DOE/ER/13369--5

DE92 016799

PROGRESS REPORT

ABSTRACT OF RESEARCH PROGRESS

We have made a great deal of progress on several research fronts during this reporting period spanning two years (July 1, 1990 to date). The lines of research investigation pursued were essentially those described in the summary which was submitted to the DOE. We have expanded the work on the *LIP* genes to *Trametes versicolor*, the second most studied white-rot fungus after *P. chrysosporium*. Six *LIP* genes have been cloned from this organism and one of these has been completely sequenced and compared to the known *LIP* genes that have been described to date. Our studies gave us further insights into the novel non-integrative transformation system of *P. chrysosporium*. Our recombinant plasmid pU*GLG1*-kan, which contains a *LIP* gene disrupted by the insertion of kan^r determinant, also failed to integrate into the chromosome. Instead, it was maintained as a circular extrachromosomal element and was recoverable as a plasmid both from the meiotic and mitotic progeny. Basic characterization of the lignin peroxidase-negative mutant (*lip* mutant) and nitrogen-deregulated mutant has been completed. We also investigated the question whether carbon, nitrogen, and Mn(II) regulate *LIP* expression coordinately or independently. Our results indicate that these three environmental controls independently regulate *LIP* and *MNP* gene expression. Furthermore, an idiophasic protease has been shown to be responsible for the sharp decline in LIP activity after day 6 of incubation in low nitrogen cultures and addition of glucose to these day 6 cultures has been shown to suppress the protease levels and maintain high levels of LIP. The results further indicated that this protease is synthesized *de novo* during the idiophase. Additional studies showed that MNPs play a dominant role in the decolorization of chlorolignols in bleached kraft pulp effluents and that LIPs play a relatively minor role in this process. These studies have since been confirmed independently by an Austrian group. As a first step in developing a mathematical model for fungal secondary metabolism, oxygen microelectrodes were used to probe fungal mycelial pellets for determining the respiration kinetics in these pellets. Based on the brief summation presented above, I believe we have made important contributions on several research fronts to a better understanding of the physiology and molecular biology of the lignin-degrading enzyme systems of the white-rot fungi.

The research described above has led to the publication of eight papers, ten abstracts, and four invited presentations at national and international meetings. Also, during the two year reporting period, two graduate students have finished their doctoral dissertation. Thus, the DOE funds have been invaluable in training this next generation of researchers on fungal biodegradation.

NARRATIVE REPORT

TRANSFORMATION. A dependable DNA transformation system is critical for the detailed molecular genetic analysis of the lignin degradation system of *P. chrysosporium*. To this end, We described a protoplast transformation system for *P. chrysosporium* using a novel extrachromosomally maintained transformation plasmid, pG12-1 (Randall et al.,1991). This

plasmid contains a *kan*^{r} selection marker, pBR322 *ori*, and a 2.2 kb fragment (designated ME-1) derived from an endogenous plasmid (pME) of *P. chrysosporium*.

Plasmid pG12-1 transforms *P. chrysosporium* to G418 resistance and is maintained in the fungal transformants in an extrachromosomal circular form. Southern blot analyses showed that pG12-1 is maintained at a low copy number in the fungal transformants as it is demonstrable in the total DNA of the individual G418-resistant basidiospore progeny of the transformants only after amplification of the pG12-1 sequences using a polymerase chain reaction. *Dam* methylation analyses and Exonuclease III digestion data, respectively, confirmed that pG12-1 does replicate in *P. chrysosporium* transformants and that it is maintained in an extrachromosomal circular form. Plasmid pG12-1 is maintained in the transformants even after long term non-selective growth. We were able to show that pG12-1 does not integrate into the chromosome of the transformants and that it can be readily rescued from the total DNA of the transformants via *E. coli* transformation(Randall et al., 1991).

The pG12-1 transformation system described here is of considerable interest because such low copy extrachromosomal maintenance of the transforming plasmids is quite rare in filamentous fungi. In fact, a careful examination of most of the previous reports on circular, extrachromosomal vectors in filamentous fungi showed that these vectors were either transiently extrachromosomal prior to integration into the chromosome or that these result from excision of the intact (or altered) form of the vector from the chromosome. It is also of interest that pG12-1, even at a low copy number, confers adequate G418 resistance to *P. chrysosporium* transformants. It is possible that the coenocytic nature of *P. chrysosporium* mycelia allows for sufficient cross feeding of the *kan*^{r} gene product to allow survival in the presence of G418. The pG12-1 based transformation system should be of value not only for the molecular genetic analysis of the lignin degradation system of *P. chrysosporium*, but also as an useful model for studying the nature of maintenance of low copy extrachromosomal vectors in filamentous fungi. Further studies to improve the copy number of this plasmid and to understand the molecular mechanisms of this novel transformation system are in progress.

More recently, we disrupted the *LIP* gene *GLG1*, that encodes the LIP protein H2, by inserting into its coding region the kan^r determinant of *TN903*. The resulting recombinant plasmid, pUGLG1-*kan* was transformed into *P. chrysosporium* with the expectation that the disrupted *LIP* gene in the transforming plasmid might integrate into the homologous site in the chromosome. The results, however, showed that pU*GLG1-kan* sequences do not integrate into the chromosome. Instead, the plasmid is maintained intact as a circular extrachromosomal element and that the plasmid was rescued intact in *E. coli*. These results lead us to the conclusion that the *GLG1* component of pU*GLG1-kan* contains as yet unidentified sequences that allow it to autonomously replicate in *P. chrysosporium* transformants.

LIP GENES. The general features of the LIP genes of *P. chrysosporium* based on the cumulative data obtained to date in our laboratory are summarized below in Table 1. These data show that each *LIP* gene encodes a mature protein that contains 344 aa and is preceded by a signal peptide that contains 27 to 28 aa. All the LIP proteins contain one potential *N*-glycosylation site except the H10 protein which contains two such sites. Each LIP protein contains a number of *O*-glycosylation sites. The *C*-terminal end of all the LIP proteins contains

a characteristic proline-rich region ending with the sequence Pro-Gly-Ala. Archetypal TATA-box-like and CAAT-box-like eukaryotic promoter elements are readily seen in the 5'-non-coding region of each gene. The coding region of each *LIP* gene is interrupted by eight or nine introns which range in size from 50 to 63 bp.

Table 1. General features of the *LIP* genes of *P. chrysosporium*

Characteristic	*GLG1*	*GLG2*	*GLG3*	*GLG6*
LIP protein encoded	H2	H10	H8	?
Coding region (aa)	344	344	344	344
Leader peptide (aa)	28	27	28	28
N-glycosylation sites	one	two	one	one
TATA box and CAAT box	+	+	+	+
Number of introns	8	9	8	9
Length of introns (bp)	50-63	50-62	50-60	50-57

These studies have now been expanded to include the *LIP* genes of *T. versicolor*. Six putative *LIP* genes were recently isolated from the white-rot fungus, *T. versicolor* (Black and Reddy,1991). Sequence analysis of one of the genes (*VLG1)* has been completed and these data showed that it encodes mature LIP protein that contains 341 aa (M_r: 36,714) that is preceded by a 25 aa signal peptide sequence. This protein has a homology of 55 to 60 % to the LIP proteins of *P. chrysosporium*.

PROTEASE-MEDIATED DEGRADATION OF LIGNIN LIPs. In nitrogen-limited liquid cultures of *P. chrysosporium*, the LIP activity peaks around day 6 of incubation and sharply declines there after regardless of the carbon source, culture conditions or the strains used. Our recent studies showed that this sharp decline in LIP activity is closely correlated with the appearance of idiophasic extracellular protease activity (Dosoretz et al.,1990), the appearance of which was triggered by glucose depletion in the medium. The contribution of the protease to the decline in the LIP activity was further supported by the fact that daily addition of glucose to the culture starting on day 6 resulted in greatly decreased protease levels and this in turn stabilized the LIP activity. Addition of cycloheximide to 6-day-old cultures resulted in negligible levels of protease activity and virtually no drop in LIP activity. These results indicate that the idiophasic protease is synthesized *de novo*. Incubation of FPLC-purified LIP isozymes with the protease-rich extracellular culture fluid of 11-day-old cultures resulted in marked time-dependent loss of LIP activity concomitant with the degradation of the LIP isozymes. In contrast to this, incubation of the LIP isozymes with boiled extracellular culture fluid of the day 11 cultures resulted in no loss of LIP activity. These results indicated that protease-mediated degradation of the LIP proteins is a major cause for the decay in LIP activity in idiophasic cultures of *P. chrysosporium* and that suppression of this protease activity may be important for stabilizing the LIP levels in such cultures.

REGULATION OF LIP AND MNP PRODUCTION. It has been well established that nitrogen starvation triggers the production of LIPs and MNPs while nitrogen excess in the medium represses LIP and MNP synthesis. Since previous results also showed that carbon and Mn(II) concentrations in the medium have a profound effect on the production of LIPs and MNPs by *P. chrysosporium*, we asked the following question: Do nitrogen, carbon, and Mn(II) independently regulate LIP and MNP production or do these act via a common regulatory mechanism? Our nitrogen-deregulated *der* mutant proved to be an invaluable tool to investigate this question. Our results showed that LIP and MNP production by the *der* mutant was not blocked in a medium containing 56 mM glucose and 72 mM $NH4^+$ whereas the production of these enzymes by *der* as well as the WT was blocked in a medium containing 168 mM glucose and 24 mM $NH4^+$. These results indicated that LIP and MNP production by the *der* mutant, while deregulated for nitrogen, is still subject to repression by high levels of carbon. High levels (100 ppm) of Mn (II) repressed LIP production by the *der* mutant in low nitrogen medium as well as in high nitrogen medium suggesting that Mn (II) exerts independent regulation of LIP production. Similar results were obtained when the nitrogen concentration in the medium was kept high and the carbon concentrations were varied from 12 mM to 56 mM. These results clearly indicated that nitrogen, carbon, and Mn(II) concentrations independently regulate LIP and MNP production in *P. chrysosporium.*

DECOLORIZATION OF PULP MILL EFFLUENTS. The pulp and paper industry releases into the environment billions of gallons of toxic and intensely colored waste streams. The primary contributors to the color and toxicity of these streams is the kraft bleach plant effluent which contains considerable levels of high molecular weight modified and chlorinated lignins. Conventional bacterial water treatment processes are relatively ineffective at removing these pollutants. Ligninolytic white-rot fungi, in particular *P. chrysosporium,* are known to efficiently decolorize and dechlorinate these effluent streams. However, the role of LIPs and MNPs to the decolorization process if, any, was not known. Therefore, we initiated the study to investigate the role of LIPs and MNPs in decolorizing BPE. The results showed negligible BPE decolorization by a *per* mutant, which lacks the ability to produce both the LIPs and MNPs. Also, little decolorization was seen when the wild type (WT) was grown in high nitrogen medium, in which the production of the LIPs and MNPs is blocked. A *lip* mutant of *P. chrysosporium*, which produces MNPs but not LIPs, showed about 80% of the activity exhibited by the WT indicating that the MNPs play an important role in BPE decolorization. When *P. chrysosporium* was grown in a medium with 100 ppm Mn (II), high levels of MNPs but no LIPs were produced and this culture also exhibited high rates of BPE decolorization lending further support to the idea that MNPs play a key role in BPE decolorization. When *P. chrysosporium* was grown in a medium with 0 ppm Mn(II), high levels of LIPs but negligible levels of MNPs were produced and the rate and extent of BPE decolorization by such cultures was quite low indicating that LIPs play a relatively minor role in BPE decolorization. Furthermore, high rates of BPE decolorization were seen on days 3 and 4 of incubation when the cultures exhibit high levels of MNP activity but little or no LIP activity. These results indicate that MNPs play a relatively more important role than LIPs in BPE decolorization by *P. chrysosporium.*

RESPIRATION KINETICS OF MYCELIAL PELLETS. Mycelial pellet cultures, which are used for the industrial scale production of products such as pharmaceuticals, organic acids and enzymes, offer the key advantages of immobilization, low medium viscosity, and simplified product isolation without the problems associated with artificial immobilization matrices. Previous studies have shown that oxygen can become mass transfer limited within mycelial pellets potentially affecting productivity. In this study, we determined the kinetic parameters of respiration in mycelial pellets of P. chrysosporium. In mycelial pellet cultures of *P. chrysosporium,* the oxygen concentration of the medium has been reported to strongly influence the production of the extracellular lignin peroxidases (LIP) and manganese peroxidases (MNP) which are key components of the lignin degrading system of this organism. To test the hypothesis that oxygen limitations in the pellets are responsible for this effect, oxygen microelectrodes were used to determine oxygen concentration gradients within mycelial pellets of *P. chrysosporium.* pellets that were removed from oxygenated cultures. The oxygen profiles were modelled assuming that respiration follows a Michaelis-Menten relationship. The V_{max} and K_m values for respiration were 0.76 ± 0.10 g/m^{3}pellet/s and 0.5 ± 0.3 g/m^3, respectively. These kinetic values were used to predict respiration rates in air-flushed cultures, oxygen-flushed cultures and in low agitation cultures in which large pellets (diameter > 6mm) form. The predicted respiration rates were independently validated by experimentally measuring the evolution of carbon dioxide from whole cultures flushed daily with pure oxygen. The results indicate that pellets of *P. chrysosporium* become oxygen limited depending on pellet size and oxygen concentration.

RENEWAL PROPOSAL

The research in the coming year will proceed essentially on the lines described by us in 1990. As described in the progress report above, we have isolated six LIP genomic clones of *T. versicolor* and have sequenced one of these genes, *VLG1*. More recent Northern blot studies indicate that *LIP* gene *VLG2* is highly expressed in low nitrogen cultures of *T. versicolor*. We have initiated sequencing of this gene and will compare its sequence with known *LIP* gene sequences to determine broad similarities and differences with these genes.

It has been well established that *LIP* genes of *P. chrysosporium* represent a closely related gene family, but there is no understanding as to why there are so many closely related genes (perhaps as many as 12 to 15) in a single organism. In the coming year, we will test the hypothesis that the *LIP* genes are differentially expressed when *P. chrysosporium* attacks different species of wood. Using probes specific for selected *LIP* genes of the latter organism, we will determine the specific patterns of expression of *LIP* genes when the organism colonizes different species of wood. Preliminary studies to date have been very promising and these will continue.

P. chrysosporium represents only one of over 1600 species of wood-rotting basidiomycetes known to date. Hence, there is a need to study the comparative biology of lignin degrading enzyme systems in selected genera of wood-rotting fungi. As a first step in this

direction, we have initiated studies to determine the extent of distribution of genes that have homology to the *LIP* genes of *P. chrysosporium*. We will then study the LIP and MNP enzyme systems of selected species and determine how these are similar to or different from those of *P. chrysosporium*. For this portion of the study, we will select white-rot fungi that contain genes with a relatively high homology to the *LIP* genes of *P. chrysosporium* as well as those that contain genes with a relatively low homology.

It is well established from previous studies that *P. chrysosporium* has the ability to degrade a broad variety of xenobiotics including PCBs, benzo(a)pyrenes, munitions wastes such as trinitrotoluene, and an array of other compounds. Our recent studies showed that *P. chrysosporium* rapidly decolorizes kraft bleach plant effluents and that , compared to LIPs, MNPs play a much more important role in this process which was quite unexpected at the time. In fact, this is the first documentation of a specific role for MNPs in the biodegradation processes catalyzed by *P. chrysosporium*. We have now initiated studies to investigate the ability of *P. chrysosporium* to degrade benzene, toluene, ethyl benzene, and xylenes (BTEX compounds) which are listed as priority pollutants by EPA. We will be investigating the biochemistry of degradation of these compounds as well as the degradation of 2,4-dichlorophenoxyacetic acid, and 2,4,5-trichlorophenoxy acetic acid.

Studies aimed at understanding the regulation of transcription of the *LIP* genes at the molecular level are also in progress. We are particularly focussing on characterization of the DNA binding proteins involved in the regulation of transcription of the *LIP* genes at this time. With the completion of these studies, we would have attained practically all the stated objectives as outlined originally by us in our research summary in the Spring of 1990.

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Papers removed and agenda by -

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8/31/92