DIVERSITY OF ASCOMYCETE LACCASE SEQUENCES AND CONTRIBUTIONS OF BACTERIA AND ASCOMYCETOUS FUNGI TO LIGNOCELLULOSE DEGRADATION IN A SOUTHEASTERN U.S. SALT MARSH

by

JUSTINE ISABELLE LYONS MORETA

(Under the direction of Dr. Mary Ann Moran)

ABSTRACT

In the first part of this study, we used molecular tools to create a database of protein sequences for laccase genes of salt marsh ascomycetes. Laccase is one of the enzymes shown to be involved in fungally-mediated lignin degradation. We then used this database to identify laccase sequences in the natural decomposer community on blades of *Spartina alterniflora* in two stages of decomposition. The sequences we collected contribute significantly to the relatively limited database of ascomycete sequences that currently exists. The second part of this study addressed the relative activity levels of bacterial and fungal decomposers by measuring rates of lignocellulose degradation in a simple microcosm system. The results of this study provide a tool for future evaluation of the effects of genetic manipulations on ecologically relevant decomposer organisms, including studies of gene function and degradative efficiency and interactions between bacterial and fungal decomposers.

INDEX WORDS: Laccase, Ascomycete, Fungal, Bacterial, Decomposition, Lignocellulose

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DEDICATION

This thesis is dedicated to my parents, Jean and Terry Krugman, with deepest gratitude for all their support during the many life changes I faced while working on this research. I can never say "thank you" enough. I love you both.

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CHAPTER 1

INTRODUCTION

The vast tidal salt marshes of the southeastern U.S. are highly productive ecosystems, dominated in many areas by the macrophyte smooth cordgrass, *Spartina alterniflora.* This high primary productivity is generally not consumed by herbivores, and instead supports an active microbial community that degrades the decaying plant material. The major portion of organic matter that enters salt marsh food webs is therefore in the form of the highly refractory polymeric complex, lignocellulose. The lignocellulose fraction constitutes 75-80% (ash-free dry weight) of the biomass of *Spartina* (Benner et al., 1988). Marsh microorganisms transform the lignocellulose into biomass that is more available to other organisms.

When examining the decomposition of emergent macrophytes, it is important to consider the spatial and temporal conditions under which plant litter decomposes. In the case of *S. alterniflora*, collapse of shoot material to the sediment surface does not occur immediately following shoot senescence and death (Newell et al., 1998). This results in the accumulation of standing dead plant matter, which undergoes considerable initial microbial decay prior to its collapse into the aquatic environment.

Intertidal marshes provide an excellent opportunity to undertake studies of the interactions of prokaryotes and eukaryotes in decomposer communities. Heterotrophic microorganisms in a detritus-based salt marsh system are a major link in the

mineralization and transformation of organic matter. Aerobic microorganisms include bacteria, fungi, protozoans, and microscopic metazoans. Although some studies have suggested that bacteria and fungi dominate different stages of decay (Pomeroy and Wiegert, 1981; Benner et al., 1986; Newell and Porter, 2000), others suggest that the two form a consortium, and may work together to break down their substrate (Benner et al., 1984; Newell and Porter, 1999).

Ascomycetous fungi are one of the principal microbial secondary producers in decaying salt marsh grasses (Newell, 1996). Fungal mycelium can be found on the surface, in the hollow center of the culm, and intra- and extracellularly within the tissues of *S. alterniflora* (Gessner, 1980). Observations of fungi on aerial parts of *S. alterniflora* and their association with detritus suggest that they are active in its decomposition. Most evidence suggests that fungi use nonspecific, extracellular enzymes to modify the lignocellulose complex (Gessner, 1980).

Bacterial assemblages attached to vascular plant litter make contributions to total particulate organic mass that are very small (Newell, 1996). However, their overall contribution to the degradation of lignocellulosic carbon has been shown to be quite high (Benner et al., 1986; Newell and Palm, 1998). A recent study using molecular techniques to characterize the taxonomy of the bacterial decomposer community in a southeastern U.S. salt marsh found that α-Proteobacteria dominate the community, although γ-Proteobacteria, Cytophaga-Flexibacter-Bacteroides, and Gram positives are also present (Buchan et al, in prep).

Few studies to date have looked at the interactions between bacteria and fungi during the decomposition of salt marsh grasses (Newell et al., 1989; Kuehn et al., 2000).

Even fewer have used molecular methods to do this (Buchan et al., 2002; Buchan et al., in prep.). However, molecular tools allow insight into genetics and enzyme pathways that otherwise would not be available. Enzymes such as the ones that fungi use to degrade salt marsh grasses are of great interest from an ecological standpoint, and may lend themselves to molecular approaches.

In the first part of this study, we used molecular tools to create a database of protein sequences for laccase genes of salt marsh ascomycetes. Laccase is one of the enzymes shown to be involved in fungally-mediated lignin degradation. We then used this database to identify laccase sequences in the natural decomposer community on blades of *Spartina alterniflora* in two stages of decomposition. The sequences we collected contribute significantly to the relatively limited database of ascomycete sequences that currently exists.

The second part of this study addressed the relative activity levels of bacterial and fungal decomposers by measuring rates of lignocellulose degradation in a simple microcosm system. The results of this study provide a tool for future evaluation of the effects of genetic manipulations on ecologically relevant decomposer organisms, including studies of gene function and degradative efficiency and interactions between bacterial and fungal decomposers.

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CHAPTER 2

DIVERSITY OF ASCOMYCETE LACCASE GENE SEQUENCES IN A SOUTHEASTERN U.S. SALT MARSH[1]

[1]Lyons Moreta, J.I., S.Y. Newell, A. Buchan, and M.A. Moran. To be submitted to *Microbial Ecology.*

Abstract

A database of salt marsh ascomycete partial laccase sequences was created using degenerate primers designed around two of the four copper binding sites conserved in fungal laccases. Laccase sequences were amplified from genomic DNA extracted from twenty four isolates representing ten ascomycete species. Twenty-one isolates yielded a PCR product of expected size (~900 bp) that was tentatively identified as laccase based on sequence similarities to previously published laccase sequences from related organisms. Overall, fifteen distinct sequence types were identified, with several species yielding multiple distinct laccase types. The database was used to identify laccases amplified directly from early and late decay *Spartina alterniflora* blades from a southeastern U.S. salt marsh. Seven laccase types were identified from the community samples. Of these, five were >96% similar to sequences from three ascomycete species previously found to dominate fungal communities on decaying *S. alterniflora* blades. Two were novel laccase sequences.

Introduction

Lignin degradation is extremely important in the global carbon cycle. This complex aromatic polymer constitutes 20-30% of wood and other vascular tissues of plants, and is one of the most abundant renewable materials on Earth. The molecular units that make up lignin are linked by nonhydrolyzable C-C and ether (C-O-C) bonds and the structure is highly irregular. These features render it resistant to attack by most organisms, and dictate that a lignin biodegradation system be relatively nonspecific and extracellular. Lignin also reduces the bioavailability of other cell wall constituents, protecting plant carbohydrates from microbial attack by physical exclusion and reducing the surface area available to enzymatic attack.

One of the principal groups of organisms responsible for lignocellulose degradation is aerobic filamentous fungi. In southeastern U.S. marshes, salt marsh fungi colonize senescing blades of the dominant primary producer, *Spartina alterniflora.* Here, fungal productivity on smooth cordgrass can average almost 550 g m^{-2} y^{-1} (Newell, 2001a). As they decompose plant matter, fungi efficiently break down the lignin polymer into smaller, low molecular weight fragments that are then available either for conversion into fungal biomass, or for further decomposition by bacteria.

Most evidence suggests that fungi use nonspecific, extracellular enzymes to modify the lignin macromolecule (Gessner, 1980). Since the lignin is interconnected by stable ether and carbon-carbon bonds, the mechanisms by which the enzymes function must be oxidative rather than hydrolytic. Enzymatic action removes various functional groups, side chains, and aromatic rings randomly from the lignin macromolecule.

Laccases are one of the three families of enzymes that are responsible for the initial fragmentation of the lignin polymer and production of low molecular weight breakdown products. This enzyme (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a member of the blue multi-copper family of enzymes, and is typically an extracellular enzyme with a molecular mass of ~65 kDa. Laccases catalyze the one-electron oxidation of a wide variety of substrates (typically mono-, di-, and polyphenols, aromatic amines, methoxyphenols and ascorbate) coupled to the four-electron reduction of dioxygen to water (Thurston, 1994). In lignin degradation, the mechanism of action of laccases is to generate free radicals which, because of their chemical instability, subsequently undergo a variety of spontaneous cleavage reactions.

The involvement of laccase in lignin degradation has been often investigated and sometimes debated. Some studies show that lignin can be degraded by basidiomycetes without laccase (Thurston, 1994). However, Ander and Eriksson (1976) demonstrated diminished ability to degrade lignin in laccase-minus mutants of the basidiomycete *Phanerochaete chrysosporium*, coupled with recovery of lignolytic ability in laccase-plus revertants. Further, Eggert et al. (1997) showed that laccase-minus mutants of another basidiomycete, *Pycnoporus cinnabarinus*, were greatly diminished in their ability to metabolize ^{14}C-ring-labeled synthetic lignins to $^{14}CO_2$. Addition of purified laccase to the mutants increased $^{14}CO_2$ evolution to rates comparable to those generated by wild-type (laccase-plus) strains. These results indicate that laccase is essential for lignin degradation in some species of fungi.

Interest in laccases has been fueled not only by their involvement in lignin degradation but also by their potential uses in detoxification of environmental pollutants

(Amitai et al., 1998), prevention of wine decoloration, paper processing (Li et al., 1999), and production of useful chemicals from lignin. The occurrence of laccase is widespread amongst fungi and higher plants, but the enzyme appears to have different functions in different organisms. For example, plant laccases have a role in the lignification of differentiating xylem tissues (O'Malley et al., 1993), while in plant-pathogenic fungi, laccases can be produced as 'attack' enzymes during the infective process (Edens et al., 1999).

In recent years, laccase gene sequences have been reported from a number of fungal species. Many of these manifest as gene families containing either several alleles of the same gene (Germann et al., 1988), or in some cases, up to five distinct laccase genes (Yaver et al., 1996; Mansur et al., 1997; Yaver et al., 1999). These different laccase genes are regulated by factors such as pH, substrate condition, and fungal metabolism (Broda et al., 1995). Multiplicity of laccase genes differing only slightly in sequence is not uncommon in fungi (Germann et al.,1988; Mansur et al., 1997)

In this study, we investigated laccases of ascomycetous fungi found on decaying *Spartina alterniflora* blades in a southeasterm U.S. salt marsh. PCR primers were designed to amplify the region of fungal laccase that lies between two of the conserved copper binding sites (Fig 2.1). We tested these primers on 24 isolates representing ten ascomycete species potentially involved in *S. alterniflora* decomposition. The sequences were then used to create a database to which laccase sequences from the natural *S. alterniflora* decomposition community were compared.

Materials and Methods

Fungal isolations

Twenty-four fungal isolates were cultured from decaying *S. alterniflora* for laccase analysis. Mycelial ascomycetes were isolated by an ascospore-drop technique (Luttrell, 1979). Wet blade pieces were positioned in a sterile plastic dish (60 x 15 mm) above dilute V8 agar (DV8; 2% V8 [Campbell Soup, Inc.], 2% agar, 1.5% sea salts). Incubation was at 25°C in indirect sunlight under natural day/night lighting. Ascospores that had been expelled onto the agar surface were removed with a flame-sterilized, flattened-tip nichrome needle under the dissecting microscope and transferred onto new plates of dilute V8 agar. A wet, autoclaved piece of senescent (yellow-green) blade of *S. alterniflora* was added to the surface of the isolation plate adjacent to the transferred ascospores to ensure availability of natural substrate. Mitosporic fungi (fungi that produce asexual spores and lack a sexual stage) that were observed directly on the incubated blade pieces were brought into culture by manipulation of conidia. Ascospores of ascomycetes that did not eject spores in spore-drop plates were also isolated by nichrome-needle micromanipulation after cutting open ascomata at the dissecting microscope using DV8 plates.

The fungal isolates were maintained in sterilized sections of blades on DV8 plates. For generation of mycelium for DNA extraction, 5-10 small pieces (≅ 1 mm^3 volume each) of DV8 culture were incubated statically at 25°C in liquid malt/yeast extract medium (2.0% malt extract, 0.2% yeast extract, 1.5% sea salts). After a substantial quantity of mycelium had been formed (after approximately two weeks of

growth), the liquid medium was withdrawn by sterile pipette in a laminar-flow cleanhood and the mycelium stored wet at room temperature until subsequent DNA extraction.

Cultures of the isolates are maintained in the laboratory of S. Y. Newell, and representatives have been submitted to ATCC (*Phaeosphaeria spartinicola* SAP132, *Phaeosphaeria spartinicola* SAP135, *Phaeosphaeria halima* SAP134, *Mycosphaerella* sp. 2 subgroup A SAP153, *Mycosphaerella* sp. 2 (Kohlmyer and Kohlmeyer, 1979) subgroup B SAP154, *Hydropisphaera erubescens* SAP145, and '4clt' SAP162).

Enzyme assay

To rapidly screen fungal isolates for laccase activity, a colorimetric plate assay was carried out using syringaldazine as the laccase substrate (Gessner, 1980). Isolates were cultured on malt/yeast extract plates containing the following: 0.1% ground plant material (sterile, dried *S. alterniflora*), 0.04% malt extract, 0.04% yeast extract, 2.0% sea salts (Sigma Chemical Co., St. Louis, MO), 2.35 mM $NaNO_3$, 1.72 mM K_2HPO_4, 1 mM KH_2PO_4, and 1.6% agar. Plates were inoculated with the following strains: *Buergenerula spartinae* (SAP12 & 124), *Phaeosphaeria spartinicola* (SAP132 & 135), *Mycosphaerella* sp. 2 subgroup B (SAP133 & 136) (Buchan et al., 2002), *Phaeosphaeria halima* (SAP134 & 137), and *Lachnum spartinae* (SAP138). After fourteen days of incubation at room temperature and natural lighting, the plates were flooded with a 0.1% syringaldazine solution (0.1% syringaldazine in 95% ethanol). A positive test for laccase was indicated by the immediate appearance of a pink color.

Community DNA samples

Decaying blades of tall-form *S. alterniflora* were collected from Dean Creek Marsh, Sapelo Island, in July 2000 for studies of laccases from natural fungal communities. Dean Creek Marsh is located within Site 6 of the Georgia Coastal Ecosystems Long Term Ecological Research sampling area and is typical of southeastern U.S. salt marshes (Chalmers, 1997) (maps available at http://gce-lter.marsci.uga.edu/lter/asp/studysites.htm).

Three 5-m diameter replicate plots (Plots 1-3) were established in Dean Creek Marsh. Dead, attached blades of *Spartina alterniflora* in two distinct stages of decomposition were collected: "early decay" blades (S) were yellow or brown in color, remained attached to the stem, and were not yet collapsed onto the sediment; "late decay" blades (L) were brown to black in color and also remained attached to the stem, but were collapsed onto the sediment surface. Ten blades in each category were collected from each plot and pooled for DNA extraction. Only *S. alterniflora* blades collected in Plot 1 were used in this study, but data on fungal community composition in all three plots are available (Buchan et al. 2002).

DNA extractions and laccase amplifications

Genomic DNA was extracted from mycelia of fungal isolates and from the pooled samples of ten blades of decaying *S. alterniflora* collected from Dean Creek Marsh, Plot 1.

DNA extractions were carried out using Soil DNA Extraction Kits (MoBio, Solana Beach, CA). The kit uses a combination of heat, detergent, and mechanical force

to lyse the microorganisms in the grass. The sample is first agitated with beads and a guanidine isothiocyanate solution, then heated for 30 minutes at 65°C with an inhibitor removal solution and an aqueous solution containing tris (hydroxymethyl) aminomethane / hydochloric acid, and sodium dodocyl sulfate. The solution is centrifuged, the supernatant is collected and mixed with an aqueous solution of acetate, and then centrifuged again. The supernatant is then mixed with an aqueous solution of guanidine HCl. The DNA is collected on a spin filter, cleaned with an aqueous solution of tris(hydroxymethyl) aminomethane/hydrochloric acid, ethylenediaminetetraacetic acid, sodium chloride, and 50% ethyl alcohol, and collected into an elution buffer of tris(hydroxymethyl) aminomethane / hydrochloric acid. The DNA can then be concentrated by ethanol precipitation.

Prior to this study, no general fungal laccase primers were available that were suitable for use with environmental samples. Therefore, primers were designed by compiling approximately twenty published laccase sequences from a mixture of organisms including plants, bacteria, basidiomycetes, and ascomycetes. The degenerate primers were designed to be specific to fungal laccase, and targeted conserved sequences around the two pairs of histidines [G140-H146 and P476- G483 of *N. crassa* strain TS, accession number M18334; (Germann et al., 1988)] involved in two of the copper binding regions (designated II and III) of known ascomycete sequences (Fig. 2.1) The forward (LAC2FOR; 5' GGI ACI WII TGG TAY CAY WSI CA 3') and reverse (LAC3REV; 5' CCR TGI WKR TGI AWI GGR TGI GG 3') primers amplified a product of ~900 bp. Ambiguous bases are defined as follows: R= A/G; W= A/T; Y= C/T; S= C/G; K= T/G; I= Inosine.

Positive controls and PCR conditions

Four ascomycete isolates whose laccase sequences were already available were used as positive controls in PCR amplifications. Strains of *Aspergillus nidulans* (accession number X52552), *Cryphonectria parasitica* (accession number S38903), *Saccharomyces cerevisiae* (accession number D50617), and a *Podospora* sp. (accession number Y08827) were obtained from the University of Georgia Department of Plant Sciences (courtesy of Dr. Richard Hanlin).

PCR was carried out using Buffer E (Epicentre Technologies, Madison, WI), with 50 µM concentration of each primer and 50-100 ng of DNA. An initial 3 min at 95°C was followed by 35 cycles of 45 sec at 95°C, 45 sec at 47°C, and 3 min at 72°C. A final step of 10 min at 72°C was included to complete any partial polymerizations.

Construction of laccase clone libraries

Since fungi can contain several laccase alleles and multiple distinct laccase genes, amplified products from isolates were cloned before sequencing. Amplified products containing mixtures of laccase genes from environmental samples were also cloned. To construct clone libraries, amplified laccase gene products were recovered from a 1% agarose gel using a QiaSpin Gel Extraction Kit (Qiagen, Valencia, CA) and cloned using a TA Cloning Kit (Invitrogen Corp., Carlsbad, CA). Clone libraries were created for all samples that yielded PCR products of appropriate size.

Sequencing and phylogenetic analysis

All samples were sequenced at the University of Georgia Molecular Genetics Instrumentation Facility. Approximately 600 bp of sequence information was obtained in each direction by sequencing purified plasmid DNA using the M-13 forward and reverse primers. Sequences were edited and assembled using the AssemblyLign program (Oxford Molecular, 1998). The forward and reverse reactions resulted in a complete sequence for the amplified region of the laccase gene (~925 bp). Sequences were analyzed using the Wisconsin Package Version 10.2 (Genetics Computer Group (GCG), Madison, Wisc.), and BlastX searches were carried out using the National Institute of Health's NCBI sequence database. A phylogenetic tree was created at the protein level using the Phylip package, using evolutionary distances (Kimura algorithm) and the neighbor-joining method.

Results

Ascomycete isolates and initial screens

The following species isolated from the Sapelo Island salt marsh were used to establish a salt marsh fungal laccase sequence library (Table 1): *Buergenerula spartinae* (3 strains), *Phaeosphaeria spartinicola* (4 strains), *Mycosphaerella* sp. 2 (5 strains), *Phaeosphaeria halima* (4 strains), *Lachnum spartinae* (1 strain), ascomycete '1ch' (1 strain), coelomycete 7 septate appendaged *Stagonospora* (1 strain), *Pleospora spartinae* (1 strain), *Halosarpheia viscosa* (1 strain), and ascomycete '4clt' (3 strains).

Representatives of the four species that typically dominate fungal communities on *S. alterniflora* in southeastern U.S. salt marshes (*P. halima*, *P.*

spartinicola, *B. spartinae*, and *Mycosphaerella* sp. 2) (Gessner et al., 1972; Newell, 2001b) were chosen for the laccase plate assay, as well another less common species, *L. spartinae* (Buchan et al., 2002). The assay gave positive results (pink color) for eight of the nine strains tested. Only in the case of *L. spartinae* was there no color change in the assay medium.

Multiple PCR products

The degenerate primers were tested on four ascomycetes known to contain a laccase gene. *Aspergillus nidulans*, *Cryphonectria parasitica*, *Saccharomyces cerevisiae*, and a *Podospora* sp. all produced PCR products of the expected size (~900 bp). However, in all positive control strains and in several of the Sapelo Island isolates, PCR amplifications also produced up to three other products that were either larger or smaller than the expected 900 bp product. These products were excised from the gel, cloned, and sequenced, but BLAST analysis indicated that they were not laccases. Adjustments to PCR conditions were unsuccessful in eliminating the non-specific amplification products. Subsequently, all PCR reaction mixtures were size-separated on a 1% agarose gel, and only the product of appropriate size (~900 bp) was excised and sequenced.

Laccase sequences from fungal isolates

Out of 24 ascomycete strains, 21 produced PCR products of the appropriate size. Of these, eighteen were identified as a putative ascomycete laccase based on the closest match in GenBank (Table 2.1). Several were highly related sequences (>97% amino acid similarity), such that overall, thirteen distinct putative laccase sequences were identified

from the isolates (Types II & III; V– XV; Fig. 2.2). Positions of putative introns were determined by comparing each deduced amino acid sequence to that of its closest identified relative.

Three strains produced PCR products of the appropriate size that were not identified as a laccase based on the most closely related sequence identified by BLAST analysis. *Buergenerula spartinae* (SAP124) produced a 900 bp product that was most similar to a mannitol transport protein from *Neurospora crassa*, SAP163 (‘4clt’) produced a product most similar to a cofilin-related protein from *N. crassa*, and SAP164 (‘4clt’) produced a product most similar to phenylalanyl t-RNA synthetase from *Saccharomyces cerevisiae*. Also, the two strains of *Mycosphaerella* sp. 2 subgroup A (SAP152 & 153) and one strain of *Buergenerula spartinae* (SAP126) did not produce a product after repeated attempts at PCR amplification, despite the fact that the colorimetric laccase screen was positive for all three of these strains.

The four *Phaeosphaeria halima* strains (SAP134, 137, 140, and 159) yielded three distinct laccase sequences (≤53.9% similar at the protein level). SAP140 yielded an example of each of the three sequence types (SAP140^{A}, SAP140^{B}, and SAP140^{C}). Additionally, each of the three sequence types was found in one of the other three *P. halima* strains. The three sequence types had distinct structures, with either one (SAP134, 137, and 140) or two (SAP159) introns, and intron positions were unique for each.

Phaeosphaeria spartinicola (SAP132, 135, 149, and 151) yielded two distinct sequences (<56.3% similar). The most prevalent sequence type was found in three of the four strains (SAP132, SAP135^{A}, SAP135^{B}, SAP149^{A}, SAP149^{B}). The other sequence

type appeared only in SAP151. Both sequence types contained only one intron, and the exon-intron structure was different between the two types.

The single *Stagonospora* species (SAP143) yielded two distinct sequence types (34.6% similar). Each sequence contained one intron, and the exon-intron structure was different between the sequences.

All other strains yielded one sequence each. Two clones of '4clt' (SAP162) produced the same sequence, which contained no introns. Three strains of *Mycosphaerella* sp. 2 subgroup B (SAP133, SAP136[A], SAP136[B], and SAP154) clustered together with no less than 96% similarity at the protein level. *Buergenerula spartinae* (SAP12) and *Halosarpheia viscosa* (SAP147) laccases each contained one intron, and the *Lachnum spartinae* (SAP138) laccase contained two. Finally, multiple clones of *Pleospora spartinae* (SAP146) and '1ch' (SAP142) produced one sequence type that clustered with >99% similarity. Neither of the laccases from these organisms contained an intron.

Laccase sequences from decaying **S. alterniflora** *blades*

The laccase regions amplified from the fungi established a database to which laccases amplified from natural community samples could be compared. Many of the laccase sequences amplified from *S. alterniflora* blades matched sequences from the isolate collection (Fig. 2.2). Eleven of 25 sequences were more than 97.5% similar at the protein level to isolates representing *P. halima* laccase type VIII and three were >95% similar to *P. halima* laccase type III. Five sequences were at least 98% similar to one of the *P.*

spartinicola sequence clusters (II), and four were more than 97.5% similar to the single *Mycosphaerella* sp. 2 subgroup B cluster (IX). One sequence clustered with *P. spartinicola* type VII (99% similar), and two sequences were novel. Of these, one (S9) identified with the published sequence from *Botryotinia fuckelania* (AY047482) and one (S2) with the published laccase sequence from *Colletotrichum lagenarium* (AB055709), although neither was a close match (≤48.9%). The novel sequence from S2 was slightly more similar (50.0%) to the laccase sequence from '4clt' determined in this study (SAP162). None of the cloned laccase sequences appeared related to '1ch', *Halosarpheia viscosa*, *Pleospora spartinae*, *Lachnum spartinae*, *Buergenerula spartinae*, or the *Stagonospora* sp., although these species have been identified by ITS sequences and direct microscopy to be a component of the *S. alterniflora* decay system (Gessner 1977, Newell 2001b, Buchan et al. 2002). However, Newell and Porter (2000) described all of these species, except for *Buergenerula spartinae*, to be rare in the system.

Overall, fifteen distinct laccase sequences were identified from the isolates and the environmental samples. Diversity was greater in the early decay stage clone library (Fig 2.3), which was dominated by two laccase types from *Phaeosphaeria halima* (III and VIII) and yielded two novel laccase sequences. The late decay stage clone library was also dominated by laccase sequences representing *P. halima*, but only one type (VIII) was represented.

Analysis of laccase genes

The overall exon-intron structures were identical within the laccase sequence clusters, but distinct between sequence clusters. Intron structure was also distinct in

strains that produced more than one distinct laccase. Sequences contained zero (SAP142, 146, 162), one (SAP 12, 132, 134, 135, 137, 140[A], 140[C], 143, 147, 149, 151), two (SAP 133, 136, 140[B], 154, 159), or three (SAP138) introns.

Discussion

Previous studies based on culturing and microscopy approaches had indicated that ascomycetes were the dominant fungal species in southeastern U.S. salt marshes (Newell, 2001a and b). We used an isolate collection containing representatives of ascomycetes most commonly observed by direct microscopy in decaying *S. alterniflora* blades from the Sapelo Island marshes (Newell, 2001a and b) to establish a laccase sequence database. Diversity of laccases was high among the isolates, with sequences from the 24 strains clustering into thirteen distinct groups based on protein sequence similarity.

Enzyme assay and PCR results

Buergenerula spartinae failed to yield a laccase PCR product, even though this species was positive for laccase production in the enzyme assay. The lack of a product in laccase-positive strains might be attributed to the presence of an intron in the regions of the gene where primer binding occurs. Consistent with this suggestion, an intron has been reported previously in the copper-binding region II of laccases from both *Agaricus bisporus* (Perry et al. 1993) and *Pleurotus ostreatus* (Giardina et al., 1995). Conversely, *Lachnum spartinae* gave a negative result in the enzyme assay but yielded a PCR product that identified with a laccase sequence. Laccase activity is known to be regulated by environmental factors, including temperature (Lang, Gonser, and Zadrazil, 2000),

substrate quality (Mansur et al., 1997), and even developmental stage (Zhao and Kwan, 1999). Thus the laccase of *L. spartinae* may not have been induced by the conditions under which the culture was grown.

Multiplicity of laccase sequences among sequence types

D'Souza et al. (1996) described multiple PCR products resulting from a single PCR reaction with basidiomycete laccase primers, and in some cases more than one product was identified as a distinct laccase. In our study, however, bands produced in initial PCR amplifications that were of a different size than expected with our primer set did not yield fragments related to laccases.

Because organisms can contain multiple distinct laccase genes or distinct alleles of a single laccase gene, PCR products of the correct size (900 bp) were cloned prior to sequencing. Using this strategy, a number of distinct laccase sequence types were recovered from the fungal isolate collection. Germann et al. (1988) compared laccase sequences from two strains of *N. crassa* and determined them to be alleles based on sequence similarities of >98% at the protein level. In this study, the *Phaeosphaeria spartinicola* type II cluster contained two subgroups with between-group similarities of >96%; the laccase genes represented by these *P. spartinicola* sequences are also likely to be alleles of the same laccase gene.

Intra-organismal heterogeneity among laccase genes in fungi has also been documented previously (Litvintseva and Henson, 2002; Mansur et al. 1997; Munoz, 1997; Yaver et al., 1999), and the multiplicity of sequence types yielded by single organisms in this study likely represents examples of distinct laccase genes within the

same isolate. Several isolates yielded two (*Mycosphaerella* sp.2, *Phaeosphaeria spartinicola*, and *Stagonospora* sp.) or even three (*Phaeosphaeria halima*) distinct laccases that exhibited protein similarities of <54%. Litvintseva and Henson (2002) identified three laccases in *Gaeumannomyces graminis* var. *tritici* that were <43% similar to each other at the deduced protein level. One of the three laccases was shown to be capable of oxidizing a lignin-like polymer (Edens et al., 1999). Evidence of unique amino acid composition in a region of the *G. graminis* laccase responsible for redox potential and enzyme specificity suggests that the three enzymes may have different substrate specificities and possibly different functions in the fungus. Still, the ability of laccase to oxidize compounds such as lignin depends on the redox potential of the surrounding environment, and multiple functions have been shown to be carried out by the same laccase in different situations (Litvintseva and Henson, 2002).

Comparison of laccase and ITS clusters

An earlier study in the Dean Creek marsh on Sapelo Island examined the taxonomic composition of the fungal community associated with decaying *S. alterniflora* (Buchan et al., 2002). In this previous study, amplified ascomycete ITS sequences identified *P. halima*, *P. spartinicola*, and *Mycosphaerella* sp. 2 as the dominant colonizers of both early and late decay stages of *S. alterniflora* blades. Laccase gene amplifications in this study were conducted using the same DNA samples (Plot 1, July 2000, early and late decay samples) as the ITS clone libraries of Buchan et al. (2002). Using protein sequence similarities of ≥96% as the criterion for assigning a laccase amplified from the natural community to a species represented in the fungal culture collection, we were able

to assign 23 of 25 laccase clones to known species. Laccases assigned to *P. halima* comprised 53% and 60% of sequences from the early and late decay stages, respectively; laccases from *P. spartinicola* contributed 20 and 30%; and laccases from *Mycosphaerella* sp. 2 contributed 13 and 10% to early and late decay stages. These findings are in agreement with the ITS taxonomic data, in which Buchan et al. (2002) found that *P. halima* made up 13% of the early decay ITS sequence library and 20% of the late decay library; *P. spartinicola* accounted for 22 and 35% of the early and late decay ITS sequences; and *Mycosphaerella* sp. 2 accounted for 59 and 20% of the early and late decay ITS sequences. Although the correlation between expected laccase representation in the natural community (calculated by multiplying the number of laccases found in each species by the abundance of that species in the ITS clone library) and representation in the actual laccase clone library was insignificant ($r=0.75$; $df=4$), results from both DNA markers indicate dominance in the *S. alterniflora* decomposer community by these three ascomycete species.

The fact that several of the dominant ascomycetes contain multiple laccases (Fig. 2.2) may explain the over-representation of laccases from *P. halima* in clone libraries of the natural community relative to ITS sequence abundance from these same organisms. Furthermore, the under-representation of *Mycosphaerella* sp. 2 may be due to the fact that only one of the subgroups (subgroup B) identified by Buchan et al. (2002) yielded an amplifiable laccase PCR product. While biases in both ITS and laccase sequences may arise from interspecies variability in DNA extraction efficiency, primer binding, and cloning efficiency, the number and diversity of laccases appears to be greater in the most successful fungal species in the *S. alterniflora* decomposer community.

Several species found in previous studies to be present in the *S. alterniflora* decay system (Buchan et al., 2002; Gessner, 1977; Newell, 2001b), including *Buergenerula spartinae* and '4clt', were not represented in our analysis of environmental samples. Possibly, the relatively small sample size of environmental laccases limited our sensitivity in detecting rarer genes. The two novel sequences found in the early decay laccase clone library may represent unidentified laccase genes from one of the less abundant ascomycete species, possibly one that was not included in our fungal isolate collection.

Although the copper binding sites of the laccase gene used as targets for PCR primers are well conserved among fungi (Fig 2.1), the region between sites is sufficiently variable to allow us to distinguish among related laccase sequences (Fig 2.2). Laccase clusters identified here coincide well with species groups identified by ITS sequence analysis (Buchan et al., 2002), although the presence of paralogous genes among the sequenced laccases limits our ability to directly map laccase sequences to ITS-based phylogeny.

Importance and diversity of fungal laccases in the environment

Ascomycetous fungi are dominant secondary microbial producers of the *Spartina alterniflora* decay system (Newell, 2001a), and have been shown to be capable of degrading both the lignin and polysaccharide moieties of lignocellulose (Bergbauer and Newell, 1992; Newell et al., 1996). Because standing crops of fungi can comprise up to 28% of living cordgrass standing crop (Newell and Porter, 2000), their ecological role in the marsh is evident.

In this study, we have shown that the most dominant species of ascomycetous fungi as shown by Buchan et al. (2002) and previous studies (Gessner, 1972; Newell, 2001b) also appear to harbor the highest density of laccase types. *Phaeosphaeria spartinicola* and *Phaeosphaeria halima* each have two and three different laccase genes, respectively. It may be that the multiplicity of laccase types, which allows for broader substrate specificity, is an evolutionary advantage in the competition for space and nutrients in the decay system. However, *Mycosphaerella* sp. 2, with only a single laccase gene in one subgroup and no amplifiable gene in the second (Table 2.1), does not appear to fit this pattern.

Although laccases have been shown to serve multiple functions, their participation in delignification has been definitively shown in several fungi (Edens et al., 1999; Eggert, 1997). We hypothesize that the diverse laccases present in the microbial community on decaying *S. alterniflora* play a role in delignification and that they are critical enzymes in salt marsh decomposition processes. However, the difficulty of determining the function of a gene that exists in multiple copies and has low substrate specificity precludes definitively assigning function.

Much previous research acknowledges the potential roles of lignin degrading enzymes and their importance not only in the natural environment, but potentially in industrial and bioremediation applications, as well. Boonchan et al. (2000) and Collins et al. (1996) describe the ability of some fungi to degrade PAHs and detoxify PAH-polluted soils and sediments through the production of extracellular lignin-degrading enzymes including laccase. Newell et al. (2000) showed that the biomass of ascomycetous fungi was not adversely affected by a series of anthropogenic toxicants. Because the existing

database of ascomycete laccase sequences for environmental and bioremediation questions is limited, the sequences assembled here from isolates and via direct amplification from the natural environment provide a valuable tool for future studies of degradative enzymes.

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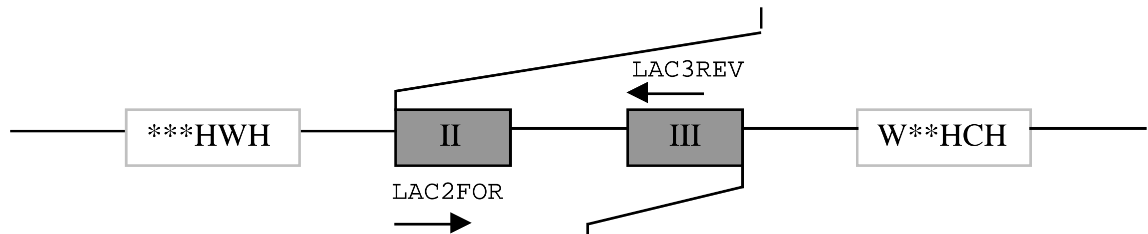
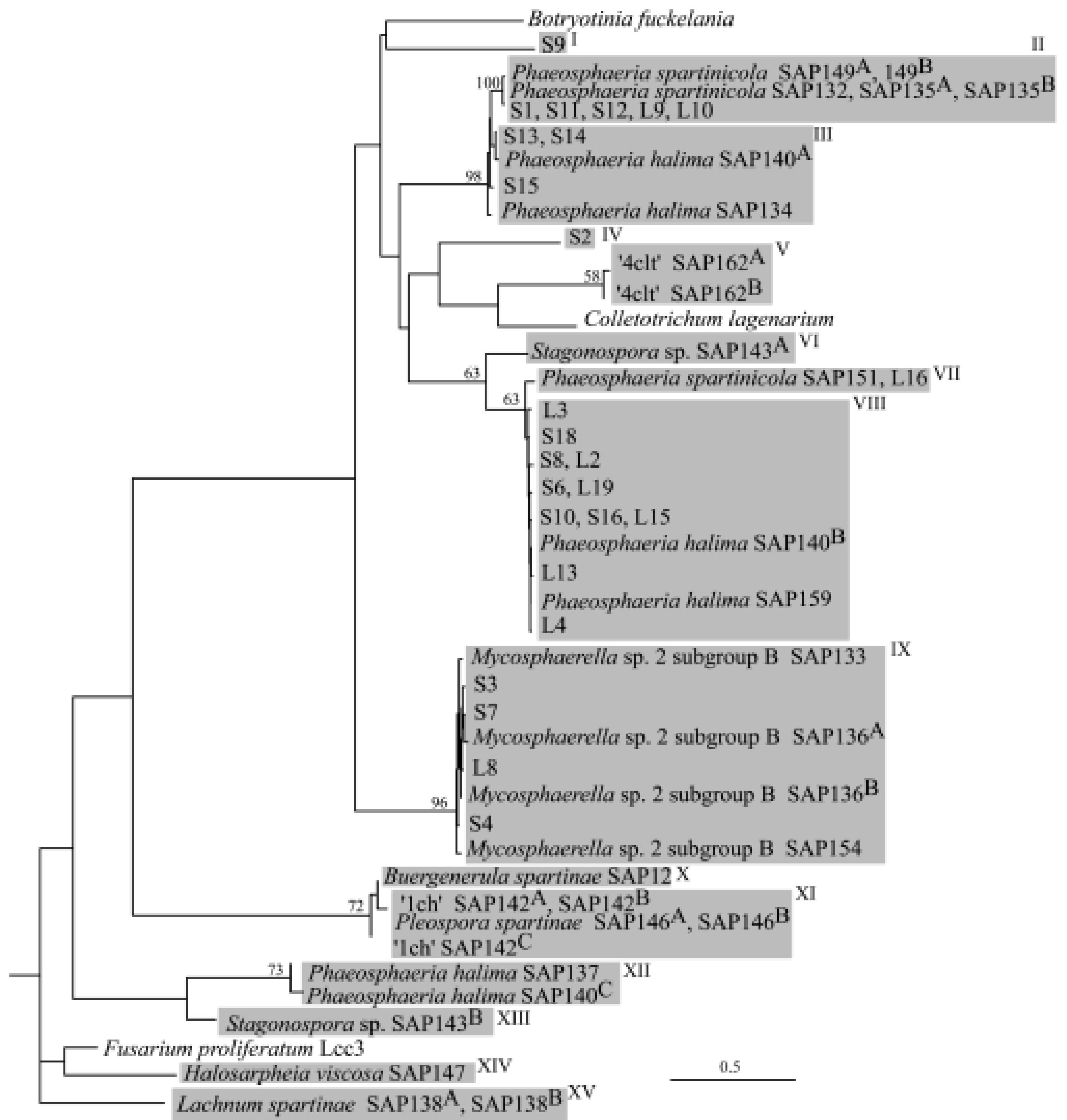
Fig 2.1 Alignment of previously published ascomycete and basidiomycete laccase sequences. Degenerate primers for this study were designed around the shaded copper binding sites. Amino acid numbers are based on the *Neurospora crassa* strain TS laccase gene. The sequences at the first and fourth copper binding regions indicate the other two areas of conservation. Asterisks (*) indicate amino acids that were not 100% conserved in the copper binding sites.

```
                    110                                                   159
M.quercophilus      ~~~~~AFVNQ CPIS.TGHAF LYDFQVPDQA GTFWYHSHLS TQYCDGLRGP
    P.ciliatus      ~~DGPAFVNQ CPIS.TGNSF LYDFTAADQA GTFWYHSHLS TQYCDGLRGP
P.cinnabarinus      ~~~~~AFVNQ CPIA.SGHSF LYDFQVPDQA GTFWYHSHLS TQYCDGLRGP
   P.ostreatus      WADGPAFVTQ CPVA.SGDSF LYNFNVPDQA GTFWYHSHLS TQYCDGLRGP
     S.commune      WADGPAGVTQ CPIA.TGDSF VYEFGVPDQA GTFWYHSHLS TQYCDGLRGA
    C.cinereus      ~~~~~~~~~~ ~~~~~~~HAF LYKFTPAGHA GTFWYHSHFG TQYCDGLRGP
   N.crassa TS      IQDGVNGVTE CPIPPRGGSK VYRWR.ATQY GTSWYHSHFS AQYGNGIVGP
    P.anserina      ~~DGANGVTE CPIPPKGGSR IYRFR.AQQY GTSWYHSHFS AQYGNGVVGT
  C.parasitica      ~~~~~~~~~~ ~~~~PNGGSK TYTFI.AHQY GTSWYHSHFS AQYGNGIVGA
  B.fuckelania      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ GTSWYHSHYS SQYGEGMLGG
    G.graminis      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ GSSWYHSHFA LQAWQGVFGG
  S.cerevisiae      ~~~~~~~VTQ CPIVP.GQTY LYNFTVPEQV GTFWYHAHMG AQYGDGMRGA
```




```
                    456                                                   505
M.quercophilus      .DIEISLPAT SA.AP...GF PHPFHLHGHT FAVVRSAGS. .STYNYANP~
    P.ciliatus      .TIELSFPIT ATNAP...GA PHPFHLHGHV FSVVRSAGS. .SEYNYVNPP
P.cinnabarinus      .SIEISFPAT A.NAP...GF PHPFHLHGHA FAVVRSAGS. .SVYNYDNPI
   P.ostreatus      .VVEISMPAL AV......GG PHPFHLHGHT FDVIRSAGS. .TTYNFDTPA
     S.commune      .TVEISIPGG SA......GA PHPFHLHGHT FDVVRSAGS. .TDYNYANPI
    C.cinereus      .VVELVVPA. ..GVL...GG PHPFHLHGHA FSVVRSAGS. .STYNFVN~~
   N.crassa TS      WLIE.NDP.. .DG.AFSL.. PHPIHLHGHD FLILGRSPD. .V..TA.ISQ
    P.anserina      WLIE.NDP.. .TG.PFSI.. PHPMHLHGHD FLVVGRSPD. .Q..PAGVPQ
  C.parasitica      WLIE.NDP.T ATG.N.AL.. PHPIHLHGHD FVVLGRSPN. .VSPTA~~~~
  B.fuckelania      WVIQ.EVPGN VNGNPVSINV PHPMHLH~~~ ~~~~~~~~~~ ~~~~~~~~~~
    G.graminis      VIIQ.T...N F.G..VA... .HPVHLHGH~ ~~~~~~~~~~ ~~~~~~~~~~
  S.cerevisiae      QLLKHNDIIE VVLNNYDSGR .HPFHLHGHN FQIVQKSPGF HVDEAYDESE
```

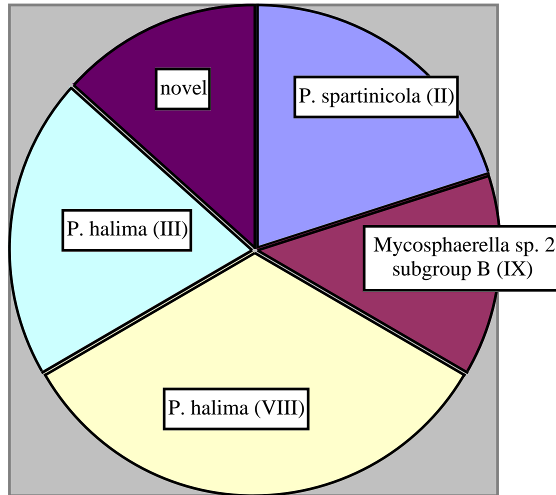
Fig. 2.2 Phylogenetic tree of ascomycete laccase sequences obtained from cultured strains and PCR amplicons from decaying *Spartina alterniflora* blades. Sequence labels are as follows: "SAP" = isolates from Sapelo Island; "S" = laccase amplicons from early decay blades; "L" = laccase amplicons from late decay blades. The tree was constructed from 257 amino acid residues. The distance bar represents Kimura distance. Boostrap values greater than 50% are indicated at branch nodes. Groups of sequences with greater than 96% similarity are identified by shading; the laccase type is given in romannumerals outside the top right corner of the shaded group.



Botryotinia fuckelania
S9 I
II
Phaeosphaeria spartinicola SAP149A, 149B
Phaeosphaeria spartinicola SAP132, SAP135A, SAP135B
S1, S11, S12, L9, L10
100
S13, S14
III
Phaeosphaeria halima SAP140A
98
S15
Phaeosphaeria halima SAP134
S2 IV
'4clt' SAP162A V
58
'4clt' SAP162B
Colletotrichum lagenarium
Stagonospora sp. SAP143A VI
63
Phaeosphaeria spartinicola SAP151, L16 VII
63
L3
VIII
S18
S8, L2
S6, L19
S10, S16, L15
Phaeosphaeria halima SAP140B
L13
Phaeosphaeria halima SAP159
L4
Mycosphaerella sp. 2 subgroup B SAP133
IX
S3
S7
Mycosphaerella sp. 2 subgroup B SAP136A
L8
96
Mycosphaerella sp. 2 subgroup B SAP136B
S4
Mycosphaerella sp. 2 subgroup B SAP154
Buergenerula spartinae SAP12 X
'1ch' SAP142A, SAP142B
XI
72
Pleospora spartinae SAP146A, SAP146B
'1ch' SAP142C
73
Phaeosphaeria halima SAP137
XII
Phaeosphaeria halima SAP140C
Stagonospora sp. SAP143B XIII
Fusarium proliferatum Lcc3
Halosarpheia viscosa SAP147 XIV
0.5
Lachnum spartinae SAP138A, SAP138B XV

Fig 2.3 PCR results of clones from salt marsh community DNA extracted from early and late decay blades of smooth cordgrass (*Spartina alterniflora*) on Sapelo Island, GA. Results are given as closest identified relative based on percent similarity at the level of deduced amino acid sequence. The number in parenthesis indicates the laccase type.

Early Decay



Late Decay

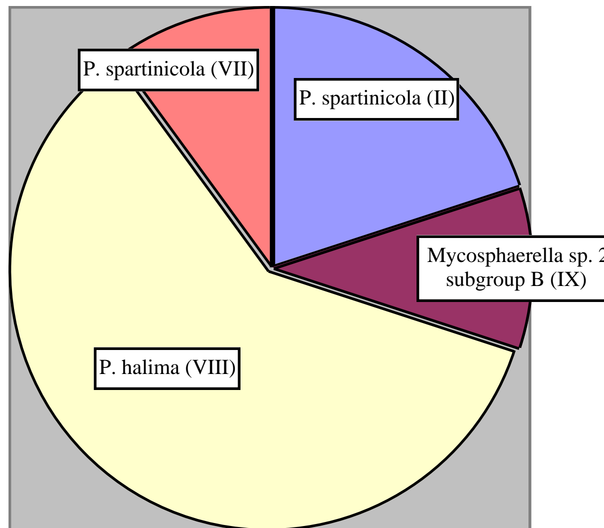


TABLE 2.1. Species, strains, and results of PCR amplifications of laccase genes from isolated ascomycetes and mitosporic fungi from smooth cordgrass (*Spartina alterniflora*). Strains were isolated from Sapelo Island, GA. The *Lachnum* species was isolated from leaf sheaths; all others from leaf blades. Best hits from a BLAST of GenBank sequences are reported unless otherwise noted. A superscripted letter next to the strain number distinguishes multiple clones from the same strain.

Species	Strains Analyzed	Closest Identified Relative and % Similarity	Laccase Type
Buergenerula spartinae	SAP012	*Botryotinia fuckelania* (46.6%)	X
	SAP124	*N. crassa* mannitol transport protein	NA
	SAP126	No PCR product	NA
Phaeosphaeria spartinicola	SAP132	*Botryotinia fuckelania* (50.2%)	II
	SAP135[A]	*Botryotinia fuckelania* (50.2%)	II
	SAP135[B]	*Botryotinia fuckelania* (49.8%)	II
	SAP149[A]	*Colletotrichum lagenarium* (50.0%)	II
	SAP149[B]	*Colletotrichum lagenarium* (49.7%)	II
	SAP151	*Botryotinia fuckelania* (52.0%)	VII
Mycosphaerella sp. 2 subgroup B[b]	SAP133	*Colletotrichum lagenarium* (50.2%)	IX
	SAP136[A]	*Botryotinia fuckelania* (46.4%)	IX
	SAP136[B]	*Colletotrichum lagenarium* (49.7%)	IX
	SAP154	*Botryotinia fuckelania* (48.1%)	IX
Phaeosphaeria halima	SAP134	*Colletotrichum lagenarium* (48.3%)	III
	SAP137	*Fusarium proliferatum*Lcc3 (41.5%)	XII
	SAP140[A]	*Colletotrichum lagenarium* (48.0%)	III
	SAP140[B]	*Botryotinia fuckelania* (50.2%)	VIII
	SAP140[C]	*Botryotinia fuckelania* (50.2%)	XII
	SAP159	*Botryotinia fuckelania* (50.4%)	VIII
Lachnum spartinae	SAP138[A]	*Fusarium proliferatum* Lcc1 (50.0%)	XV
	SAP138[B]	*Fusarium proliferatum* Lcc1 (36.6%)	XV
'1ch'[a]	SAP142[A]	*Fusarium proliferatum* Lcc1 (35.7%)	XI
	SAP142[B]	*Fusarium proliferatum* Lcc1 (34.3%)	XI
	SAP142[C]	*Fusarium proliferatum* Lcc1 (36.2%)	XI
Stagonospora sp. (8-celled)	SAP143[A]	*Botryotinia fuckelania* (46.4%)	VI
	SAP143[B]	*Fusarium proliferatum* Lcc3 (42.4%)	XIII
Pleospora spartinae	SAP146[A]	*Fusarium proliferatum* Lcc3 (31.6%)	XI
	SAP146[B]	*Fusarium proliferatum* Lcc3 (46.8%)	XI
Halosarpheia viscosa	SAP147	*Fusarium proliferatum* Lcc3 (43.8%)	XIV
Mycosphaerella sp. 2 subgroup A[b]	SAP152	No PCR product	NA
	SAP153	No PCR product	NA
'4clt'[a]	SAP162[A]	*Colletotrichum lagenarium* (62.9%)	V
	SAP162[B]	*Colletotrichum lagenarium* (63.8%)	V
	SAP163	Cofilin related protein from *N. crassa*	NA
	SAP164	Phenylalanyl t-RNA synthetase from *S. cerevisiae*	NA

[a] Nicknames for undescribed ascomycetes ('1ch' is the "one-celled haustorial" of Newell [2001b]; '4clt' is briefly described in Buchan et al. (2002)

[b] See Buchan et al. (2002) for description of *Mycosphaerella* subgroups

CHAPTER 3

CONTRIBUTIONS OF BACTERIA AND ASCOMYCETOUS FUNGI TO LIGNOCELLULOSE DECOMPOSITION

Abstract

Microcosms were designed to measure rates of lignocellulose degradation by decomposer communities consisting of marine fungi and bacteria. Twenty-four microbial communities consisting of either three species of ascomycete fungi, a natural bacterial assemblage, or a combination of fungi and bacteria were incubated in glass microcosms containing ^{14}C-labeled *Spartina alterniflora* lignocellulose. Lignocellulose degradation was monitored by measuring $^{14}CO_2$ evolution over a six week period. Dissolved organic carbon was also measured at the end of the experiment. We found that fungi alone on a moist substrate were the most efficient degraders of *S. alterniflora* lignocellulose. Fungi on submerged substrate degraded lignocellulose at approximately the same rates as fungi and bacteria together. Moisture regime did not affect the degradation capabilities of the marine bacterial assemblage. Dissolved organic carbon accumulation was greatest in microcosms containing only fungi, whereas all samples containing bacteria showed little measurable accumulation of dissolved degradation products from lignocellulose.

Introduction

Along the east coast of the U.S., primary production in salt marshes is dominated by the macrophyte smooth cordgrass, *Spartina alterniflora* (Pomeroy and Wiegert, 1981). Degradation of *S. alterniflora* is an important aspect of salt marsh ecosystem dynamics because of the large reservoir of organic matter represented by *S. alterniflora* biomass and the recalcitrance of the lignins that make up the biomass. Microbial degradation represents the major mechanism for the breakdown of vascular plant lignins. While both fungi and bacteria have been shown to be capable of lignin degradation in coastal marshes (Benner et al., 1988; Newell et al., 1996), the potential physical and physiological interactions between these two decomposer groups have not been well studied.

Since many fungi contain extracellular degradation enzymes, they are well equipped to degrade macromolecules and make the substrate more available to other decomposers. Fungi are invasive, and begin the degradation process by using their hyphae to penetrate the substrate. The hyphae then excrete degradation enzymes into the substrate. It has been suggested that fungi are responsible for breaking down the lignin macromolecule into smaller fractions that are more readily metabolized by bacteria (Newell et al., 1995; Newell and Porter, 2000). In this case, bacteria may benefit from the presence of fungi during decomposition.

Lignin-degrading enzymes produced by fungi may also oxidize precursors to other fungal enzymes that function as antibacterial agents (Eggert, 1997). This would support the idea that competition may be occurring between the two groups of organisms. Similarly, some bacteria produce fungicidal enzymes (Nielsen et al., 1998), and therefore

the presence of these bacteria in a mixed microbial community could affect fungal activity. Although the specific interactions between lignocellulolytic bacteria and fungi are primarily speculation at this point, it is clear that they may be highly complex.

A molecular approach examining the genes involved in lignocellulose degradation could provide valuable information regarding enzyme function and degradation in both bacteria and fungi. For example, Eggert et al. (1997) created laccase-minus mutants of the basidiomycete *Pycnoporus cinnabarinus* and demonstrated that they had diminshed ability to metabolize a synthetic lignin (DHP). A laboratory system for measuring rates of lignocellulose degradation by defined microbial communities would be very useful for assessing effects of genetic manipulations of bacterial or fungal genes. This study investigated a technique in which degradation of ^{14}C-labeled lignocellulose preparations is used to screen fungal and bacterial contributions to lignocellulose degradation in a laboratory microcosm.

Materials and methods

Fungal and bacterial cultures

Three ascomycete fungi were isolated from *Spartina alterniflora* blades as described previously (Chapter 2). *Phaeosphaeria spartinicola* (SAP132, 135, 149, 151), *Phaeosphaeria halima* (SAP134, 137, 140, 159), and *Mycosphaerella* sp. 2 (SAP133, 136, 154) were shown by Buchan et al. (2002) to dominate the fungal biomass of decaying *S. alterniflora* in coastal Georgia salt marshes, and previous research has demonstrated that these three species possess enzymes likely to be involved in lignocellulose degradation (Chapter 2).

Fungal isolates were cultured on 1-cm-long pieces of sterile blades of *Spartina alterniflora* on the surface of DV8 plates (dilute V8 agar (DV8; 2% V8 [Campbell Soup, Inc.], 2% agar, 1.5% sea salts). Each blade piece was inoculated with one isolate, taken as an agar plug from malt/yeast extract plates containing the following: 0.1% ground plant material (sterile, dried *S. alterniflora*), 0.04% malt extract, 0.04% yeast extract, 2.0% sea salts (Sigma Chemical Co., St. Louis, MO), 2.35 mM $NaNO_3$, 1.72 mM K_2HPO_4, 1 mM KH_2PO_4, and 1.6% agar. Isolates were incubated at room temperature under ambient daylight conditions until the fungus had visibly attached to the *S. alterniflora* blade.

The bacterial inoculum for the microcosms was a natural assemblage collected from Dean Creek on Sapelo Island, Georgia, in July 2001. Dean Creek Marsh is located within Site 6 of the Georgia Coastal Ecosystems Long Term Ecological Research sampling area and is typical of southeastern U.S. salt marshes (Chalmers, 1997) (maps available at http://gce-lter.marsci.uga.edu/lter/asp/studysites.htm). One liter of water was filtered through a 1.0 µm polycarbonate membrane filter to remove nonbacterial particles, and then through a 0.2 µm membrane filter (Poretics Corp., Livermore, CA). When approximately fifty milliliters of water was left above the filter, the water containing concentrated bacteria was collected into a sterile container.

Five ml of the bacterial inoculum was reserved for counting and preserved with 2% buffered formalin. Bacterial counts were determined by epifluorescence direct count microscopy after staining with 4'6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980). Water that passed through the 0.2 µm filter was collected and autoclaved. The

autoclaved, filtered, seawater (AFSW) was used to provide sterile seawater for the microcosms. The salinity of this water was 15 ppt.

Lignocellulose preparation

Unlabeled *S. alterniflora* was collected from a salt marsh on Sapelo Island, GA, and dried for two days at 50˚C. Dried plant material was ground to <250 µm diameter using a Wiley Mill (Arthur Thomas Co., Philadelphia, Pa). The ground material was sterilized by autoclaving.

For radiolabeling, living *S. alterniflora* plants were collected from the Sapelo marsh and grown in natural light for three months. At three week intervals, plants were enclosed in gas impermeable bags and exposed to an atmosphere of $^{14}CO_2$. Following labeling, plants were dried, ground, and extracted as described previously (Benner et al., 1986).

Microcosm design

Twenty-four sterile milk dilution bottles were ashed at 550ºC. To each bottle, 10 mg of radiolabeled lignocellulose and 90 mg of unlabelled lignocellulose were added.
Three treatments were established consisting of the three fungal isolates only, a bacterial assemblage only, or a combination of fungi and bacteria. A fourth set of bottles remained uninoculated (negative controls). All inoculations were performed using aseptic technique in a laminar flow hood.

To establish the fungal-only microcosms, three 1-cm-long blades of *S. alterniflora*, each inoculated with one of the three fungal isolates, were added to six

bottles. Two moisture regimes were also established. To create a 'moist' substrate in three of the fungal microcosms, 1 ml of AFSW was added to the milk dilution bottle. To create a 'submerged' substrate fungal treatment in the remaining three replicates, 10 ml of AFSW was added.

To establish the bacteria-only microcosms, 1 ml of the bacterial assemblage inoculum was added to six bottles. Again, two moisture regimes were created. For the 'moist' substrate treatment in three of the bacterial microcosms, no other additions were made. For the 'submerged' substrate treatment, an additional 9 ml of the AFSW was added.

To establish the microcosms containing both organisms, three blades of *S. alterniflora*, each inoculated with one of the three fungal isolates, and 1 ml of bacterial inoculum were added to six bottles. For the 'moist' treatments, no additional liquid was added. For the 'submerged' treatment, an additional 9 ml of ASFW was added to the bottle.

The negative control treatments contained three blades of sterile *S. alterniflora* and either 1 ml or 10 ml of AFSW.

The microcosm treatments were designated as follows: FM (contained fungi and 1 ml AFSW); FS (contained fungi and 10 ml AFSW); BM (contained 1 ml of bacterial inoculum); BS (contained 1 ml of bacterial inoculum and 9 ml AFSW); FBM (contained fungi and 1 ml of bacterial inoculum); FBS (contained fungi, 1 ml of bacterial inoculum, and 9 ml of AFSW); NM (contained three 1 cm sterile *S. alterniflora* blades and 1 ml of AFSW); and NS (contained three 1 cm sterile *S. alterniflora* blades and 10 ml of AFSW) (Table 1).

Each bottle was sealed with a sterile rubber stopper, from which a small glass vial was suspended. The vial contained 2 ml of a 3 mM solution of NaOH (Sigma Corp., St. Louis, Mo.) which was used to trap evolved $^{14}CO_2$. The NaOH solution was made fresh on the morning of each sampling day. The seal between the bottle and the rubber stopper was wrapped in parafilm to minimize contamination and evaporation. Microcosms were incubated at ambient temperature (~24°C) and light conditions (approximately 12 hours light and 12 hours dark).

Microcosm sampling

Microcosms were sampled once per week, with the first sampling occurring seven days after the microcosms were inoculated. To sample, the rubber stopper was removed from the bottle and 1 ml of the NaOH solution was collected into a glass scintillation vial. Approximately 10 ml of scintillation cocktail (Sigma Corp., St. Louis, Mo.) was added. The other 1 ml of NaOH was discarded, 2 ml of fresh NaOH were added, and the bottles were resealed until the next sample date.

In some bottles containing the moist substrate, evaporation was evident. To compensate for this, 1 ml of sterile deionized water was added at sampling time to any of the microcosms that appeared to be approaching dryness. The liquid level of the substrate in the 'submerged' microcosms did not noticeably change.

After six weeks, all liquid remaining in the microcosms was collected, filtered through a 0.2 µm membrane filter (Poretics Corp., Livermore, CA), and the volume was measured. A 1 ml subsample was placed in a scintillation vial with 10 ml of scintillation cocktail. Radioactivity in the NaOH solutions (representing radiolabel mineralized to

CO_2) and in the microcosm liquid (representing radiolabel present as dissolved organic and inorganic carbon) was measured in a Beckman LS6500 scintillation counter.

Statistical comparisons were carried out using SAS software (SAS Institute, Cary, NC) using a general linear model with post-test comparisons based on least squares means. To account for the differences in number of replicates in control treatments after NM01 and NM03 were removed, only p-values <0.001 were considered significant.

Results

DAPI counts indicated a high concentration of bacteria in the inoculum ($1.2 * 10^9$ cells/ml); starting concentrations in the microcosms were therefore approximately $6.0 * 10^8$ cells/ml in the moist treatment and $1.0 * 10^8$ cells/ml in the submerged treatment. One replicate from each of the moist and submerged control microcosms showed visible fungal growth and $^{14}CO_2$ evolution a few weeks into the study, indicating contamination. Replicate NM01 was eliminated from the experiment during week three and replicate NS03 was removed during week five.

Cumulative lignocellulose degradation and carbon mineralization are reported as the average percent lignocellulose degradation for the three replicates (Fig. 3.1). Kinetics of mineralization over the six-week study indicated that rates were gradually slowing over time in most treatments (Fig. 3.1).

The four highest rates of lignocellulose mineralization were found in the four treatments containing fungi (FM, FBM, FBS, and FS) (Fig. 3.1). Fungi alone on a moist substrate (FM) mineralized significantly more lignocellulose than did any of the other treatments ($p<.0001$), reaching 22% by the end of the experiment. When bacteria were

added to the fungal treatment (FBM), the lignocellulose mineralization was no longer significantly different from the other treatments ($p=.0043$). With or without bacteria, fungal treatment microcosms with a moist substrate supported more lignocellulose mineralization than did those with a submerged substrate ($p<.0001$). The two treatments containing only the bacterial assemblage (i.e. both moist and submerged) showed the same amount of lignocellulose mineralization (~9%; $p=0.63$), and neither of the negative control treatments showed any significant $^{14}CO_2$ evolution.

The percentage of dissolved organic carbon (DOC) remaining in the AFSW in the microcosms was highest in the treatments containing only fungi (FM, FS) (Fig. 3.2). All microcosms containing a bacterial assemblage (BM, BS, FBM, FBS) showed a lower percentage of DOC accumulation ($\leq 1.2\%$) that was not significantly different from that of the negative controls ($p \geq 0.19$).

Discussion

The relative contributions of bacteria and fungi to the degradation of plant detrius have long been debated. It is generally agreed that fungi initially dominate microbial assemblages on decaying leaves as long as the tissue is intact, while bacterial activity tends to increase when leaves become partially broken down. There is only a little quantitative data to address this hypothesis, however. Baldy et al. (1995) found that fungi play an important role in the biological transformation of leaf litter in rivers, while the bacterial contribution is relatively small. Conversely, Benner et al. (1986) showed bacteria are the predominant degraders of lignocellulose in aquatic systems. More recently, Newell and Palm (1998) suggested that the bacterial assemblages interact

significantly (and in a noncompetetive manner) with fungal decomposers on vascular plant material.

In this study, fungi were more successful than the bacterial community at mineralizing lignocellulose in both moist and submerged conditions (Fig. 3.2). While fungi alone degraded 25 and 19% of lignocellulose on moist and submerged substrates, respectively, bacterial assemblages alone mineralized the lowest percentages of lignocellulose among the inoculated treatments (~10%) regardless of whether they were presented with a moist or submerged substrate. Other studies have found conflicting results, and it is clear that experimental methods and incubation conditions can affect relative decomposition rates. For example, Benner et al. (1986) concluded that bacteria were the predominant degraders of lignocellulose in *S. alterniflora* decay systems although that study differed from the present work in that fungi were not inoculated as established mycelia and microcosms were shaken, a factor that may favor growth of nonfilamentous organisms. In other studies, environmental conditions (i.e. pH, light, temperature) have been found to affect the growth and activity of the microbial communities (Benner et al., 1985).

Fungi alone on a moist substrate (FM) mineralized significantly higher percentages of *S. alterniflora* lignocellulose (22%) than did fungi submerged in liquid (FS; 14%) ($p=0.0007$). The submerged fungal decomposer communities with and without bacteria were similar in their mineralization abilities (FS and FBS=14%). These findings agree well with those of previous studies, which have shown fungi to be most effective in the early stages of *S. alterniflora* decay (Newell et al., 1995; Newell, 1996), when the grass has not yet collapsed onto the sediment.

The treatments which included both fungi and bacteria on a moist substrate (FBM) degraded significantly less lignocellulose than fungi acting alone on a moist substrate (FM) ($p=0.0009$). Because fungal degradation in the same moisture treatment was more effective in the absence of bacteria, this suggests that fungal degradation is inhibited in the presence of bacteria. It has been shown that some fungi are capable of producing antibacterial metabolites (Eggert, 1997), and it is possible that bacteria are capable of producing antifungal metabolites. Competition may also be due to space limitations or utilization of limiting nutrients. But while the three saltmarsh fungi appear to be more successful at mineralizing lignocellulose, the natural bacterial community may be better able to use all the components of the heterogenous lignocellulose polymer. Treatments containing fungi alone (FM and FS) accumulated more dissolved decomposition products (^{14}C-DOC) than did incubations containing bacteria (FBM, FBS, BM, BS) ($p<0.0001$). Presumably, diverse members of the bacterial community are utilizing a wider variety of chemical moieties and allowing less material to accumulate as unincorporated dissolved products. In this sense, a mixed fungal/bacterial decomposer community may be more efficient at using lignocellulosic material, although overall rates of degradation are nonetheless greater for fungi in the absence of a bacterial community.

The relative contributions of bacteria or fungi to lignocellulose degradation reported here do not consider the incorporation of lignocellulosic carbon into cell biomass. Nonetheless, bacterial growth efficiencies would have to be many-fold higher than for fungi to make up for the inequity in mineralization observed between bacterial and fungal treatments. For example, if the fungi in the FM treatment degraded lignocellulose with an average growth efficiency of 25%, bacteria would have to

incorporate lignocellulosic carbon into biomass at an efficiency of ~75% in the BM treatment to match the fungal decomposition rate. Such a high bacterial yield is unlikely for growth on vascular plant detritus.

Results of this study provide evidence that differences exist between the rates and mechanisms of lignocellulose degradation by fungi and bacteria, and that important interactions may occur between the two types of organisms at either the physical or physiological level. This microcosm design serves as an effective method for screening test organisms or communities for their degradative capabilities and will be valuable for assaying for changes in those capabilities as environmental or genetic factors are manipulated.

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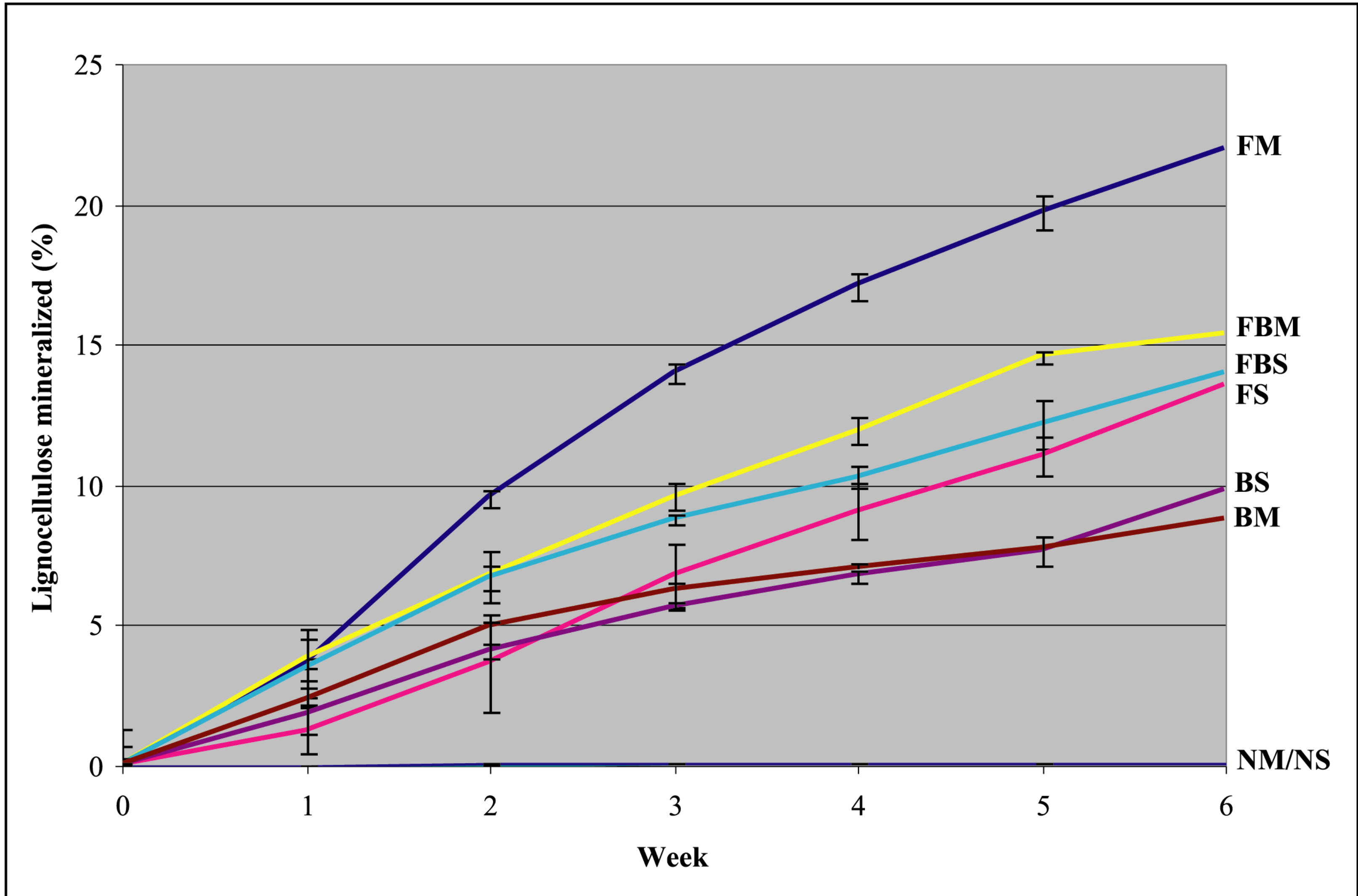
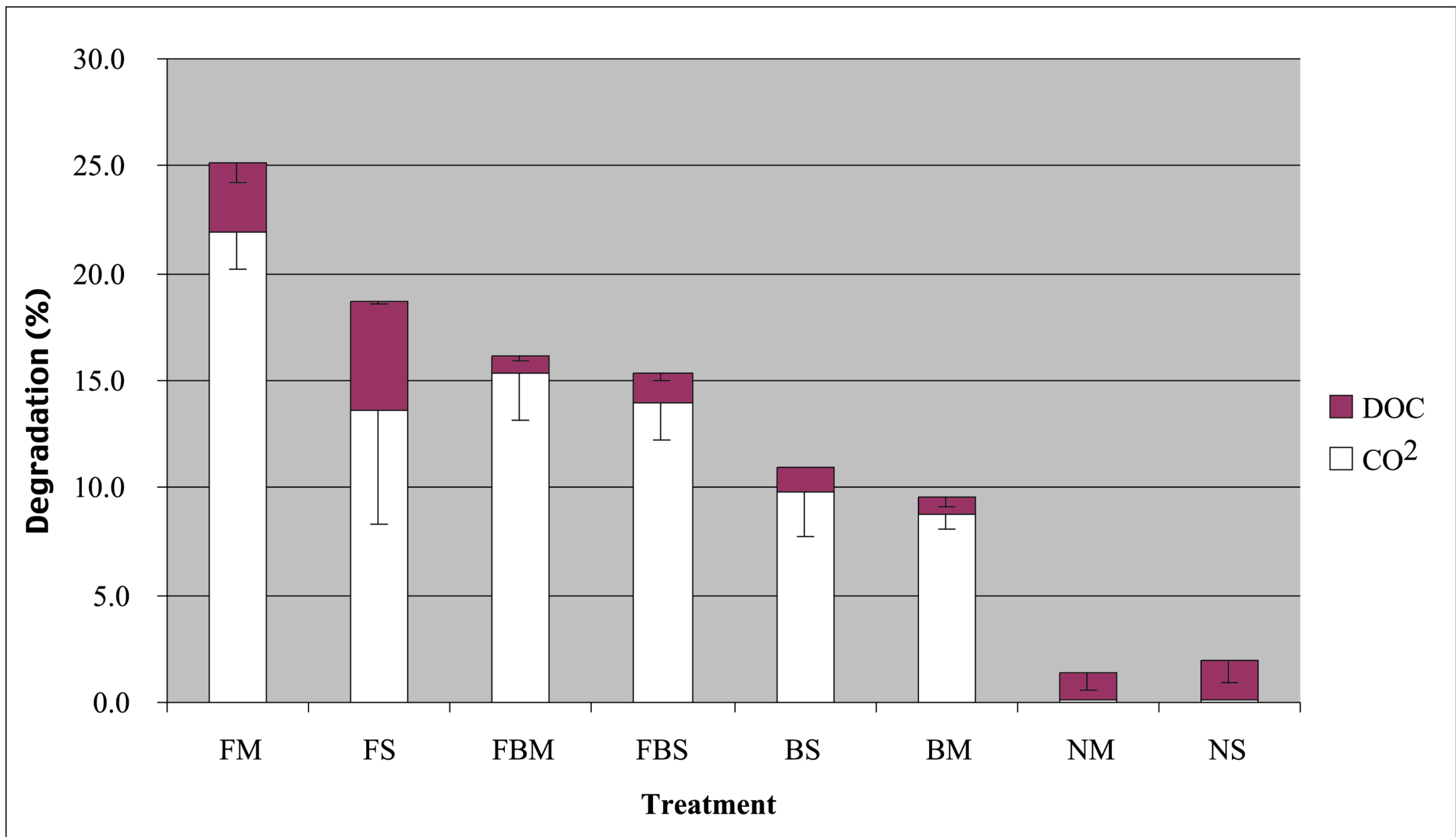
Fig 3.1. Percentage of ^{14}C-labeled lignocellulose degraded in fungal and bacterial microcosms was measured by $^{14}CO_2$ evolution. Bottles contained either three species of ascomycete fungi (F), a bacterial assemblage (B), a mixture of fungi and bacteria (FB), or neither fungi nor bacteria (N). Two moisture regimes were tested: a ‘moist’ substrate (M) and a ‘submerged’ substrate (S). n=2 or3.



Lignocellulose mineralized (%)
25
20
15
10
5
0
0
1
2
3
4
5
6
Week
FM
FBM
FBS
FS
BS
BM
NM/NS

Fig 3.2. Percentages of ^{14}C-labeled lignocellulose degraded in fungal and bacterial microcosms was measured by $^{14}CO_2$ evolution and ^{14}C-DOC production at the end of six weeks of degradation. Bottles contained either three species of ascomycete fungi (F), a natural bacterial assemblage (B), a mixture of fungi and bacteria (FB), or neither fungi nor bacteria (N). Two moisture regimes were tested: a 'moist' substrate (M) and a 'submerged' substrate (S). n= 2 or 3. Error bars represent standard deviations.


30.0
25.0
20.0
15.0
10.0
5.0
0.0
Degradation (%)
FM
FS
FBM
FBS
BS
BM
NM
NS
Treatment
DOC
CO2

CHAPTER 4

SUMMARY

The intent of these studies was to investigate degradation of vascular plant lignocellulose by fungi and bacteria in salt marsh ecosystems. To this end, we looked at both the general question of rates of lignocellulose degradation by both types of organisms, and at the more specific question regarding the presence of lignin degrading genes in ascomycetous fungi. The relevance of these investigations is illustrated by the predominance of bacterial and fungal communities on decaying salt marsh grass, and by the ability of ascomycetes to degrade the lignin and cellulose components of *Spartina alterniflora.*

The question of the relative roles of bacteria and fungi in vascular plant decomposition has not been adequately addressed in any ecosystem, and traditionally there has been little interaction between mycologists and bacteriologists in this regard. Intertidal salt marshes are an excellent site to undertake a detailed study of the interactions between prokaryotes and eukaryotes in decomposer communities, both because of the significant previous research on the separate contributions of each group to plant decomposition in these marshes (Newell and Porter, 2000), and because of the availability of techniques to target the mechanisms used by both fungal and bacterial communities.

Lignins constitute the most abundant natural aromatic polymer in the biosphere; thus, their biodegradation occupies an important position in the global carbon cycle.

Studies of lignin biodegradation are also of great importance for possible biotechnical applications, since lignin polymers are a major obstacle to the efficient use of lignocellulosic materials in a wide range of industrial processes (Eriksson et al., 1990).

Ascomycetous fungi are predominant secondary producers of the *Spartina alterniflora* decay system (Newell, 2001), and it has been shown that they are capable of degrading the lignin moiety of *S. alterniflora* lignocellulose (Bergbauer and Newell, 1992; Newell et al., 1996). Because of the abundance of fungi in the salt marsh decay system, their ecological role in the marsh is significant. Many fungi degrade not only lignins but also a broad range of diverse aromatic pollutants (Yadav, 1995; Collins et al., 1996). This degradation is accomplished by oxidative extracellular enzymes that are increasingly incorporated into commercial processes (Amitai, et al., 1998; Li et al., 1999).

Solubilization of organic matter by bacteria is also an important feature of carbon cycling in marine ecosystems. While it is well established that bacteria contribute significantly to the transformation of this plant-derived material (Moran and Hodson, 1989; Moran and Hodson, 1990; Moran and Hodson, 1994), the organisms responsible for catabolism of the chemically stable aromatic structures and the enzymatic pathways involved have yet to be properly characterized. The mechanism of transformation of the large variety of bonds in lignin by bacteria is thought to be more specific than in fungal systems (Crawford et al., 1982). Degradation of lignin by bacteria relies on the attack of enzymes to specific bonds, as opposed to fungal degradation where radicals are involved (Perestelo et al., 1999). Therefore, it is intuitive that lignin degradation would be more

efficient when both types of organisms are involved, as multiple types of bonds are attacked.

In this study, we showed that although fungi degrade lignocellulose at a higher rate than do bacteria in laboratory microcosms (Fig 3.1), environments containing bacteria more effectively utilize dissolved organic materials (Fig. 3.2). As an initial exploration into the genes involved in lignocellulose degradation, we also showed that the majority of fungi cultured from decaying *Spartina alterniflora* possessed laccases (Fig. 2.2), and some had up to three distinct laccase genes (Table 2.1). Eighteen of twenty fungal laccase sequences retrieved directly from decaying *Spartina alterniflora* blades matched sequences from three salt marsh ascomycetes previously implicated in lignin degradation. The data presented here will prove valuable to future studies regarding the dynamics of microbial degradation in salt marshes and the mechanisms used to break down complex molecules.

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