ELSEVIER

FEMS Microbiology Letters 199 (2001) 91–96

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Isolation and characterization of homokaryotic strains from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*

Mario Tello [a], Daniela Seelenfreund [a], Sergio Lobos [a], Jill Gaskell [b], Daniel Cullen [b], Rafael Vicuña [c,d,*]

[a] *Laboratorio de Bioquímica General, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile*

[b] *USDA Forest Products Laboratory, Madison, WI 53705, USA*

[c] *Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile*

[d] *Millenium Institute for Fundamental and Applied Biology, Santiago, Chile*

Received 30 January 2001; received in revised form 22 March 2001; accepted 24 March 2001

First published online 12 April 2001

Abstract

Genetic analyses of the lignin-degrading fungus *Ceriporiopsis subvermispora* is complicated by a dikaryotic nuclear condition and the absence of spore forms. Previous investigations had identified a family of closely related sequences encoding manganese peroxidase (MnP), but the relationship between genes and allelic variants could not be experimentally established. Addressing this issue, homokaryotic derivatives of *C. subvermipora* strain FP105752 were isolated from regenerated protoplasts. Designated CsA and CsB, their homokaryotic nature was established by polymerase chain reaction amplification and sequence analysis of the allelic variants of three MnP genes. Isoelectrofocusing revealed fewer MnP isoenzymes in filtrates of homokaryon cultures relative to the parental strain. The homokaryotic strains will simplify genetic analyses, particularly the identification of new genes.

Keywords: Homokaryon; Basidiomycete; Manganese peroxidase; Ligninolytic; *Ceriporiopsis subvermispora*

1. Introduction

White-rot basidiomycetes such as *Ceriporiopsis subvermispora* selectively and efficiently delignify woody tissue [1]. Components of the ligninolytic system include manganese peroxidase (MnP) and a copper-containing phenol oxidase termed laccase [2]. The former is a heme-containing glycoprotein that oxidizes Mn(II) to Mn(III), which then acts as an oxidant of phenolic residues present in the lignin macromolecule [3]. MnP is also able to mediate the oxidation of non-phenolic lignin structures through a novel mechanism involving peroxidation of unsaturated ra has demonstrated considerable promise in biomechanical pulping processes [5], and cultures efficiently transform an array of recalcitrant xenobiotics [6,7].

When grown in either solid or liquid cultures, *C. subvermispora* produces a family of MnP isoenzymes within a wide range of isoelectric points [8]. Heterogeneity in N-terminal sequences of isoenzymes suggested multiple genes [8], and differences in their kinetic properties imply distinct roles in lignin degradation [9]. MnP isoenzyme multiplicity has been observed in related white-rot fungi [10–13].

C. subvermispora sequences encoding a laccase [14] and four MnPs [15,16] have been reported. The MnP-like sequences designated *Csmnp2a* and *Csmnp2b* are 97% identical [16]. This observation, together with the probable dikaryotic nature of *C. subvermispora* [17], suggests an allelic relationship.

* Corresponding author at address c. Tel.: +56 (2) 686 2663; Fax: +56 (2) 222 5515; E-mail: rvicuna@bio.puc.cl

0378-1097/01/$20.00
PII: S0378-1097(01)00156-2

Allelism among the closely related sequences can be experimentally resolved by analyses of homokaryotic progeny. For example, basidiospores are the homokaryotic products of meiosis in *Phanerochaete chrysosporium* [18], another white-rot fungus marked by families of closely related genes. Segregation analysis of these fully viable haploid progeny provides direct evidence of allelic relationships [19,20] as well as genetic linkage [21,22]. Unfortunately, *C. subvermispora* does not sporulate under laboratory conditions and therefore it has not been possible to carry out classical genetic analysis with the natural isolate. Circumventing this limitation, we regenerated cultures from protoplasts and demonstrated their homokaryotic condition by segregation analysis of *Csmnp2a* and *Csmnp2b*. Results confirmed the sequences are allelic variants of the same gene. Extension of the analysis identified alleles of *Csmnp1* and *Csmnp3*. Future genetic investigations of *C. subvermispora* will be substantially simplified by analysis of these homokaryotic derivatives.

2. Materials and methods

2.1. Isolation of homokaryon strains from protoplasts

Homokaryotic strains of *C. subvermispora* were isolated from regenerated protoplasts of the dikaryotic strain (FP105752, Forest Products Laboratory, Madison, WI, USA). Protoplast preparation followed the method of Brody and Carbon [23] with modifications. *C. subvermispora* was grown for 3 days at 25°C in 10 ml of YMPG medium [8]. The mycelium was minced, pelleted and the media replaced with 20 ml of fresh YMPG medium. The fungus was grown for an additional 18 h at 25°C. Mycelium was harvested by filtration through Miracloth (Calbiochem) and washed with MgOsm solution (0.5 M $MgSO_4$, 0.05 M maleic acid, pH 5.9). The washed mycelium was then resuspended in 10 ml of MgOsm solution containing 10 mg ml^{-1} of Novozyme 234 (Novo Nordisk, Denmark). After 90 min at 37°C, the digest was filtered through Miracloth and the protoplasts were collected by centrifugation at low speed. Dilutions containing approximately 10^3 protoplasts ml^{-1} were plated on PDA medium and incubated at 28°C. After 4 days, colonies were transferred to fresh PDA plates.

Dikaryotic and homokaryotic strains were differentiated by polymerase chain reaction (PCR) amplification of *Csmnp2a* and *Csmnp2b* sequences followed by restriction enzyme analysis and direct sequencing. Primers (1546: 5′·GCC·GTT·CTT·CCA·CGC·GAA·TGC·T-3′ and 1547: 5′-GTG·TCG·TTG CCG CCC TGC·TTG·T·3′) were designed to amplify *Csmnp2a* and *Csmnp2b*. PCR reactions were performed in 50 μl with 5 μl (approximately 100 ng) of DNA, 7 pmol of each primer and 1 U of Taq polymerase (Promega) under standard PCR conditions. To discriminate *Csmnp2a* and *Csmnp2b* amplicons, we took advantage of a *Kpn*I site present in *Csmnp2b* but not in *Csmnp2a* [16]. The digested PCR products were fractionated on agarose gels. The identity of each PCR product was further confirmed by direct sequencing using an ABI Prism Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with an ABI377 DNA sequencer.

The haploid condition of the putative homokaryotic isolates was also established by PCR amplification and sequence analysis of two additional genes. *Csmnp1* [15] was PCR-amplified using oligonucleotides 1520 (5′-CTC GCG CAA GAC CTA CAG TCT-3′) and 1523 (5′-GAA TGG AAG CGG GGA CGC G-3′) and *Csmnp3* [16] with oligonucleotides 1550 (5′-ATT·GCC·ATC·TCC·CAA·TCT·TTG-3′) and 1551 (5′-GTG·ACG·GGC·CCG·ACG GAT·AG·-3′). These PCR products were also directly sequenced.

2.2. Culture conditions and enzyme assays

For enzymatic studies either homokaryons or the wild-type strain were grown in liquid salts medium [2] at 30°C for 10 days. MnP and laccase were assayed as previously described [2] and activities were corrected by dry weight of the mycelium.

2.3. Growth rate

Glass petri plates containing 20 ml of YMPG liquid medium were inoculated with *C. subvermispora* homokaryons (CsA and CsB) and also with the wild-type strain (CsWT) and grown for 3 or 6 days at 25°C. The biomass in each culture was estimated from the dry weight of mycelium. The growth rate was calculated as $(\ln(B_1/B_2))/\Delta T$, where B_1 and B_2 correspond to the biomass of cultures at days 3 and 6, respectively, and ΔT is the time (days) elapsed between them.

2.4. Isoelectrofocusing (IEF)

After 10 days growth, culture filtrates were concentrated approximately 100-fold in dialysis bags using powdered polyethylene glycol-32 000. The concentrated fluid was then extensively dialyzed against 20 mM sodium acetate pH 5.0 at 4°C. IEF analyses were performed over the pH range 4–6 on 5% polyacrylamide gels containing 5% glycerol and 2% ampholytes (Bio-lytes, Bio-Rad). Samples were focused on a refrigerated plate and the gel stained for MnP activity, as previously described [8].

2.5. DNA extraction

PDA plates with regenerated colonies of putative homokaryotic strains of *C. subvermispora* were scraped with a spatula and the mycelium transferred to Eppendorf tubes with 1 ml of lysis solution (Tris–HCl, 10 mM (pH 8);

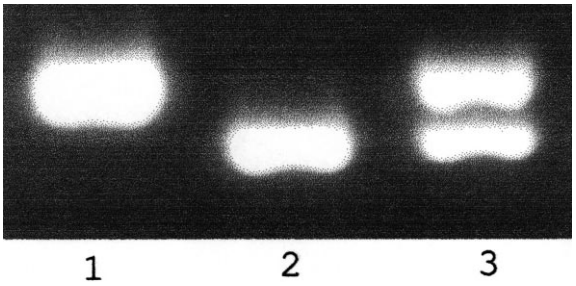

Fig. 1. Identification of the PCR products of *Csmnp2* genes. The PCR reactions were conducted using the specific primers 1546 and 1547. The PCR products were digested with *Kpn*I and analyzed by agarose gel electrophoresis. Lanes 1, 2 and 3 contain the digested PCR products of genomic DNA from CsA, CsB and the wild-type strain, respectively.

EDTA, 50 mM; β-mercaptoethanol, 3%) and quickly frozen with liquid nitrogen. After freezing, each tube was incubated at 65°C for 1 h. The DNA was purified by phenol–chloroform extraction and isopropanol precipitation. The pellet was resuspended in 500 μl of TE buffer and 5 μl were used per PCR reaction.

2.6. Statistical analyses

Statistical analyses were performed using the STATISTICA (1997) package for Windows 95. Data were analyzed by one-way ANOVA and Tukey tests ($\alpha = 0.05$) for multiple comparisons.

A

```
Csmnp1a  AAGATGgtgtgcacaagtcgaatgctctgcggtgctccaagtgctgactttcattgattttagCTCACGAGATCATCCGTCTCACTTTCCgtcagtaacaattctaaatcgattgttctcc 120
Csmnp1b  AAGATGgtgtgcacaagtcgaatgctctgcggtgctccaagtgctgactttcattgattttagCTCACGAGATCATCCGTCTCACTTTCCgtcagtaacaattctaaatcgattgttctcc 119
           D  A                                                                                   H  E  I  I  R  L  T  F  H

Csmnp1a  gaatttatagtaacctctagATGATGCCATTGCCATCTCCCAATCTCTGGGTCCTTCTGCgtatgtccttcacttcttttgtagcacacgagtcgcgtggctaacattattcgatctaca 240
Csmnp1b  gaatttatagtagcctctagATGATGCCATTGCCATCTCCCAATCTCTGGGTCCTTCTGCgtatgtccttcacttcttttgtagcacacgagtcgcgtggctaacattattcgatctaca 239
                                 D  A  I  A  I  S  Q  S  L  G  P  S  A

Csmnp1a  gGGGAACCGGAGCTGACGGTTCCATGCTTCTGTTCCCGCTCGTTGAGCCCGAATTCCAGGCCAGCAACGGGATTGACGACTCCGTGAACAACTTGATCCCCTTCCTATCGAGCCACCCGA 360
Csmnp1b  gGGGAACCGGAGCTGACGGTTCCATGCTTCTGTTCCCGCTCGTTGAGCCCGAATTCCAGGCCAGCAACGGGATTGACGACTCCGTGAACAACTTGATCCCCTTCCTATCGAGCCACCCGA 359
           G  T  G  A  D  G  S  M  L  L  F  P  L  V  E  P  E  F  Q  A  S  N  G  I  D  D  S  V  N  N  L  I  P  F  L  S  S  H  P  N

Csmnp1a  ACATCACCGCTGGTGATCTCGTCCAGTTCGCTGGTGCAGTCGCCCTCACGAACTGTCCTgtcagtaccggtgttccgaggtccattgaaccggtttgctcacatgtcttttttgtttgcag 480
Csmnp1b  ACATCACCGCTGGTGATCTCGTCCAGTTCGCTGGTGCAGTCGCCCTCACGAACTGTCCTgtcagtaccggtgttccgaggtccattgaaccggtttgctcacatgtcttttttgtttgcag 479
           I  T  A  G  D  L  V  Q  F  A  G  A  V  A  L  T  N  C  P

Csmnp1a  GGCGCTCCGCGCGTGAACTTCCTGGCGGGCCGCAAGAACGCGGTTGCCCCGGCCATTGATGGTCTGATCCCCGTGCCGCAGGACAACGTCTCCACCATTCTTGCTCGCTTTGCCGATGCT 600
Csmnp1b  GGCGCTCCGCGCGTGAACTTCCTGGCGGGCCGCAAGAACGCGGTTGCCCCGGCCATTGATGGTCTGATCCCCGTGCCGCAGGACAACGTCTCCACCATTCTTGCTCGCTTTGCCGATGCT 599
           G  A  P  R  V  N  F  L  A  G  R  K  N  A  V  A  P  A  I  D  G  L  I  P  V  P  Q  D  N  V  S  T  I  L  A  R  F  A  D  A

Csmnp1a  GGCAACTTCTCGCCGTTCGAGGTCGTCTCGCTCCTGGCCTCGCACAGTGTGGCGCGGGCCGACAAGGTCGACCCGACCCTTGATGCCGCTCCCTTCGACACCgtgagtgccttccatcctt 720
Csmnp1b  GGCAACTTCTCGCCGTTCGAGGTCGTCTCGCTCCTGGCCTCGCACAGTGTGGCGCGGGCCGACAAGGTCGACCCGACCCTTGATGCCGCTCCCTTCGACACCgtgagtgccttccatcctt 719
           G  N  F  S  P  F  E  V  V  S  L  L  A  S  H  S  V  A  R  A  D  K  V  D  P  T  L  D  A  A  P  F  D  T

Csmnp1a  tgagaccgaggattgcctgctgatacgtgctgcatcttctagACTCCCTTCACTTTCGATACCCAGATTTTCCTCGAAGTTCTTCTCAAGGGCGTCGGCTTC 821
Csmnp1b  tgagaccgaggattgcctgctgatacgtgctgcatcttctagACTCCCTTCACTTTCGATACCCAGATTTTCCTCGAAGTTCTTCTCAAGGGCGTCGGCTTC 820
                                                  T  P  F  T  F  D  T  Q  I  F  L  E  V  L  L  K  G  V  G  F
```

B

```
Csmnp3a  gcagcaaactaacgcatcattgacattatatcggatctgcagGGGTACCGGAGCTGACGGGTCCATGCTTCTGTTCCCGCTCGTTGAGCCCGAGTTCGCGGCCAGCAACGGTATCGACG 120
Csmnp3b  gcagcaaaccaacgcatcattgacattatatcggatctgcagGGGTACCGGAGCTGACGGGTCCATGCTTCTGTTCCCGCTCGTTGAGCCCGAGTTCGCGGCCAGCAACGGTATTGACG 119
                                                  G  T  G  A  D  G  S  M  L  L  F  P  L  V  E  P  E  F  A  A  S  N  G  I  D  D

Csmnp3a  ACTCCGTGAACAACTTGATCCCCTTCTTATCGAGCCACCCGAACATCTCTGCTGGTGATCTGTCCAGTTCGCTGGTGCCGTTGCCCTCACGAACTGCCCTgtcagtacca-----gcat 235
Csmnp3b  ACTCCGTGAACAACTTGATTCCCTTCTTGTCGAGCCACCCGAACATCTCTGCTGGTGATCTGTCCAGTTCGCTGGTGCCGTTGCCCTCACGAACTGCCCTgtcagtaccatacca gcat 239
           S  V  N  N  L  I  P  F  L  S  S  H  P  N  I  S  A  G  D  L  V  Q  F  A  G  A  V  A  L  T  N  C  P

Csmnp3a  gctagcgcccattgtatctgttttctcacatatttccttctttgtagGGCGCTCCACGCGTGAACTTCCTGGCGGGCCGCAAGAACGCGGTTGCCCCGGCCATTGATGGTCTGATCCCCG 355
Csmnp3b  gctagcgcccattgtatctgttttctcacatatttccttctttgtagGGCGCTCCACGCGTGAACTTCCTGGCGGGCCGCAAGAACGCGGTTGCCCCGGCCATTGATGGTCTGATCCCCG 359
                                                  G  A  P  R  V  N  F  L  A  G  R  K  N  A  V  A  P  A  I  D  G  L  I  P  E

Csmnp3a  AGCCGCAGGACAACGTCACGAGCATCCTTGCTCGGTTTGCTGATGCTGGCAACTTCTCGCCGTTCGAGGTCGTCTCGCTCCTGGCTTCGCACAGCGTAGCCCGTGCCGACAAGGTCGACC 475
Csmnp3b  AGCCGCAGGACAACGTCACGAGCATCCTTGCTCGGTTTGCTGATGCTGGCAACTTCTCGCCGTTCGAGGTCGTCTCGCTCCTGGCTTCGCACAGCGTAGCCCGTGCCGACAAGGTCGACC 479
           P  Q  D  N  V  T  S  I  L  A  R  F  A  D  A  G  N  F  S  P  F  E  V  V  S  L  L  A  S  H  S  V  A  R  A  D  K  V  D  P

Csmnp3a  CGACCCTCGATGCCGCTCCTTTCGACAGTgtcagtgcctttttccttgagaccgaggcttaacgtgctgacgcatagtccaacccctagACGCCGTTCACTTTCGACACCCAAGTCTTCC 595
Csmnp3b  CGACCCTCGATGCCGCTCCTTTCGACAGTgtcagtgcctttttccttgagaccgaggcttaacatgctgacgcatagtccaacccctagACGCCGTTCACTTTCGACACCCAAGTCTTCC 599
           T  L  D  A  A  P  F  D  S                                                                T  P  F  T  F  D  T  Q  V  F  L

Csmnp3a  TCGAGGTCCTCCTCAAGGGTGTCGGCTTCCCCGGAACCGACAACAACACTGGCGAGGTTGCGTCTCCGCTTCCGTTCGGCGATACCTCGACTGGCGGAAACGACACCGGCATGATGCGTC 715
Csmnp3b  TCGAGGTTCTCCTCAAGGGTGTCGGCTTCCCCGGAACCGACAACAACACTGGCGAGGTTGCGTCTCCGCTTCCGTTCGGCGATACCTCGACTGGCGGAAACGACACCGGCATGATGCGTC 719
           E  V  L  L  K  G  V  G  F  P  G  T  D  N  N  T  G  E  V  A  S  P  L  P  F  G  D  T  S  T  G  G  N  D  T  G  M  M  R  L

Csmnp3a  TCCAGTCCGACTTCGCCCTCGCGCGCGACGAGCGCACTGCTTGCTTCTGGCAGAGCTTCGTCGACCAACAGGACTTGATGGCGCAGAGTTTCCAGGCCGCTTTCGAGAAGCTCGCCAT 836
Csmnp3b  TCCAGTCCGACTTCGCCCTCGCGCGCGACGAGCGCACTGCTTGCTTCTGGCAGAGCTTCGTCGACCAACAGGACTTGATGGCGCAGAGTTTCCAGGCCGCTTTCGAGAAGCTCGCCAT 838
           Q  S  D  F  A  L  A  R  D  E  R  T  A  C  F  W  Q  S  F  V  D  Q  Q  D  L  M  A  Q  S  F  Q  A  A  F  E  K  L  A  I
```

Fig. 2. Alignment of the nucleotide sequences of the PCR products amplified from the genomic DNA of homokaryons. (A) Alignment of *Csmnp1* from products obtained using DNA from CsA (*Csmnp1a*) and CsB (*Csmnp1b*). (B) Alignment of *Csmnp3* products obtained using DNA from CsA (*Csmnp3a*) and CsB (*Csmnp3b*). Allelic differences are indicated by open boxes, and introns are in lower case. Sequences *Csmnp1*, *Csmnp2a*, *Csmnp2b* and *Csmnp3* have been assigned GenBank accession numbers AF013257, AF161078, AF161584 and AF1615, respectively.

Table 1
Ligninolytic enzyme production and growth rates of homokaryotic (CsA and CsB) and dikaryotic parent (CsWT)

	CsWT	CsA	CsB
MnP (U g^{-1})[a]	4.64 ± 0.23	5.35 ± 0.11	2.70 ± 0.19
Laccase (U g^{-1})[b]	2.11 ± 0.04	2.44 ± 0.50	1.68 ± 0.06
Growth ($days^{-1}$)[b]	0.69 ± 0.008	0.48 ± 0.016	0.29 ± 0.064

[a]The activity of ligninolytic enzymes was measured after 10 days of culture and corrected for dry weight.
[b]Growth rates evaluated as $(\ln(B_1/B_2))/\Delta T$ where B_1 and B_2 correspond to the biomass of cultures of 3 and 6 days, respectively, and ΔT to the elapsed time between them.

3. Results

Colonies regenerated from protoplasts showed a low rate of growth, which was visible after 4 days. Approximately 100 colonies were analyzed by PCR and only 10 showed one band after *Kpn*I digestion; five corresponding to *Csmnp2a* and five to *Csmnp2b* (Fig. 1). Of these colonies, one of each group was selected for further studies. Designated CsA and CsB, they harbored the *Csmnp2a* and *Csmnp2b* sequences, respectively. The identity of each PCR product was unambiguously established by direct sequencing and comparison to previously reported sequences [16].

When *Csmnp1* and *Csmnp3* genes were PCR-amplified from genomic DNA of CsA, CsB and CsWT, a single band was obtained in each case (data not shown). Direct sequencing of these PCR products showed a small number of distinct differences. Alignment of the PCR products obtained with primers designed to amplify the *Csmnp1* gene showed seven mismatches, five of which were located within introns. Differences within exons were confined to the third base of codons and were silent changes. These results strongly suggest that the two PCR products derived from allelic variants of *Csmnp1*. A similar alignment of the PCR products obtained with the primers complementary to *Csmnp3* revealed 13 differences in 838 nucleotides, eight of which were within introns. Considering that they correspond also to allelic variants, *Csmnp3b* contained a perfect repeat of 5 bp. As in the case of *Csmnp1* allelic variants, differences within exon sequences were in the third position of codons and would not alter translation (Fig. 2). Previous attempts to directly sequence the *Csmnp3* PCR products from the parental dikaryon had yielded illegible electropherograms, probably due to the presence of the two templates (data not shown).

Phenotypic features such as growth rate and ligninolytic enzyme production were compared among homokaryotic and wild-type strains (Table 1). Growth rate showed significant differences (one-way ANOVA: $F_{2,30} = 257.75$, $P \ll 0.0001$) among strains. The a posteriori Tukey test revealed that all strains differed, being higher in CsWt and lower in CsB. Isoenzyme patterns of MnP also showed marked differences between strains. At least seven isoenzymes were apparent in the parent culture, while CsA and CsB cultures showed only four or three isoforms, respectively (Fig. 3). Homokaryotic cultures contained MnP isoenzymes with a low isoelectric point that are typical of cultures of wild-type strains grown on wood [8]. In contrast, the laccase pattern did not differ between homokaryotic and dikaryotic strains (data not shown).

4. Discussion

The non-spore forming basidiomycete *C. subvermispora* is of considerable interest for its efficient ligninolytic system(s), but it is limited as an experimental system. Genetic analysis has revealed two MnP sequences, *Csmnp2a* and *Csmnp2b*. An allelic relationship between these sequences seemed plausible on the basis of sequence comparisons (95 and 97% identity at nucleotide and protein level, respectively) [16]. However, large gene families are common in white-rot fungi, and extreme sequence conservation has been observed among members of the peroxidase gene families [24]. Consequently, the possibility that *Csmnp2a* and *Csmnp2b* were separate genes could not be formally excluded. The segregation of these sequences into protoplast-derived cultures strongly supports an allelic relationship.

The homokaryotic nature of CsA and CsB was confirmed by PCR amplification and sequence analysis of alleles of *Csmnp3* and *Csmnp1*. The *Csmnp3* gene was found in its previously reported form in homokaryon CsA and a novel allelic version was discovered in CsB. Comparison of *Csmnp3a* and *Csmnp3b* showed 13 differences of which eight were transitions, two were transversions and three were deletions. This is in general agreement with the estimated frequency of transitions in relation to transversions [25]. A duplicated sequence of five nucleotides was detected in the fifth intron (Fig. 2).

PCR products of *Csmnp1* from CsA and CsB showed seven differences of which five were transitions and only one was a transversion. The relative frequency is similar to the changes shown by *Csmnp3* genes and the majority of these differences also occur within introns. The allelic variant found in the fourth intron changes the first base of the consensus lariat [26] sequence by a T. However, this new sequence (TTAAC) within the fourth intron of *Csmnp1b* is in agreement with a lariat variant recently reported in yeast [27]. Usually, nucleotide mismatches are approximately six times more abundant within introns relative to exons. Differences in exons fall on the third base of their respective codons, which do not change the coded amino acid.

The phenotypic differences between both homokaryotic strains and the wild-type strains were quite striking. Whether derived from protoplasts or basidiospores, homokaryotic derivatives of basidiomycetes often vary substantially with respect to growth rate and/or enzyme produc-

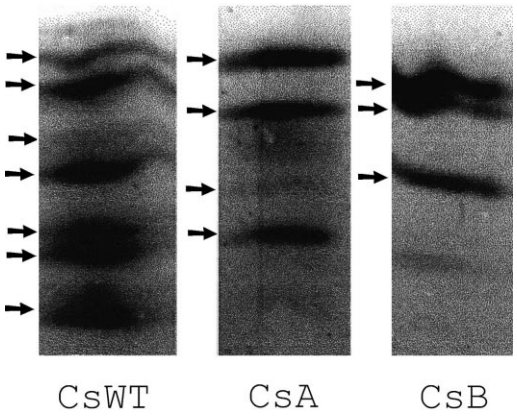

Fig. 3. IEF analysis of MnPs produced by CsA, CsB and the wild-type strain. MnP activity (0.09 U) was applied to a gel possessing a pH range from 4 to 6 and electrophoresis was conducted as indicated in Section 2. The gel was stained for MnP activity using 4-chloro-naphtol. The arrows denote the position of the various isoenzymes in the electrofocusing gels.

tion [28–31]. In the case of *P. chrysosporium*, single basidiospore cultures generally exhibit low growth rate and enzyme production [32,33], but a protoplast-derived homokaryon was markedly similar to the parent [34].

An interesting feature is that the CsA strain showed a higher ratio of ligninolytic enzymes versus biomass than the wild strain, which could reflect a higher ligninolytic potential. Conversely, the CsB strain showed a lower enzymatic activity versus biomass and an even slower growth rate. The ligninolytic enzyme production of the wild strain was nearly an average between the homokaryotic strains and may correspond to a combination of both homokaryons.

The MnP isoenzyme pattern differed among strains, with fewer bands visible in the homokaryon cultures relative to the parent (Fig. 3). Altered patterns of α-esterase isoenzymes were also observed among homokaryotic strains of *Agaricus bisporus* [31]. Fewer bands for homokaryons seems in agreement with their reduced genomic complexity. However, it has been shown that a single MnP gene may give rise to multiple isoforms [35], and interactions among allelic forms during glycosylation might generate complex banding patterns.

Homokaryotic cultures provide a useful tool for genetic analysis. Here we have shown how allelic relationships can be resolved. In addition, analysis of genomic libraries, particularly assembly and genome walking, is greatly simplified by the absence of alleles.

Acknowledgements

We thank Dr. G. Hermosilla and G. Ebensperguer for the DAPI staining and some experiments to induce sporulation. This work was supported by grants from FONDECYT 1971239 and FONDECYT 8990004 to R.V., by an Eduardo Neale-Silva scholarship to M.T., and by US Department of Energy Grant DE-FG02-87ER13712 to D.C.

References

[1] Otjen, L., Blanchette, R., Effland, M. and Leatham, G. (1987) Assessment of 30 white rot basidiomycetes for selective lignin degradation. Holzforschung 41, 343–349.
[2] Ruttimann, C., Schwember, E., Salas, L., Cullen, D. and Vicuña, R. (1992) Ligninolytic enzymes of the white rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. Biotechnol. Appl. Biochem. 16, 64–76.
[3] Wariishi, H., Valli, K. and Gold, M.H. (1991) In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. 176, 269–275.
[4] Jensen, K., Bao, W., Kawai, S., Srebotnik, E. and Hammel, K. (1996) Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis subvermispora* in the absence of lignin peroxidase. Appl. Environ. Microbiol. 62, 3679–3686.
[5] Akhtar, M., Blanchette, R., Myers, G. and Kirk, T.K. (1998) An overview of biomechanical pulping research. In: Environmentally Friendly Technologies for the Pulp and Paper Industry (Young, R. and Akhtar, M., Eds.), pp. 309–340. Wiley, New York.
[6] Garon, D., Krivobok, S. and Seigle-Murandi, F. (2000) Fungal degradation of fluorene. Chemosphere 40, 91–97.
[7] Krivobok, S., Miriouchkine, E., Seigle-Murandi, F. and Benoit-Guyod, J.L. (1998) Biodegradation of anthracene by soil fungi. Chemosphere 37, 523–530.
[8] Lobos, S., Larrain, J., Salas, L., Cullen, D. and Vicuña, R. (1994) Isozymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. Microbiology 140, 2691–2698.
[9] Urzua, U., Fernando Larrondo, L., Lobos, S., Larrain, J. and Vicuña, R. (1995) Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Ceriporiopsis subvermispora*. FEBS Lett. 371, 132–136.
[10] Johansson, T. and Nyman, P.O. (1993) Isozymes of lignin peroxidase and manganese(II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. I. Isolation of enzyme forms and characterization of physical and catalytic properties. Arch. Biochem. Biophys. 300, 49–56.
[11] Martinez, M.J., Ruiz-Duenas, F.J., Guillen, F. and Martinez, A.T. (1996) Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii*. Eur. J. Biochem. 237, 424–432.
[12] Perie, F.H., Sheng, D. and Gold, M.H. (1996) Purification and characterization of two manganese peroxidase isozymes from the white-rot basidiomycete *Dichomitus squalens*. Biochim. Biophys. Acta 1297, 139–148.
[13] Pease, E. and Tien, M. (1992) Heterogeneity and regulation of manganese peroxidases from *Phanerochaete chrysosporium*. J. Bact. 174, 3532–3540.
[14] Karahanian, E., Corsini, G., Lobos, S. and Vicuña, R. (1998) Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochim. Biophys. Acta 1443, 65–74.
[15] Lobos, S., Larrondo, L., Salas, L., Karahanian, E. and Vicuña, R. (1998) Cloning and molecular analysis of a cDNA and the Cs-mnp1 gene encoding a manganese peroxidase isoenzyme from the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. Gene 206, 185–193.
[16] Tello, M., Corsini, G., Larrondo, L.F., Salas, L., Lobos, S. and Vicuña, R. (2000) Characterization of three new manganese peroxi-

dase genes from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochim. Biophys. Acta 1490, 137–144.
[17] Nakasone, K.K. (1981) Cultural studies on *Poria cinerascens*, *P. rivulosa*, and *P. subvermispora* (Aphyllophorales, Basidiomycotina). Mycotaxon 13, 105–111.
[18] Alic, M., Letzring, C. and Gold, M.H. (1987) Mating system and basidiospore formation in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microb. 53, 1464–1469.
[19] Gaskell, J., Vanden Wymelenberg, A., Stewart, P. and Cullen, D. (1992) Method for identifying specific alleles of a *Phanerochaete chrysosporium* encoding a lignin peroxidase. Appl. Environ. Microbiol. 58, 1379–1381.
[20] Kersten, P.J., Witek, C., Vanden Wymelenberg, A. and Cullen, D. (1995) *Phanerochaete chrysosporium* glyoxal oxidase is encoded by two allelic variants: structure, genomic organization and heterologous expression of *glx1* and *glx2*. J. Bact. 177, 6106–6110.
[21] Gaskell, J., Stewart, P., Kersten, P., Covert, S., Reiser, J. and Cullen, D. (1994) Establishment of genetic linkage by allele-specific polymerase chain reaction: application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. BioTechnology 12, 1372–1375.
[22] Raeder, U., Thompson, W. and Broda, P. (1989) RFLP-based genetic map of *Phanerochaete chrysosporium* ME446: lignin peroxidase genes occur in clusters. Mol. Microbiol. 3, 911–918.
[23] Brody, H. and Carbon, J. (1989) Electrophoretic karyotype of *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 86, 6260–6263.
[24] Cullen, D. (1997) Recent advances on the molecular genetics of ligninolytic fungi. J. Biotechnol. 53, 273–289.
[25] Topal, M.D. and Fresco, J.R. (1976) Complementary base pairing and the origin of substitution mutations. Nature 263, 289–293.
[26] Padgett, R.A., Konarska, M.M., Grabowski, P.J., Hardy, S.F. and Sharp, P.A. (1984) Lariat RNAs as intermediates and products in the splicing of messenger RNA precursors. Science 225, 898–903.
[27] Spingola, M., Grate, L., Haussler, D. and Ares, M. (1999) Genome-wide bioinformatic and molecular analysis of introns in *Saccharomyces cerevisiae*. RNA 5, 221–234.
[28] Prokop, A., Rapp, P. and Wagner, F. (1992) Production of extracellular β-1,3/β-1,6-glucan by mono- and dikaryons of *Schizophyllum commune*. Exp. Mycol. 16, 197–206.
[29] Addleman, K. and Archibald, F. (1993) Kraft pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. Appl. Environ. Microbiol. 59, 266–273.
[30] Eichlerova-Volakova, I. and Homolka, L. (1997) Variability of ligninolytic enzyme activities in basidiospore isolates of the fungus *Pleurotus ostreatus* in comparison with that of protoplast-derived isolates. Folia Microbiol. 42, 583–588.
[31] Sonnenberg, A., Wessels, J.G. and van Griensven, L.J. (1988) An efficient protoplasting/regeneration system for *Agaricus bisporus* and *Agaricus bitorquis*. Curr. Microbiol. 17, 285–291.
[32] Wyatt, A.M. and Broda, P. (1995) Informed strain improvment for lignin degradation by *Phanerochaete chrysosporium*. Microbiology 141, 2811–2822.
[33] Raeder, U., Thompson, W. and Broda, P. (1989) Genetic factors influencing lignin peroxidase activity in *Phanerochaete chrysosporium* ME446. Mol. Microbiol. 3, 919–924.
[34] Stewart, P., Gaskell, J. and Cullen, D. (2000) A homokaryotic derivative of a *Phanerochaete chrysosporium* strain and its use in genomic analysis of repetitive elements. Appl. Environ. Microbiol. 66, 1629–1633.
[35] Larrondo, L.F., Lobos, S., Stewart, P., Cullen, D. and Vicuña, R. (2000) Isozyme multiplicity and characterization of recombinant manganese peroxidases (rMnPs) from *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 67, in press.