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FEMS Microbiology Letters 242 (2005) 37–44

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Incomplete processing of peroxidase transcripts in the lignin degrading fungus *Phanerochaete chrysosporium*

Stuardo Macarena [a], Larrondo Luis Fernando [a], Vásquez Mónica [b], Vicuña Rafael [a], González Bernardo [a,*]

[a] *Laboratorio de Microbiología, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, and Millennium Institute for Fundamental and Applied Biology, Santiago, Chile*

[b] *Laboratorio de Biotecnología. Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Chile*

Received 14 July 2004; received in revised form 6 October 2004; accepted 19 October 2004

First published online 3 November 2004

Edited by B.A. Prior

Abstract

Phanerochaete chrysosporium has been thoroughly studied as a microbial model for lignin degradation. The enzymes lignin peroxidase (LiP) and manganese peroxidase (MnP), both encoded by several genes, play the main role in the cleavage of different lignin substructures. In this work, the expression of specific LiP and MnP transcripts in liquid medium and in a wood-containing soil system was studied by reverse transcription-PCR and subsequent cloning and sequencing of the products obtained. Splice variants of different LiP and MnP transcripts were observed in wood-containing soil incubations and in liquid cultures. The processed transcripts contained different numbers of complete introns. Since the presence of stop codons in several of these introns would prevent the synthesis of active enzyme, we propose that these transcripts arise as a result of incomplete processing rather than alternative splicing. Interestingly, analysis of splice variants from *mnp* genes led to the identification of a fourth actively transcribed gene coding for MnP in *P. chrysosporium*.
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Keywords: Altered splicing; *Phanerochaete chrysosporium*; Manganese peroxidase; Lignin peroxidase

1. Introduction

Lignin-degrading fungi produce different non-specific, oxidative extracellular enzymes which include lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, among others [1,2]. The white-rot basidiomycete *Phanerochaete chrysosporium* has become the foremost model for studies of the biochemistry and genetics of lignin degradation [1,2]. In this fungus, a family of 10 structurally related genes designated *lipA* through *lipJ* encodes LiP [3]. The reason for the multiplicity of *lip* genes is unclear, although differences in the oxidation–reduction potential among LiP isoenzymes have been observed [4]. In turn, the presence of three genes encoding MnP (*mnp1*, *mnp2* and *mnp3*) has been reported in *P. chrysosporium* [5]. In contrast to most white rot fungi, it does not produce laccase [6], although a cluster comprising four multicopper oxidase genes (*mco1* to *mco4*) has been recently reported [7].

Previous work has shown differential expression of *lip* and *mnp* genes in response to culture conditions. These studies have been conducted with cultures grown both in chemically defined media [5,8] and on solid substrates [9–11]. Thus, the pattern of *lip* transcripts in solid

* Corresponding author. Tel.: +56 2 6862845; fax: +56 2 2225515. *E-mail address:* bgonzale@bio.puc.cl (G. Bernardo).

0378-1097/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Microbiological Societies.
doi:10.1016/j.femsle.2004.10.037

cultures is significantly different from that observed in defined media [8]. The same appears to be the case of *mnp* transcripts [5,10]. Studies on transcripts produced by *P. chrysosporium* have shown alternative splicing of introns in exocellobiohydrolase and in cytochrome P450 monooxygenase genes [12,13]. On the other hand, incomplete splicing of *mco* transcripts has been recently reported in *P. chrysosporium* [7]. The presence of in frame stop codons within introns in these transcripts would prevent synthesis of an active enzyme. Therefore, this phenomenon has been called 'altered splicing' as opposed to the concept of 'alternative splicing', which leads to translation into functional protein variants.

In this report, we explored the possibility that transcripts from ligninolytic genes in *P. chrysosporium* might also be incompletely spliced. For this purpose, mRNA was isolated from cultures of this fungus grown in both liquid and solid state cultures. Using reverse transcription (RT)-PCR and clone analysis of the PCR products we detected and sequenced partial *P. chrysosporium lip* and *mnp* transcripts that contained complete introns in them. We believe based on the evidence presented below, that this phenomenon is due to incomplete processing of peroxidase transcripts.

2. Materials and methods

2.1. Culture conditions

For solid-state incubations, *P. chrysosporium* BKM-1767 spores (2×10^5 per plate) were inoculated on potato-dextrose agar [14] and grown for one week at 39 °C, and then, wood chips sterilized by autoclave were added (1 g per plate) to obtain colonized wood-chips. Six gram of non sterile *Nothofagus* forest soil containing 1 g of *P. chrysosporium*-colonized wood chips was used as a wood-containing soil system. Incubations were conducted in triplicate and maintained at room temperature (20–25 °C). Wood-containing soil samples were harvested after 0 and 20 days of incubation and stored at −80 °C. For liquid cultures, *P. chrysosporium* was grown at 39 °C from a spore inoculum in 20 ml C-limited stationary cultures in Erlenmeyer flasks (250 ml) as described before [15]. Samples were harvested after seven days of incubation when the cultures showed maximal LiP activity (0.5 U/ml) [16]. Cultures were filtered through Miracloth, washed with distilled water, quick-frozen in liquid N_2, and stored at −80 °C.

2.2. RNA isolation

RNA isolation from soil was carried out with 2 g of frozen sample ground with mortar and pestle. One gram of frozen powder was mixed with 1 ml of a buffer containing 4 M guanidinium thiocyanate, 0.1 M sodium citrate pH 8.0, 1% β-mercaptoethanol and 0.5% sarkosyl. The mixtures were centrifuged (10,000*g*) for 2 min and the polyA RNA was magnetically captured from the supernatants with the Dynabeads mRNA kit (Dynal, Norway), following the instructions of the supplier. The eluted mRNA was treated with DNase and stored at −80 °C. RNA isolation from liquid cultures was performed as previously reported [17]. Briefly, frozen mycelia (1 g) were ground to a powder under liquid N_2 and homogenized by the addition of 2 vol. of ice-cold guanidine buffer. The homogenate was extracted twice with 1 vol. of phenol:chloroform:isoamyl alcohol (25:24:1) and the RNA was precipitated by the addition of 2 vol. of cold absolute ethanol. After centrifugation, the RNA pellet was washed with cold 70% ethanol and dissolved in DEPC treated water. The eluted RNA was treated with DNase and the mRNA was obtained from 10 μg of total RNA using the Dynabeads mRNA kit.

2.3. RT-PCR

RT reactions were conducted with SuperScript II RT-PCR System (Invitrogen) and were primed with oligo (dT). RNA preparations not subjected to RT were used as amplification controls in PCR. The PCR reactions were performed in 25 μl of 1 × PCR buffer, 1 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, five pmols of each forward and reverse primers, 2% DMSO, 0.5 unit of *Taq* DNA polymerase (Invitrogen, Carlstad, CA) and 2 μl of cDNA. PCR conditions for reactions with *lip* and *mnp* consensus primers (Table 1), were performed as follows: initial denaturation at 94 °C for 5 min, 30 cycles of amplification at 95 °C for 30 s, annealing at 56 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 15 min. PCR conditions for reactions with isoenzyme specific primers pairs lipA 5′/lipA 3′, lipB 5′/lipB 3′, lipG 5′/lipG 3′ and lipJ 5′/lipJ 3′ (Table 1), were performed as follows: initial denaturation at 94 °C for 6 min, 35 cycles of amplification at 95 °C for 1 min, annealing at 54 °C for 1.3 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 15 min. The PCR reactions were performed in a Perkin–Elmer GeneAmp PCR System 2400. As a control, PCR primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase of *P. chrysosporium* were used [5]. PCR products were separated by electrophoresis in 1.5% agarose and then stained with ethidium bromide.

2.4. Cloning and sequence analysis of the RT-PCR products

The RT-PCR products were cloned into pGEM-T easy vector (Promega) and electroporated into *E. coli* Sure cells (Stratagene) following standard procedures

Table 1
Oligonucleotide primers used in this study

Gene	5′ end	3′ end	cDNA[d]	gDNA[d]
lip[a]	ccc gaa cat cgg tct cga cga	ggg atc aca tcc gag cag tcg	620	800–900
lipA[b]	tcc atc gca att tcg ccc	aca cgg ttg atg att tgg	320	500
lipB[b]	gct att gcc atc tct cct	aca cga gcg atg atc tgg	320	500
*lip*G[b]	tcg atc gcc atc tcg ccc	aca cgc tcg atg agc tgg	320	500
lipJ[b]	gcc atc gcg atc tct ccc	acc cga gcc agg att tga	320	500
mnp[c]	gac ggc tcc atg ctc ctg ttc cc	tgc cag aag cac gcc gtg cg	570	700

[a] *lip* consensus primers sequence based on the 10 reported *lip* gene sequences from *P. chrysosporium* [20].
[b] *lip* isoenzyme specific primer sequences [9].
[c] *mnp* consensus primers sequence based on six *mnp* gene sequences reported for *P. chrysosporium* and *Ceriporiopsis subvermispora* [20].
[d] Approximated size (bp).

[18]. *Hae*III restriction patterns were done in silico for the published *P. chrysosporium lip* and *mnp* sequences, using the DNASTAR software (DNASTAR, Madison, WI) [Gene Bank Accession No. m27401 (*lipA*), m37701 (*lipB*), m63496 (*lipC*), × 15599 (*lipD*), m92644 (*lipE*), m77508 (*lipF*), af140063 (*lipG*), m24082 (*lipH*), [19] (*lipI*), af140062 (*lipJ*), m77513 (*mnp1*), s69963 (*mnp2*), u70998 (*mnp3*)], and were compared with the *Hae*III restriction pattern obtained with each clone. PCR products and *Hae*III digested products were separated by electrophoresis in 3% agarose and then stained with ethidium bromide. The clones obtained with *lip* and *mnp* consensus primers that showed different restriction patterns, as well as the isoenzyme specific *lip* clones with different sizes were sequenced using a capillary Applied Biosystems model 3100 sequencer (Perkin–Elmer Applied Biosystems). The PCR product sequences were analyzed using the DNASTAR software (DNASTAR, Madison, WI). Blast analyses were performed using the program "BLAST 2 sequences" of NCBI Blast Home Page and the *P. chrysosporium* genome home page (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html).

3. Results

3.1. Incomplete processing of lip transcripts

To study the possible presence of splice variants in peroxidase transcripts in *P. chrysosporium*, we carried out a RT-PCR analysis using *lip* consensus primers (Table 1). These primers were designed to target *lip* gene sequences including 3–5 introns, depending on the *lip* gene (Fig. 1). Although originally designed to amplify *lip* peroxidases from *P. chrysosporium* and other ligninolytic fungi, no RT-PCR products could be obtained in non inoculated, non sterile soils [20]. mRNA was obtained from cultures of *P. chrysosporium* grown under three different conditions: a wood-containing soil system at zero (WT_0) and 20 days of incubation (WT_{20}), and a 7 days liquid culture (LC_7).

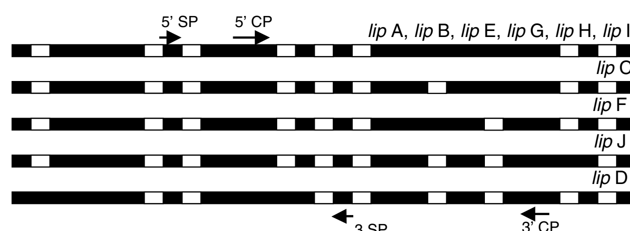

Fig. 1. Position of PCR primers targeting *P. chrysosporium* lip genes. The exon/intron organization of *lip* genes was adapted from Stewart and Cullen [8], with exons represented in black and introns in white boxes. The consensus primers (CP) as well as the isoenzyme specific primers (SP) are indicated.

Soil incubations were performed at room temperature, closer to normal soil conditions, whereas liquid cultures were carried out at 39 °C, a temperature where optimal expression of peroxidases is observed. RT-PCR products obtained from the corresponding mRNA samples were cloned to make cDNA *lip* clone libraries. Fifty colonies from each of these three libraries were chosen and screened with *lip* consensus primers by PCR amplification. Restriction fragment length polymorphism (RFLP) analyses were performed using *Hae*II restriction enzyme and the clones with distinctive RFLP patterns were sequenced. In the WT_0 clone library, we detected *Hae*III restriction patterns corresponding to the nucleotide coding sequence of *lipA* cDNA (92% of clones) and *lipB* cDNA (8%). In the WT_{20} clone library, we found restriction patterns corresponding to the nucleotide coding sequence of immature *lipA* and *lipJ* cDNAs (40% of the clones), and to mature *lipB* cDNA (60%). Sequence analysis of the RT-PCR clones corresponding to the nucleotide coding sequence of immature cDNAs clearly showed the presence of the complete introns 4 and 5 in the *lipA* cDNA, and of intron 5 in *lipJ* cDNA. In the LC_7 clone library, we found restriction patterns corresponding to the nucleotide coding sequence of *lipD*, *lipJ* and *lipA* cDNAs (92% of clones) and two immature *lipA* cDNAs (8%). Sequence analyses demonstrated that the immature cDNA clones correspond to a *lipA*

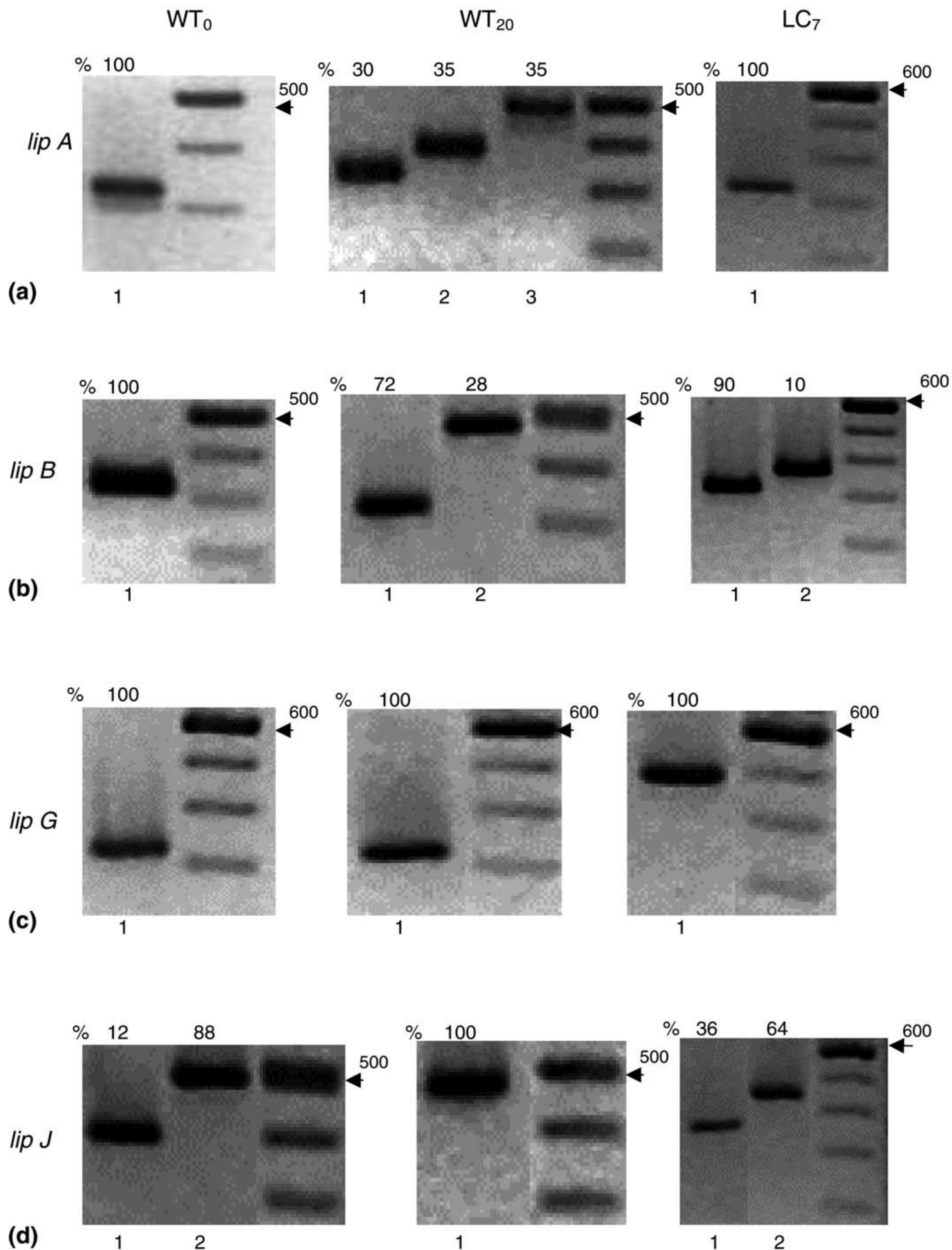

Fig. 2. Analyses of the RT-PCR products obtained with lip isoenzyme specific primer pairs. Electrophoreses of the PCR products obtained with the *lip* A (a), *lipB* (b), *lipG* (c), and *lipJ* (d) specific primers. cDNA from clone libraries prepared with mRNA from: (left) the wood-containing soil system at zero (WT_0); (center) at 20 days (WT_{20}); and (right) the 7-day old C-starved liquid culture (LC_7) were used as template. Numbers above indicate the percentage of clones with that restriction pattern in the library. Arrows indicate sizes of DNA markers for reference.

cDNA with the complete sequence copy of either intron 4 or intron 6.

To confirm the results obtained with the *lip* consensus primers, the mRNA extracted from the three different growth conditions was analyzed by RT-PCR with *lip* isoenzyme specific primers that amplify *lipA*, *lipB*, *lipG* or *lipJ* genes (Table 1) comprising introns 3–5 (Fig. 1). mRNA extracted from WT_0, WT_{20} and LC_7 cultures were used to amplify *lipA*, *lipB*, *lipG* and *lipJ* gene specific fragments to construct specific clone libraries. Twenty colonies of each one of the *lipA*, *lipB*, *lipG* and *lipJ* clone libraries were selected. These were amplified with the corresponding isoenzyme specific *lip* primers and subsequently sequenced. In the *lipA* libraries, all

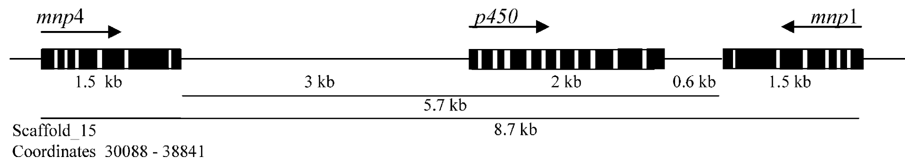

Fig. 3. Physical map of the mnp gene cluster located in scaffold_15 of the *P. chrysosporium* genome. The figure depicts the physical relation between *mnp* 4 and *mnp* 1, as well as with a gene encoding for a putative cytochrome P450 located between these genes [22]. The map is at scale (distances are in kb). Transcriptional orientation is indicated by the arrows. Gene models are represented in rectangles, whereas black and white symbolize exons and introns, respectively.

clones from the WT_0 (Fig. 2(a), WT_0, lane 1) and LC_7 clone libraries (Fig. 2(a), LC_7, lane 1), corresponded to the coding sequence of *lipA* cDNA. However, in the *lipA* WT_{20} clone library we detected three different sizes of PCR products (Fig. 2(a), WT_{20}, lanes 1–3). Sequencing of the corresponding PCR products revealed that 30% of the clones corresponded to mature *lipA* cDNA, whereas the remaining clones were immature *lipA* cDNA possessing intron 5 (Fig. 2(a), WT_{20}, lane 2) or introns 3–5 (Fig. 2(a), WT_{20}, lane 3). The WT_0 *lipB* clone library showed 100% of the clones corresponding to mature cDNA (Fig. 2(b), WT_0, lane 1). However, in the *lipB* WT_{20} clone library, two different length products were found. Sequencing of both PCR products indicated that they correspond to mature *lipB* cDNA and a splice variant having introns 3–5 (Fig. 2(b), WT_{20}, lanes 1 and 2, respectively). Two clone sizes were found in the *lipB* LC_7 clone library: a mature cDNA and a splice variant possessing intron 4 (Fig. 2(b), LC_7, lanes 1 and 2, respectively). In the *lipG* clone libraries, 100% of the clones from WT_0 and WT_{20} corresponded to mature cDNA (Fig. 2(c), WT_0 and WT_{20}, lanes 1, respectively). However, in the *lipG* LC_7 clone library, 100% of the RT-PCR product corresponded to a splice variant of *lipG* cDNA possessing introns 3–5 (Fig. 2(c), LC_7, lane 1). Finally, in the *lipJ* WT_0 clone library, we observed a splice variant with intron 5 and another one with introns 3–5 (Fig. 2(d), WT_0, lanes 1 and 2, respectively). In the *lipJ* WT_{20} clone library, 100% of the clones corresponded to a splice variant with introns 3–5 (Fig. 2(d), WT_{20}, lane 1), whereas in the *lipJ* LC_7 clone library, splice variants having intron 4 (36%) and introns 4 and 5 (64%) were found (Fig. 2(d), LC_7, lanes 1 and 2, respectively).

3.2. Incomplete processing of novel mnp *transcripts*

We extended these experiments aiming at the detection of splice variants in *mnp* transcripts. The *mnp* consensus primers (Table 1) target *P. chrysosporium mnp* genes including introns 4 and 5. RT-PCR experiments with these primers were conducted over mRNA samples from cultures WT_0 and WT_{20} and the PCR products were cloned to prepare cDNA *mnp* clone libraries. In this case, only six colonies from cultures WT_0 and WT_{20} were chosen by screening by PCR amplification with *mnp* primers and subjected to both *Hae*III RFLP analyses and DNA sequencing. The restriction patterns of these clones and Blast sequence analyses with the *P. chrysosporium* genome home page of the JGI showed that four *mnp* WT_0 clones corresponded to a splice variant of a *mnp* sequence (scaffold_15: 30519-31237) having introns 4 and 5 (GenBank Accession No. AY442335) and two clones corresponded to a mature cDNA (AY442338) from a *mnp* sequence found in scaffold_15: 37693-38410. In the WT_{20} library, we observed four clones of a mature cDNA (AY442337) corresponding to a *mnp* sequence found in scaffold_15: 30519-31237 and two clones of a splice variant of the same *mnp* sequence, which contained intron 4 (AY442336). Since none of the sequences present in scaffold_15 matched exactly the *mnp* sequences found in GenBank, we analyzed them in more detail. Including six introns each, the sequences between coordinates 31 and 38 kb on scaffold_15 exhibit 94.6% and 96.1% identity with *mnp1*, respectively [21]. The deduced as a sequence of the sequence encoded between coordinates 37328 and 38841 matches exactly MnP1 [21], whereas the one located between coordinates 30088 and 31602 encodes for a protein which differs in only one aminoacid from MnP1. Based on the work of Martinez et al. [22], they correspond to *mnp1* and *mnp4*. As observed in Fig. 3, *mnp1* and *mnp4* are clustered in an 8.7 kb segment, which in addition contains an ORF encoding a putative cytochrome P450 protein located between both *mnp* genes. This cytochrome P450 DNA sequence is interrupted by 10 introns and its predicted mature protein holds an identity of 47.7% with a recently reported cytochrome P450 sequence from *Coriolus versicolor* [23].

4. Discussion

Results reported here show that splice variants of *lip* and *mnp* genes contribute to transcript levels in liquid and solid-state cultures of *P. chrysosporium*. These incompletely processed mRNA species, which contain one or more introns, could be detected by analyses of the RT-PCR product clones. The pattern of splice variants in the clones from the three libraries obtained with

lip consensus primers were consistent with those obtained with the clones from the libraries prepared with *lip* specific primers. This observation supports the notion that splice variants do not correspond to artifacts generated in the PCR methodology. Some spliced variants were found with *lip* consensus primers and were not observed using *lip* specific primers, and *viceversa*. As the primer pairs used for RT-PCR analyses can only allow detection of splice variants produced inside a particular transcript sequence, it is conceivable that splice variants of introns 1 and 2, and 7–9 (Fig. 1) were also present in liquid and soil incubations. Although the presence of introns 3–5 in some splice variants may be explained by contamination with genomic DNA, such possibility is very unlikely. RNA preparations were treated with DNase and RNA preparations not subjected to RT did not give PCR products. In addition, unspliced PCR products were never observed with the housekeeping gene used as control. Moreover, if genomic material would have been present in the mRNA extracts, we should have found splice variants containing all introns in most (if not all) culture and time conditions tested. Analyses of the sequences of all *lip* and *mnp* splice variants showed that the exon and intron sequences exactly matched those of the genomic sequence DNA. This fact rules out the possibility that these splice variants are not correctly processed due to point mutations in the intron–exon boundaries (GT-AG). Also, it indicates that alternative donor or acceptor sites [13,24] were not used in these splicing variants.

There are reports of alternative splicing in fungi. For example, differential splicing has been reported in the glucoamylase gene of *Aspergillus niger* [25], the *cox-5* gene in *Neurospora crassa* [26], two genes (YKL186C/MTR2 and YML034W) in *Saccharomyces cerevisiae* [27], the inosine 5′- monophosphate dehydrogenase gene in *Pneumocystis carinii f. sp. carinii* [28], and *ctrI* and *ctrYB* genes in *Xanthophyllomyces dendrorhous* [24]. In addition, there are three genes where alternative splicing has been detected in *P. chrysosporium*: the *pc-1* gene encoding cytochrome P450 monooxygenase [13], and the *cbhI.1* and *cbhI.2* genes [12,29]. What we report here does not correspond to alternative splicing, since all the detected splice variants had several stop codons in frame with the initiation codon. Therefore, we refer to this phenomenon as "altered" splicing, as has been previously proposed [7]. Altered splicing was observed in both liquid and solid state cultures, with patterns that varied with the growth conditions. Thus, *lipA* and *lipB* transcripts were found as mature mRNAs at the initial time of incubation in a wood-containing soil system, whereas splice variants from both transcripts were detected after twenty days of incubation.

Initially, analyses of *mnp* clone sequences failed to show high identity with the *mnp1*, *mnp2* or *mnp3* genes [5]. Further inspection of the sequences revealed that one of them encodes for a protein that fully matches MnP1, whereas the other differs in only one aminoacid residue with this enzyme, as previously described by Martinez et al. [22]. The sequences previously reported for the *mnp* 1 gene [21] and the corresponding *mnp* 1 cDNA [30] were obtained with the strain OGC101, which derives from ME-446 [31]. Inasmuch as the *P. chrysosporium* genome contains sequence information from strain RP-78, a homokaryotic derivative of BKM-1767 [32], differences with sequences from OGC101 are not unexpected. At the nucleotide level, both *mnp* 1 and *mnp* 4 RP-78 alleles share extended homology with the known *mnp1* and *mnp4 cDNA* sequences [21,30]. Therefore, to identify the *mnp* genes of strain RP-78 we used as a criterion the deduced aminoacidic sequences as it was done by Martinez et al. [22]. On the other hand, the high nucleotide identity between *mnp* 1 and *mnp* 4, and the fact that both genes are located in a cluster in transcriptionally inverted orientation, suggests a likely recent duplication. Moreover, the presence of a cytochrome P450 encoding gene between both *mnp* genes, which could be involved in lignin-degradation, suggests a possible "functional clustering" In addition, a cluster of multicopper oxidase genes has been recently reported in *P. chrysosporium* [7].

The annotation of the *P. chrysosporium* genome has revealed a total of five *mnp* genes [22], two of which (*mnp* 4 and *mnp* 5) have not been previously reported. In this work, we detected transcripts corresponding to *mnp4*, suggesting that this gene is expressed in the culture conditions employed. MnP5 matches the N-terminal sequence of a peroxidase partially purified from colonized wood pulp, which seems to be the dominant isoenzyme in solid lignocellulosic conditions [33]. The forward primer used in this study presents two mismatches with *mnp5*, which are located 6 and 8 nts from its 3′ end. In turn, the reverse primer possesses mismatches at positions 6 and 14 from its 3′ end with *mnp5*. These mismatches should not preclude the amplification of this gene. Nevertheless, under our experimental settings, we did not find transcripts corresponding to *mnp* 5 or *mnp* 3. This does not imply that these genes are not expressed, since the efficiency of the primers employed to amplify transcripts deriving from them is unclear. Now that the sequences for all five *mnp* genes are available, a more detailed analysis could be conducted in the future, in order to correlate the expression of these five genes under different conditions. To date, we do not know the physiological significance of altered splicing. In any event, since it has been observed in genes that are present in multiple copies in the genome, it would be expected to have a limited deleterious effect relative to single genes.

Acknowledgements

This work was funded by FONDECYT Grants 8990004 and 1030495, and by the Millennium Institute for Fundamental and Applied Biology, Chile. L.F.L. was supported by a predoctoral fellowship from Fundacion Andes.

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