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Section 2 **Test Methodology and Assessment**

What molecular biology can tell us about the biodegradation of lignocellulose: the utilization of molecular techniques for the detection, identification and enhanced understanding of wood degrading organisms

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What molecular biology can tell us about the biodegradation of lignocellulose: the utilization of molecular techniques for the detection, identification and enhanced understanding of wood degrading organisms

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ABSTRACT

Molecular techniques are now routinely used in the identification, detection and analysis of wood degrading organisms. An overview of some of the early work on nucleic acid isolation and characterization will be followed by a discussion of the power of sequencing and other procedures for better understanding: the mechanisms involved in the biological degradation of wood, the metabolic basis of preservative function and ultimately the evolutionary history and ecological function of some of these unique organisms.

Keywords: biodegradation, PCR, genomics, sequencing, epigenetics, phylogeny, detection, evolutionary, molecular, decay fungi, termites, *Limnoria*

INTRODUCTION

The development of molecular methods provides an array of tools and opportunities within the field of wood protection. The following review is not meant to be inclusive of the wide range of molecular work done but rather to give a broad overview of selected previous studies and a brief introduction to on-going work which may have relevance to the wood protection community.

DETECTION AND IDENTIFICATION OF DECAY FUNGI

Microbiological decay of wood caused by fungi can cause rapid structural failure. Traditional methods for evaluation of decay and performance such as those based on mass loss or mechanical strength evaluation in the laboratory and on visual inspection and pick-test in field trials, are useful but can lack sensitivity and may provide limited information on the nature of decay. Isolation of fungi from degrading wood and the subsequent identification of the fungal species based upon cultural characteristics can provide useful information but is slow, and often difficult. The use of molecular techniques can provide researchers with a rapid and reliable research tool. Early researchers identifying fungi in pure culture often focused on characteristics of the portion of the genome associated with the ribosomal DNA and in particular on the internal spacer regions ITS1 and ITS2. These regions do not code for proteins and therefore tend to be less conserved than many of the constrained coding regions and have therefore proven useful in distinguishing among fungal species. Researchers have used basidiomycete specific primers in conjunction with RFLP analysis (Schmidt and Moreth 2002) and/or sequencing to amplify and identify fungal DNA and have also designed numerous species-specific primers for use in fungal identification. The utility of these techniques in the field however is somewhat limited. The ability to isolate DNA directly from wood without culturing, and/or laborious cloning, was developed in the late 1990's and the improvements in DNA extraction and purification, DNA sequencing technology, and large amounts of sequence and primer design information available have made this technology more accessible (Glaeser and Linder 2010). Identification and quantification of fungi from environmental samples remains challenging with issues ranging from nucleic acid degradation and interference of wood decay products and wood extractives, to the prevalence of mixed microbial samples in the analysis. Researchers have

approached some of these challenges by developing specialized extraction techniques and protocols ranging from species-specific primers to utilization of rDNA T-RFPL (Raberg *et al.* 2005). Despite remaining technical challenges nucleic acid-based assays are becoming increasingly prevalent and are capable of detecting and identifying degradative organisms early in the decay process, in some cases down to 0.3% weight loss (Jasalavich *et al*. 2000).

GENOMICS AND MECHANISMS

In 2004 the genome sequence was published for the white rot *Phanaerochaete chrysosporium* (Martinez *et al.* 2004). The sequence was obtained using a whole genome shotgun approach and was the first published for the Basidiomycota. Numerous genes were identified including sequences for secreted oxidases, peroxidases, and hydrolytic enzymes. Extracellular alcohol oxidases, major components of the lignin degradative system including isozymes of lignin peroxidase, manganese-dependent peroxidase, glyoxyl oxidase, copper radical oxidases (although no conventional laccase) and more than 240 putative carbohydrate enzymes were encoded. Since that time genomes of multiple other degradative organisms have become available. The 2009 sequencing of *Postia placenta* provided an informative comparison between a model white and brown rot organism (Martinez *et al.* 2009). The sequence of *Postia* showed an assortment of extracellular glycoside hydrolases but lacked both genes encoding exocellobiohydrolases and cellulose binding domains, confirming metabolic and molecular observations that the brown rot fungi employ a cellulose-degrading mechanism much different from many of the other cellulytic organisms. *Postia* also lacked most class II fungal peroxidases. Sequences for iron reductases, perhaps related to previously observed glycoproteins and genes involved in iron homeostasis were detected. Wymelenberg et al. 2010, showed upregulation of ferroxidase and iron permease. Genomic and transcriptomic analysis has also demonstrated a glyoxylate shunt in *Postia*. Previous work had suggested that a metabolic shunt between the citric acid and glyoxylate cycles may be central to oxalic acid accumulation by the brown rot *Fomitopsis palustris*. Subsequent sequencing efforts have included *Serpula lacrymans* (Eastwood *et al*. 2011) providing further insight into brown rot metabolism and fungal evolution and *Cerioporis subvermispora* (Fernandez-Fueyo *et al.* 2012) allowing a comparison between the genome of a selective and non-selective ligninolysis in a white rot organism.

Currently around 80 genomes are publically available from the Basidiomycota including around 37 from decay fungi. Numerous additional genomes have been sequenced but have not yet been publically released. The availability of genomic data allows researchers to better understand and explore changes in RNA, protein and fungal metabolites as a function of various environmental factors including growth on simple vs. complex carbon sources. Combining genomic studies with protein and metabolite analysis in response to varying environmental conditions provides a more complete picture of decay mechanisms and the differential capabilities of white and brown rot species. A comparative transcriptome analysis in 2010 between *P. placenta* and *P. chrysosporium* looked at gene expression patterns on glucose vs. aspen wood, supplementing the nucleic acid data with extensive LC-MS/MS to identify induced and repressed proteins (Wymelenberg *et al.* 2010). Differences were seen in the composition and levels of expression for extracellular glycosyl hydrolases, hemicellulases and in the patterns of expression for oxidoreductase –encoding genes. In *P. placenta* the patterns were consistent with an extracellular Fenton system (Goodell *et al.* 1997). Protein analysis was also important in identifying the significant involvement of hemicellulose hydrolysis in *P. placenta*. Up-regulation of 1,4 benzoquinoe reductase in wood cultures was consistent with observations of 1,4-benzoquinone reductase previously reported to be inducibly expressed in *G. trabeum* (Qi and Jellison 2004).

Extensive work has been done involving the study of specific genes and gene products. For example the work on oxidoreductases as possible enzymatic extracellular sources of hydrogen peroxide for driving ligninolytic and Fenton reactions in white and brown rot fungi (Daniel *et al.* 1994, 2007). Studies on alcohol oxidase from *G. trabeum* as an extracellular source of hydrogen peroxide (Daniel *et al.* 2007) included purification of a homooctameric nonglycoslated protein which catalysed short chain primary aliphatic alcohols with methanol as the preferred substrate. This work involved cDNA analysis and the localization of the purified oxidase in the hyphal periplasmic space and fungal wall and on the extracellular tripartite membranes and slime layer. The preference for methanol, available from demethylation of lignin, suggested not only a possible role for this enzyme in the production of hydrogen peroxide but also the coupling of lignin-and carbohydrate biotransformation. Up-regulation of transcripts associated with methanol oxidase in wood vs. glucose based culture was also noted in subsequent genomic work (Wymelenberg *et al*. 2010) for both *P. placenta* and *P. chrysosporium*. Focused gene expression studies are important because they contribute to annotation, to the broader genomic overview and allow for a deeper mechanistic understanding of the expression, control and degradative role played by specific gene products.

RELEVANCE TO WOOD PROTECTION

Issues of sustainability, carbon sequestration and performance converge in a continuing search for environmentally benign methods of wood protection (Rowell *et al.* 2009). In order to optimize new wood protection systems one needs to understand their mode of action against wood deteriorating organisms. Current wood protection systems are often based on a toxic mode of action, like copper based systems, or a non-toxic mode of action, like modified wood. Based on the literature currently available, the degree of cell wall bulking in combination with lowering of the equilibrium moisture content seems to be a primary mode of action but the mechanism by which modified wood is protected from microbiological decay is still not completely understood. The use of molecular methods will be important in enabling us to understand the mechanistic basis of new wood protection systems and can also provide insight into where resistance in wood decay fungi might emerge, providing opportunities for mitigation strategies before problems arise.

In recent work, Tang *et al.* 2013, used high throughput transcriptomics to identify *Fibroporia radiculosa* genes that were differentially regulated on wood treated with copper preservative. Highly expressed genes included those with putative functions related to oxalate production/degradation, ATP production, and cytochrome P450 activity. Selected genes including those coding for isocitrate lyase, glyoxylate dehydrogenase and oxalate decarboxylase showing differential expression on preservative treated wood were further examined using RT-PCR. Tang *et al.* 2011, 2013). Previous qRT-PCR studies with *P. placenta* had examined the response *oah, glx* and *icl* at different growth stages and in response to varying nutrient compositions had identified OAH as the dominant oxalate producing step but had not shown a consistent response to metal concentrations (Carlson 2011). Examining genes related to oxalate metabolism and the flux of carbon through the GLOX system, has relevance for both understanding potential means of preservative tolerance and also may provide clues to differences in energy metabolism between brown and white rot fungi.

EVOLUTIONARY AND ECOLOGICAL STUDIES

Research initiated by Hibbett and Donoghue, 2001, provided a correlation among wood decay mechanisms, mating systems and substrate preferences among the homobasidiomycetes. The work indicated that the loss of exoglucanases and of lignin degrading enzymes is an evolutionary advancement in the brown rot fungi allowing them to reduce reliance on metabolically-expensive enzyme systems in favor of simpler free-radical based systems. This work, important from both a taxonomic and evolutionary standpoint showed that brown rot genera have repeatedly evolved from the ancestral white rots with diversification of fungal nutritional modes occurring concomitant with the diversification of angiosperms and gymnosperms.

The ability of wood decaying fungi to break down lignocellulose is linked to the co-evolution of boreal forests and fungi. Work by Eastwood *et al.* 2011, on the comparative and functional genomics of the dry rot fungus *Serpula lacrymans*, further examined the evolution of the brown rot life style. Transcriptome and proteome analysis of *S. lacrymans* identified differences in wood decomposition relative to *P. placenta*, as might be expected in cases of a diverse phylogenetic origin of the brown rots. Convergent changes in enzyme complement were also seen in the two independently evolved rot species. Brown rot oxidoreducatses included iron and quinone reductases and multicopper oxidases. Work on a comparative analysis of 31 fungal genomes suggested that the origin of lignin degradation may have coincided with the sharp decrease in the rate of organic carbon burial at the end of the Carboniferous period and provided further evidence suggesting that lignin degrading peroxidases present in ancestral white rots contracted in parallel brown rot and mycorrhizal lineages (Eastwood *et al.* 2011; Floudas *et al.* 2012)

BEYOND BROWN AND WHITE ROTS

Biological degradation of wood is not limited to the brown and white fungi. Cellulolytic activity is present in many other organisms, including bacteria, stain fungi, soft rots and common wood inhabitants such as *Trichoderma* and molds such as *Aspergillus*, many of which have genes coding for numerous glycolytic, oxidative and other relevant enzymes involved in processes ranging from iron metabolism to oxalate production. Sequence data for many of these organisms is already available and will allow us to better understand the genetic and mechanistic basis for the “life style” differences among these organisms and their differing capacity to degrade the wood cell wall.

Other interesting organisms with cellulolytic abilities include successful wood degraders such as the termites which are capable of efficient wood degradation enabled by a special complement of gut symbionts. The scope of this review does not allow an extensive discussion but molecular techniques have been used to assist in developing a better understanding of the microbial interactions within the termite gut allowing for lignocellulose degradation (Honogoh 2011) and also to allow differentiation of termite colonies and to contribute to control strategies (Simms and Husseneder 2009). Early molecular studies focusing on the highly destructive Formosan subterranean termite *Coptotermes formosanus* established the utility of a molecular approach (Husseneder and Grace 2001; Husseneder *et al.* 2003). The lack of genetic variation including low mtDNA variability in populations of *C.*

formosanus has been challenging but researchers have overcome this limitation through the use of microsatellites, which have higher variability than mtDNA and a different mode of inheritance (Husseneder *et al*. 2003). Microsatellite studies have yielded information on patterns of invasion biology, population diversity and breeding structure, allowing colony assignment and suggesting multiple introductions into the US (Husseneder *et al*. 2012). The number of termite species considered to be globally invasive has increased from 17 in 1967 to 28 today. Molecular tools have become increasingly important for identifying new invaders and tracing their paths of introduction, and for both discriminating among and for synonymizing morphologically similar species occurring together or in different locations (Evans *et al*. 2013). Interestingly, symbiont diversity does not seem to be affected by the introduction of the termites to new habitats (Husseneder *et al*. 2010). Additional studies looking at the lower termite *Reticulitermes flavipes* have used translational genomics to examine the synergism among host and microbial enzymes to allow lignocellulose digestion (Scharf *et al.* 2011).

Another interesting group of wood degrading organisms currently being examined using molecular techniques is group of the marine isopods from the family Limnoriidae, colloquially known as gribble. These organisms are of particular interest because the gut of the organism is sterile (itself a remarkable situation) (Boyle & Mitchell 1978) and their capacity to digest wood does not appear to be dependent upon symbiotic microbes. EST's from the digestive gland (hepatopancreas) of *Limnoria quadripuctata* showed a transcriptome dominated by glycosyl hydrolases including CBH's (not previously reported in animal genomes). In-situ hybridization demonstrated that the genetic code for these enzymes is transcribed in the cells of the hepatopancreas. A possible role for hemocyanins in lignocellulose degradation was also suggested (King *et al.* 2010). Phylogenies including GH5, 7 and 9 enzymes from *Limnoria* indicate that the GH9 is of a type found in a wide range of animal taxa, but that the GH7 sequences are found in a range of crustaceans, but in no other animal group. Evidence of marine wood degrading communities extends back to the early Jurassic and the possibility of ancestral horizontal gene transfer of GH7 and other sequences is an intriguing possibility. This genome is currently being sequenced and epigenetic studies to detect markers for the upregulation of specific enzymes is underway. Another group of marine organisms, wood boring molluscs of the family Teredinidae (shipworms), also degrade wood but unlike the gribble, have resident gut microorganisms that may play a significant role in wood digestion (Betcher *et al*. 2012). Shipworms also have a remarkable symbiosis with nitrogen fixing and cellulose producing bacteria, one species of which has been fully sequenced to generate a full genome (Yang *et al.* 2009).

CONCLUSION

Molecular techniques and the continuing development of useful databases including CAZyme (cazy.org), the genomic encyclopedia of fungi (genome.jgi.doe.gov), the fungal genome initiative (broadinstitute.org) and numerous others, provide a useful set of tools to enable us to better understand and control biological degradation of lignocellulose. Whole genome studies enable the regulatory mechanisms of genes of interest to be probed more effectively and for associations among genes to be detected. Needed developments include: a commitment to sharing genomic data, additional annotations that describe gene function, and substantial work better characterizing gene products and secondary metabolites relative to their function, localization in the cell, and metabolic control. An enhanced understanding of important epigenetic and other regulatory mechanisms of degradative systems, including the mechanisms involved in preservative tolerance, along with a better understanding of the biological diversity and complex interactions often associated with 'real life' decay scenarios will contribute to our ability to protect wood in effective and environmentally appropriate ways.

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