators, and washed with TE buffer.

Partially digested, size-fractionated genomic DNA (3 µg) prepared as described in Section A2.2.3 was dephosphorylated in a 60 µl reaction using shrimp alkaline phosphatase (United States Biochemicals) for 30 min at 37°C The phosphatase was subsequently denatured by heating the reaction to 65°C for 15 min, then the dephosphorylated DNA was concentrated and washed using Microcon-30 concentrators. The genomic DNA (3 µg) was ligated overnight to *Bam*HI-cut λGEM-12 vector arms (1.5 µg) in a 60 µl reaction volume using 12 U of DNA ligase (United States Biochemicals). The ligation reaction was concentrated to 15 µl using Microcon-30 concentrators and an aliquot corresponding to approximately 1 µg of the concentrated DNA was packaged using a commercially available system (GigaPack® Gold, Stratagene) and following recommended procedures. The packaging reaction was titrated using *E. coli* NM538 and *E. coli* NM539 (Promega) and the number of plaques generated on each strain was compared to determine the percentage of recombinant phage in the library. The initial packaging reaction was amplified once using *E. coli* NM539 and the amplified library was collected by layering 10 ml of suspension medium

(Appendix 1) on each plate and allowing the phage to diffuse into the liquid overnight at 4°C. The liquid from each plate was combined and chloroform was added to a final concentration of 5% (v/v). The suspension was incubated for 15 min at ambient temperature, then centrifuged for 10 min at approximately 1000 g to pellet the cell debris. The supernatant was decanted and chloroform was added to a final concentration of 0.3% (v/v). Aliquots of this phage suspension were stored at -70°C in 7% DMSO. The amplified library was again titrated on *E. coli* NM539.

A2.3 Results

To generate a library containing at least one copy of all *T. versicolor* genes, genomic DNA was partially digested with *Sau*3A and ligated into the cloning vector λGEM-12. The ligation reaction was packaged into viable phage particles and used to infect susceptible *E. coli* cells. The titre of this initial packaging reaction was determined, and the results are shown in Table A2.1. This figure, 1.4 x 10^5 pfu, represented the pre-amplification base of the library. The cloning vector chosen for these experiments uses a Spi (Sensitive to P2 Inhibition) selection procedure for differentiating recombinant from non-recombinant phage. The central "stuffer" fragment of this vector contains λ genes that are not essential for phage propagation and are replaced with recombinant DNA. Included among these genes are functional copies of the λ *red* and *gam* genes, which inhibit propagation on any strain of *E. coli* which is lysogenic for phage P2. Recombinant phage do not have the stuffer fragment and are therefore *red gam* (Spi^-); such phage can grow on a P2 lysogen, while non-recombinant phage (Spi^+) will not be propagated. The proportion of recombinant phage in this mixture was therefore determined by comparing the titre observed using *E. coli* NM538 cells with that obtained using *E. coli* NM539 cells. The latter strain is a P2 lysogen and only forms plaques with recombinant phage (i.e., with Spi^- phage), while the former strain will propagate either recombinant or non-recombinant phage. Since the titres obtained with the two strains were nearly identical, the library was shown to be close to 100% recombinant (a more accurate assessment of the percentage of recombinant phage

was made using the amplified library and a figure of 89.1% was obtained). This library was amplified once on *E. coli* NM539 in order to obtain a stable, high-titre phage population for use in cloning experiments. The results shown in Table A2.2 show that the final titre obtained was 2.2 x 10^9 pfu/ml.

Dilution	Number of plaques on		Pfu/ml	Pfu in packaging reaction
	E. coli NM538	*E. coli* NM539		
10^{-2}	277	281	$2.8x10^5$	$1.4x10^5$
10^{-3}	34	24	ND	ND
10^{-4}	4	4	ND	ND

Table A2.1. Titre of initial packaging reaction. An aliquot of 100 µl out of the 500 µl packaging reaction was plated. ND = not determined since sample size (number of plaques) was too small.

Dilution	number of plaques	Pfu/ml	average
10^{-6}	225	$2.25x10^9$	$2.2x10^9$
10^{-6}	229	$2.29x10^9$	
10^{-6}	203	$2.03x10^9$	
10^{-7}	20	ND	---
10^{-7}	21	ND	
10^{-7}	17	ND	

Table A2.2. Titre of the amplified library. An aliquot of 100 µl of each dilution was plated on *E. coli* NM539 and the resultant number of plaques determined. ND = not determined since the number of plaques was too small.

A2.4. Discussion

The objective of these procedures was to produce a high-titre gene library from *T. versicolor* 52J that includes the entire genome. The total number of recombinant phage that must be generated in order to obtain such an inclusive library depends upon the average phage insert size as well as on the size of the genome under analysis, according to the following formula (217):

$$N = \ln(1-P)/\ln[1-(I/G)]$$

where N = number of recombinant phage required

P = probability of the library including a given gene

I = average size of insert

G = size of genome

The size of the *T. versicolor* genome (G) is unknown, but assuming that it is of the same order of magnitude as that of other white-rot fungi such as *Schizophyllum commune* and *P. chrysosporium*, an estimate of $3x10^7$ bp may be made (311). The average size of insert (I) taken off of the sucrose gradient may be estimated to be 15 kb (the midpoint of the size range chosen, 12-18 kb). Thus, for a probability (P) of 0.999 of finding a given clone, we need to generate and screen at least $1.4x10^4$ recombinant phage. From Table A2.1 we can see that the unamplified base of the library was more than sufficient for our purposes; even if the estimate of the genomic complexity of *T. versicolor* is low by an order of magnitude, the library's base of $1.4x10^5$ phage is adequate. This library was successfully used to isolate a genomic clone containing the gene encoding cellobiose dehydrogenase (Chapter 3).

APPENDIX 3 CHARACTERIZATION OF A BIOBLEACHING MUTANT OF *TRAMETES VERSICOLOR* 52J

A3.1. Preface

Early investigations into the mechanisms of delignification by *T. versicolor* 52J indicated that MnP was important to the process (234). To investigate this further, mutant strains of this fungus were sought that lacked MnP activity. Accordingly, protoplasts of *T. versicolor* 52J were produced, mutagenized with ultraviolet light, and screened on nutrient plates containing guiaicol (3). Colonies which did not produce a dark halo of polymerizate were chosen for further analysis; one such strain, named M49, was shown to produce no detectable MnP and very low laccase (3). This mutant showed similar growth characteristics to the wild-type strain, but remarkably less delignification and biobleaching, providing strong evidence that both MnP and laccase are important to the process. As a supplement to the extensive characterization of this mutant undertaken by Addleman et al. (1)(3), we investigated several characteristics of mutant M49 in comparison to the wild-type *T. versicolor* 52J. Specifically, we examined the ability of M49 to decolorize and dechlorinate kraft bleachery effluent, to produce ethylene from α-keto-γ-methiolbutyric acid (KMB), and to decolorize the polymeric dye poly B-411. All of these activities have been reported for wild-type *T. versicolor* 52J. The data appearing in this appendix were included in the original publication describing the isolation of mutant M49 (3).

A3.2 Materials and Methods

A3.2.1. Decolorization and dechlorination of the E-stage effluent

The brown organochlorine-containing effluent from the E_1- (alkaline extraction) stage of a hardwood kraft pulp mill bleach plant was filter sterilized (0.22 μm), set to pH 7.0, and shaken (200 rpm, 25°C, 5 days) in 200 ml aliquots (500 ml flasks) with 6-day fungal inocula prepared in mycological broth (15% of final volume). The inocula were prepared by adding 5 one cm diameter punchouts from the growing edge of a fungal colony on MB agar plates to a flask containing 100 ml MB and two

glass marbles and incubating at 25°C with shaking at 200 rpm. E_1 effluent decolorization was measured after 3 and 5 days as the decrease in absorbance of the effluent at 465 nm. AOX was quantitated using a Euroglas ECS-1000 AOX analyzer and the DIN 38409 H14 procedure (283). To monitor possible AOX adsorption to the fungal mycelium, controls with heat-killed and briefly incubated viable mycelium were run. In parallel with live mycelium inocula, 6 day cultures of 52J and M49 in mycological broth were boiled with constant stirring for 15 min and used to inoculate (at 15% v/v) triplicate flasks containing sterile E_1 effluent. In addition, the short-term effects of live fungal cultures on AOX values were monitored by washing the inoculum twice with water, incubating live mycelia with E_1 effluent, and measuring the AOX at 10, 30, and 90 min, and 3 h and comparing it to a control lacking fungal mycelium.

A3.2.2. Ethylene production from KMB

Supernatants (5 ml) from 2 and 5-day pulp bleaching or effluent dechlorination trials with the wild-type *T. versicolor* 52J, or the mutant M49, were incubated (27°C, 200 rpm) in low light with 2 mM α-keto-γ-methiolbutyric acid (KMB) in sealed 50 ml flasks. After 1 h, samples (1.0 ml) of the headspace gas from control and sample flasks were analyzed for ethylene by gas chromatography using a flame ionization detector (120°C; injector at 90°C) and a Porapak-Q packed column (70°C), with helium as the carrier gas. Evolved ethylene was quantitated against standards using an integrator to monitor peak area, and the total amount (μmol) of ethylene produced per flask was calculated.

A3.2.3. Effects on Poly B-411

The decolorization of the dye Poly B-411, an indicator of white-rot (delignification) activity (66, 75, 243) was monitored in liquid cultures. An inoculum consisting of a 6-day liquid culture of *T. versicolor* 52J or M49 in MB was added at 15% (v/v) to a sterile solution of 0.02% Poly B-411 in water; a control with 15% (v/v) MB with no fungus was also included. The cultures were incubated at 25°C with shaking at 200 rpm for 5 days. At various times, 2 aliquots were removed from each culture and

centrifuged for 5 min at 12,500 g. The A_{597} of the supernatants were measured against a water blank.

A3.3 Results and discussion

Kraft bleach plant effluents are dark brown due to some of the water-soluble, chlorinated, and oxidized lignin degradation products of the pulp bleaching process. These dark chromophores are very resistant to microbial breakdown during secondary treatment of waste waters, and consequently are routinely released into waterways. Oxygen-dependent *Trametes*-mediated decolorization of these bleachery effluents, as well as of the black liquor from kraft pulping is well documented (22, 31, 108, 195, 206, 317). Although enzyme-mediated decolorization of bleachery effluents has been achieved using horseradish peroxidase and immobilized lignin peroxidase in the presence of H_2O_2 (111), as well as immobilized laccase (86), color reduction was not as extensive as with the *T. versicolor* fungal system (111, 233). MnP from *P. chrysosporium* can also decolorize these effluents (214). However, with *T. versicolor* neither addition of active oxygen species scavengers nor induction or inhibition of secondary metabolism blocks effluent decolorization (15). In this study, mutant M49 was unable to decolorize kraft mill effluent, while after 5 days wild type 52J removed about 60% of the color (Table A3.1). Similar results have been found with laccase-less mutants of *Pleurotus sajor-caju* and *P. eryngii*, which had lost the ability to decolorize various substrates (101).

"High molecular weight chlorolignins", which are largely aggregates of low molecular weight chlorolignins (161), comprise the majority of the organically bound chlorine (AOX) present in modern kraft pulp mill bleaching effluents. Although these high molecular weight AOX aggregates are very resistant to bacterial degradation (272), various white-rot fungi, including *T. versicolor* and *P. chrysosporium*, are known to dechlorinate them (15, 31, 193, 241, 317). Laccases and LiP secreted by *T. versicolor* (271) and MnP and possibly LiP from *P. chrysosporium* (193, 241) have been shown to partially dechlorinate low MW phenolic AOX species. In the present work mutant M49

displayed almost no ability to dechlorinate E_1 effluent AOX, compared to wild-type 52J (Table A3.1). In keeping with earlier results demonstrating time-and light-dependent breakdown in kraft bleachery effluent AOX under similar conditions (272), the untreated control effluent AOX also fell, but only slightly over the 5-day incubation period. This partially explains the decrease in AOX that was observed in the M49-treated effluent. Also, although AOX adsorption to fungal mycelium has been reported to be low (317), in our 5-day heat-killed controls some non-metabolically-mediated AOX decrease did occur (Table A3.1). The short-term AOX assays with live mycelium also suggested that some passive binding occured (Table A3.1). Therefore, considering the controls in Table A3.1, AOX binding and non-biological AOX degradation would appear to account for all the disappearance of AOX seen in the M49 culture, indicating that, along with delignification, AOX (chlorolignin) degradation is missing from mutant M49.

The production of ethylene from KMB has been suggested as a good assay for the presence of active delignification, since the ethylene-producing reaction is a high energy single electron abstraction which can be carried out by lignin- and manganese peroxidases, Mn(III) complexes and the •OH radical but not by horseradish or other peroxidases from non-delignifying organisms. *P. chrysosporium* has been shown to produce ethylene from KMB only during delignification, which occurs during secondary metabolism (13, 170). In contrast, the presence of LiP and secondary metabolism is not necessary in *T. versicolor* for pulp bleaching, or the production of ethylene from KMB (13). Compared to 52J, ethylene production from KMB was much lower in mutant M49 (Table A3.1), further evidence that the mutant is deficient in activities related to the delignification of pulp and the decolorization of E_1 effluent.

The dye Poly B-411 was decolorized > 97% by 52J over 5 days, while M49 removed 77% of the color over the same time period (not shown). However, at 30 minutes after addition of 0.02% Poly B-411 to the cultures, 52J had removed nearly 50% of the color from the dye, while M49 had removed less than 7% (not shown). Interestingly, although both cultures decolorized the dye to some extent, different hues

were evident in the products; 52J changed the dye from a deep blue to a faint red, while M49 changed the dye to a dark purple colour, suggesting a qualitatively different degradation mechanism or subsequent step. The decolorization of the Poly B-411 dye that was observed with mutant M49 could be attributed to the low amount of laccase that is produced by this strain, as laccase alone is known to mediate this effect (50).

Trial	Color (OD_{465})		AOX (mg/L)		ethylene (μmol/flask)		laccase (U/L)		MnP (U/L)		pH
	3d	5d	3h	5d	2d	5d	3d	5d	3d	5d	5d
no fungus (control)	0.530	0.567	59.0 ±3.0	49.2 ±5.9	0.006	0.0	0	0	0	0	7.2
52J	0.281 ±0.019	0.255 ±0.030	53.8 ±0	33.3 ±1.9	1.09 ±0.46	0.14 ±0.07	321 ±54	464 ±37	1052 ±192	891 ±195	5.6
boiled 52J	0.543 ±0.004	0.585 ±0.040	nd	43.8 ±4.5	0.014 ±0.001	0.008 ±0.001	0	0	0	0	6.9
52J (M49)	0.474 ±0.070	0.663 ±0.020	53.3 ±1.3	45.2 ±1.0	0.013 ±0.004	0.049 ±0.010	24 ±2	33 ±1	0	0	7.1
boiled 52J (M49)	0.549 ±0.020	0.571 ±0.020	nd	41.8 ±2.0	0.014 ±0.006	0.004 ±0.004	0	0	0	0	7.0

[1]Cultures with E1 effluent and 15% (v/v) fungal inoculum in mycological broth were prepared as described in the text. Values are expressed as the mean of 3 determinations ± standard deviation (nd = not determined). At day 5, the average dry weights of the fungal mycelia were: 52J, 0.268 g; M49, 0.204 g.

Table A3.1. Characteristics of *T. versicolor* mutant M49.

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