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# Saline-Dependent Regulation of Manganese Peroxidase Genes in the Hypersaline-Tolerant White Rot Fungus *Phlebia* sp. Strain MG-60▽

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**The expression pattern of manganese peroxidases (MnPs) in nitrogen-limited cultures of the saline-tolerant fungus *Phlebia* sp. strain MG-60 is differentially regulated under hypersaline conditions at the mRNA level. When MG-60 was cultured in nitrogen-limited medium (LNM) containing 3% (wt/vol) sea salts (LN-SSM), higher activity of MnPs was observed than that observed in normal medium (LNM). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis demonstrated that two MnP isoenzymes were de novo synthesized in the culture of LN-SSM. Three MnP-encoding genes (MG*mnp1*, MG*mnp2*, and MG*mnp3*) were isolated by reverse transcription (RT)-PCR and rapid amplification of cDNA ends PCR techniques. The corresponding isozymes were identified by peptide mass fingerprinting analysis. MnP isozymes encoded by MG*mnp2* and MG*mnp3* were observed mainly in LN-SSM. Real-time RT-PCR analysis revealed high levels of MG*mnp2* and MG*mnp3* transcripts in LN-SSM 48 h after the addition of 2% NaCl. The induction of MnP production and the accumulation of gene transcripts by saline were well correlated in the presence of $Mn^{2+}$. However, in the absence of $Mn^{2+}$, there was no clear correlation between *mnp* transcripts levels and MnP activity, suggesting posttranscriptional regulation by $Mn^{2+}$.**

Mangroves are trees that grow in saline habitats in the tropics and subtropics. Plants in mangrove forests have developed a set of physiological adaptations in response to frequent tidal inundation. Mangroves and sea grasses provide a natural habitat for marine fungi. Marine fungi are often found on decayed lignocellulosic substrates such as prop roots, pneumatophores, branches, leaves, and driftwood in the intertidal region of mangrove stands. They are thought to play an important role in lignocellulosic degradation in such marine ecosystems (9). Commonly isolated marine fungi belong to ascomycetes and deuteromycetes, while basidiomycetes are relatively rarely reported (22, 27, 36). The lignocellulolytic enzymes of marine fungi have potential industrial and environmental applications (22, 27, 29).

White rot fungi have a unique ability to decompose wood lignin via the secretion of extracellular lignin-degrading enzymes such as manganese peroxidase (MnP), lignin peroxidase, versatile peroxidase, and laccase. MnP is considered to be one of the key enzymes involved in lignin degradation caused by white rot fungi. MnP oxidizes $Mn^{2+}$ to $Mn^{3+}$ in an $H_2O_2$-dependent reaction, and $Mn^{3+}$ organic acid chelates oxidize monomeric phenol, phenolic lignin dimers, and synthetic lignin via the formation of a phenoxy radical (7, 19). Additionally, MnP participates in lignin biodegradation via thiol and lipid-derived free radicals that are able to oxidize a variety of nonphenolic aromatic compounds (1, 35). Although many genes encoding MnP have been cloned from several white rot fungi, there has been no report focusing on marine fungi.

The marine fungus *Phlebia* sp. strain MG-60 was selected from 28 mushrooms and driftwoods collected from mangrove stands in Okinawa, Japan, based on PolyR-478 decolorization and lignin biodegradation under hypersaline conditions (15). *Phlebia* sp. strain MG-60 produces MnP mainly under hypersaline conditions. It was able to brighten the unbleached hardwood kraft pulp extensively even under conditions of 5% (wt/vol) sea salts. In contrast, pulp was only slightly brightened by the widely studied white rot fungus *Phanerochaete chrysosporium* at 3% (wt/vol) and 5% (wt/vol) sea salt concentrations (15, 16). Thus, MG-60 has significant bleaching ability, especially in a hypersaline environment.

To clarify the effect of hypersaline conditions on MnP production from *Phlebia* sp. strain MG-60, herein, we compare the productions of MnP activity and different MnP isozymes under normal and hypersaline conditions. We also provide the full sequences of three new MnP-encoding genes, MG*mnp1*, MG*mnp2*, and MG*mnp3*, which are differentially regulated in response to saline stress.

## MATERIALS AND METHODS

**Fungal cultures.** *Phlebia* sp. strain MG-60 TUFC40001 (Fungus/Mushroom Resource and Research Center, Tottori, Japan), *Trametes versicolor* NBRC6482, and *Phanerochaete chrysosporium* ATCC 34541 were maintained on potato dextrose agar (PDA) plates. Mycelium mats on an agar plate were transferred into a sterilized blender cup containing 50 ml of sterilized water and were homogenized with a Waring blender for 30 s. To monitor the MnP activity and isozyme expression pattern, 5 ml of homogenate was inoculated into a 500-ml Erlenmeyer flask containing 300 ml of low-nitrogen basal III medium (LNM) that contained 1.0% (wt/vol) glucose as a carbon source, 1.2 mM ammonium tartrate as a nitrogen source, and 20 mM sodium acetate at pH 4.5 (33) or LNM containing 3% (wt/vol) sea salts (LN-SSM) (Sigma). One gram of sea salts contained 482.25 mg of chloride, 269.5 mg of sodium, 66.5 mg of sulfate, 33 mg of magnesium, 10.5 mg of potassium, 10 mg of calcium, 5 mg of carbonate/bicarbonate, 0.22 mg of strontium, 0.14 mg of boron, 1.4 mg of bromide, and <0.5 mg of other total trace element (manufacturer's analysis). Flasks were incubated on a rotary shaker at 150 rpm in the dark at 30°C. After the prescribed incubation period, the whole

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culture was separated into biomass and extracellular fluid by centrifugation (12,000 rpm for 10 min). The mycelial dry weight and extracellular MnP activity were then measured. In order to investigate the effect of sea salt and its main component, NaCl, on the transcription of MG*mnp* genes, LNM containing 2% NaCl (LN-NaClM) was also used.

**Enzyme activity and SDS-PAGE.** MnP activity was determined spectrophotometrically at 270 nm by monitoring the formation of the $Mn^{3+}$-malonate complex at pH 4.5 in 50 mM sodium malonate buffer (34). For analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), culture fluids were concentrated in an Amicon ultrafiltration unit with a 10-kDa-cutoff Omega membrane filter (Filtron), and the concentrates were desalted using a PD-10 column (GE Healthcare). An equal amount of protein of the resulting fraction was loaded onto each lane and separated by 10% (wt/vol) SDS-PAGE. The proteins were visualized by staining with 0.2% (wt/vol) Coomassie brilliant blue R-250.

**Isolation of nucleic acid and cDNA preparation.** The mycelia from 10-ml liquid cultures were filtered through a Miracloth (Calbiochem), semidried with a sterile paper towel, frozen rapidly in liquid nitrogen, and stored at −80°C. The frozen mycelium was ground into a powder in a mortar containing liquid nitrogen. DNA was isolated from the mycelium powder with an extraction buffer (2% CTAB [*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide], 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, and 0.2% β-mercaptoethanol) and purified with chloroform-isoamyl alcohol (24:1). Total RNA was prepared by a combination of plant RNA isolation reagent (Invitrogen Corp., Carlsbad, CA) and TRIzol reagent (Invitrogen Corp., Carlsbad, CA). The obtained RNA was washed with 75% ethanol and dissolved in diethylpyrocarbonate-treated water. The amount and quality of the RNA were calculated by measuring the absorbances at 260 and 280 nm.

cDNA was synthesized in a 20-μl reaction mixture that included 1 μg of total RNA, 1 μM oligo(dT) adapter primer containing an M13 primer M4 sequence, 10 U of RNase inhibitor, and 10 U of avian myeloblastosis virus reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. The reaction was carried out for 60 min at 45°C, and the samples were the heated for 5 min at 95°C to terminate the reaction. Finally, the reaction mixture was diluted 1:100 with diethylpyrocarbonate-treated water, and a 1-μl sample was used for real-time reverse transcription-PCR (RT-PCR) analysis as described below.

**Gene identification and characterization.** To isolate the partial sequence of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) in *Phlebia* sp. strain MG-60, a 629-bp fragment of *gpd* cDNA was amplified with a pair of degenerate primers (forward primer 5′-GGTCGYATYGGCCGYATYGT-3′ and reverse primer 5′-ATRACCTTKCCGACRGCCTT-3′) (5). The PCR amplification was performed with a denaturation step at 94°C for 3 min, followed by 35 cycles that consisted of 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min and, finally, an extension step at 72°C for 5 min. To identify the MnP-encoding genes, 1,075-bp and 1,081-bp fragments of MG*mnp2* and MG*mnp3* cDNA containing a poly(A) sequence were amplified with a degenerate sense primer (5′-TSCGYCTYAYK TTCCACGA-3′) and M13 primer M4 (5′-GTTTTCCCAGTCACACGAC-3′). The degenerate sense primer was designed according to the amino acid sequence conserved in the Mn-binding region (RLTFHD). A fragment of MG*mnp1* cDNA (909 bp) was amplified with a degenerate primer (5′-AACTGYCCYGGYGCD CCMCR-3′) and M13 primer M4. The degenerate sense primer was designed according to the conserved sequence (NCPGAP) among *mnp* genes. Each consensus amino acid sequence was searched from alignment data reported previously (19). The PCR amplification was performed with a denaturation step at 94°C for 3 min, followed by 35 cycles that consisted of 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min and, finally, an extension step at 72°C for 5 min.

A 5′ rapid amplification of cDNA ends system (Invitrogen) was used to amplify the missing 5′ ends of the transcripts according to the manufacturer's instructions. The full-length genomic DNA and open reading frame of cDNA of the *mnp* genes were amplified with primers designed according to the nucleotide sequence data obtained from the rapid amplification of cDNA ends PCR fragments.

Ex-*Taq* polymerase (TaKaRa, Japan) or *Pfu* polymerase (Stratagene, La Jolla, CA) was used for PCR amplifications. The PCR products were subcloned into vector pXcmkn12, and the resulting ligation products were transformed into *Escherichia coli* strain DH5α according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The clones were sequenced by a dideoxy method (Thermo Sequence cycle sequence kit; Amersham Bioscience) with a sequencer (LIC-4000; Aloka, Japan).

**Peptide mass fingerprinting analysis.** To identify the *mnp* gene encoding the isozyme observed by SDS-PAGE, gel tryptic digestion was performed as previously described (30). The target band was excised and cut into 2-mm cubes, and the gel pieces were then transferred into a 200-μl microcentrifuge tube and washed with 40% (vol/vol) 1-propanol in water at room temperature for 15 min. After removal of the 1-propanol solution, 200 mM ammonium bicarbonate in 50% (vol/vol) acetonitrile in water was added, and the sample was incubated at room temperature for 15 min. The gel pieces were then dried and covered with 20 ng/μl modified trypsin (Promega) in a minimal volume of 100 mM ammonium bicarbonate to rehydrate the pieces. After a 12-h incubation, the supernatant was collected, and the gel pieces were extracted with 100 mM ammonium bicarbonate, followed by two extractions with 80% (vol/vol) acetonitrile containing 0.05% trifluoroacetic acid. The supernatant and extracts were combined, and the acetonitrile was allowed to evaporate in a desiccator at room temperature. The resulting peptide mixtures were desalted using $C_{18}$ ZipTips (Millipore) and eluted onto a 96-well matrix-assisted laser desorption ionization target plate. A 1-μl sample on the plate was mixed with 1 μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid solution in $H_2O$-acetonitrile (1:1) containing 0.1% trifluoroacetic acid. Samples were then dried at room temperature. Mass spectral data were obtained using a Voyager DE mass spectrometer equipped with a 337-nm $N_2$ laser in the positive-ion reflectron mode (Applied Biosystems). Spectral data were obtained by averaging 64 spectra, each of which was the composite of 64 laser firings. The internal mass calibration was performed using bradykinin (904.45 Da) and adrenocorticotropic hormone (2,465.75 Da).

**Incubation for transcriptional analysis.** Expression of the MnP-encoding genes was assessed under conditions using several media. Mycelium was preincubated in 500-ml Erlenmeyer flasks containing 300 ml of LNM without $MnSO_4$ for 5 days as described above. Preincubated mycelium was transferred into a sterilized blender cup and homogenized with a Waring blender for 30 s. One milliliter of homogenate was inoculated into 100-ml Erlenmeyer flasks containing 10 ml of LNM, LN-SSM, LN-NaClM, or $MnSO_4$-free versions of each medium. Flasks were incubated statically at 30°C in the ambient atmosphere. After incubation, the whole culture was separated into biomass and extracellular fluid by centrifugation. The total RNA was then extracted from mycelium, and the extracellular MnP activity was measured as described above.

**Real-time RT-PCR.** Real-time, fluorescence-based RT-PCR was performed using a final volume of 10 μl with a Line Gene apparatus (Bio Flux Corporation, Japan). The total RNA was prepared from the mycelium harvested from the above-mentioned 10-ml cultures of LNM, LN-SSM, or LN-NaClM. cDNA was synthesized in a final volume of 20 μl that included 1 μg of total RNA, 1 μM oligo(dT) 18-mer primer, 10 U of RNase inhibitor, and 10 U of avian myeloblastosis virus reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. After RT for 60 min at 45°C, the samples were heated for 5 min at 95°C to terminate the reaction. The Sybr Premix Ex-*Taq* kit (TaKaRa, Japan) was used for real-time PCR according to the manufacturer's instructions, with a final concentration of 0.2 μM for each gene-specific primer. The PCR amplification was performed as follows: (i) an initial denaturation step at 95°C for 1 min and (ii) 45 cycles, with 1 cycle consisting of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and elongation at 72°C for 30 s. Amplicon specificity was verified by melting-curve analysis conducted at 65°C to 95°C with stepwise fluorescence acquisition and by 2% (wt/vol) agarose gel electrophoresis staining with ethidium bromide. No fluorescence was detected from real-time RT-PCR amplification without a template. Primer sequences and amplicon lengths for the MnPs and reference gene are listed in Table 1. In the cases of MG*mnp2* and MG*mnp3*, the forward primer sequence was designed from a coding region as close as possible to the stop codon. The reverse primer was constructed in the 3′ untranslated region to achieve specificity. Although the possibility of the existence of alternative splicing cannot be completely excluded, gel electrophoresis of PCR products using each forward primer and a poly(A) primer confirmed that a single product was amplified with each primer pair. Besides that of the melting curve, gel electrophoresis analysis confirmed single-product amplification for *gpd*, MG*mnp1*, MG*mnp2*, and MG*mnp3* under all conditions tested here. The ratio of the gene-specific expression was defined as expression relative to *gpd* gene expression. Data are presented as means of triplicate PCRs, each containing an aliquot of the same template.

**Transcriptional induction of MG*mnp* genes in *Phlebia* sp. strain MG-60.** To monitor the expression of the MnP-encoding genes after the addition of NaCl to $MnSO_4$-free LNM, mycelium was preincubated statically at 30°C in 100-ml Erlenmeyer flasks containing 10 ml $MnSO_4$-free LNM for 6 days, at which time the maximum activity of MnP in LNM was produced. After this preincubation period, 0.2 g of solid NaCl (equivalent to 2%) was added to the culture, mixed gently, and then incubated. Every day, each culture was harvested, and the whole culture was separated into biomass and extracellular fluid by centrifugation. Total RNA was extracted from mycelium, and the extracellular MnP activity was measured.

TABLE 1. Gene-specific PCR primers used for real-time RT-PCR

| Target gene | GenBank accession no. | Primer | Amplicon size (bp) |
|---|---|---|---|
| MG*mnp1* | AB360587 | 5′-CTATCCTTGGTCACTCTGAG-3′[a]<br>5′-TCATGAGTATCAACGATCTAA-3′[b] | 227 |
| MG*mnp2* | AB360588 | 5′-TTCCCCACGCTGACTACGGA-3′[a]<br>5′-CATTGCCCATATATGGAATTCGA-3′[b] | 219 |
| MG*mnp3* | AB360589 | 5′-GTTTCCCTACGCTTTCTGTTTC-3′[a]<br>5′-GTGTAGCGATAGATAGAATGTG-3′[b] | 197 |
| *gpd* | AB360638 | 5′-GCTTCAAGGTCATCTCGAAC-3′[a]<br>5′-GACGGGATGTTGTTACC-3′[b] | 199 |

[a] Forward primer.
[b] Reverse primer.

## RESULTS

**Effect of 3% (wt/vol) sea salts on MnP production.** *Phlebia* sp. strain MG-60, *P. chrysosporium*, and *T. versicolor* were cultured in LNM (with $MnSO_4$) with or without sea salts. The time course of MnP production is shown in Fig. 1. In the case of *P. chrysosporium*, MnP production in LN-SSM (with $MnSO_4$) was decreased to about one-half of the level of MnP production in LNM (with $MnSO_4$) (Fig. 1A). There was no growth of *T. versicolor* in LN-SSM (data not shown). On the other hand, when MG-60 was cultured in LN-SSM (with $MnSO_4$), the MnP activity was four times higher than that in LNM (with $MnSO_4$) (Fig. 1B). When this fungus was incubated in LNM (without $MnSO_4$) or LN-SSM (without $MnSO_4$), there was no MnP activity in the extracellular fluid under stationary conditions (data not shown). SDS-PAGE analysis of the extracellular proteins that were harvested from the 10-day shaking culture gave one main band (45 kDa) in the LNM (with $MnSO_4$) culture and two main bands (47 kDa and 50 kDa) in LN-SSM (with $MnSO_4$) (Fig. 2B).

*Phlebia* sp. strain MG-60 was incubated in LNM (with $MnSO_4$) with shaking for 10 days, and sea salts at 3% (wt/vol) of the final concentration were then added to the culture. The MnP activity was increased rapidly 48 h after the addition of sea salts (Fig. 2A). SDS-PAGE analysis of the extracellular concentrates revealed that two de novo-synthesized proteins (47 kDa and 50 kDa) appeared within 48 h of the addition of sea salts (Fig. 2B). Primary protein (45 kDa) disappeared, coinciding with the induction of the other two proteins (47 kDa and 50 kDa). These proteins are identical to the main proteins observed in the 10-day LN-SSM (with $MnSO_4$) culture.

**Cloning of *mnp* genes from *Phlebia* sp. strain MG-60.** Three full-length cDNA clones of *mnp* genes, MG*mnp1* (1,095 bp), MG*mnp2* (1,173 bp), and MG*mnp3* (1,170 bp), were obtained based on the PCR strategy. The nucleotide sequence of MG*mnp1* predicts a 365-amino-acid (aa) sequence containing a putative signal peptide (26 aa) at the N terminus. The predicted amino acid sequence of MG*mnp1* was 92% and 80% identical to *Phlebia radiata* MnP3 and *Trametes versicolor* MP2, respectively. The cDNA sequences of MG*mnp2* and MG*mnp3* were 77% identical, and their predicted amino acid sequences were 80% identical. MG*mnp2* and MG*mnp3* encode 390- and 389-aa sequences, respectively, and each one features a 23-aa putative secretion signal at the N terminus. The amino acid sequence of MG*mnp2* was 74%, 72%, and 65% identical to *P. radiata* MnP2, *Dichomitus squalens* MnP1, and *Ceriporiopsis subvermispora* MnP-2, respectively. The amino acid sequence of MG*mnp3* was 82%, 77%, and 70% identical to *P. radiata* MnP2, *D. squalens* MnP2, and *C. subvermispora* MnP-2, respectively. Multiple alignments revealed conserved catalytic and Mn binding residues (19; data not shown). The genomic nucleotide sequences of MG*mnp1*, MG*mnp2*, and MG*mnp3* were also obtained by PCR using specific primer sets constructed on the 5′ and 3′ untranslated region sequences. Comparisons of cDNA and genomic sequences showed 100% identity within coding regions and allowed the unambiguous assignment of exon-intron boundaries. MG*mnp1* contains 10 short introns, whereas MG*mnp2* and MG*mnp3* contain seven short introns. The intron-exon organizations of genes were compared with other selected genes encoding MnP (data not shown). The intron positions of MG*mnp2* and MG*mnp3* corresponded with that of Pr*mnp2* completely. The intron position of MG*mnp1* closely resembled that of Pr*mnp3*, although the eighth intron of Pr*mnp3* was absent in MG*mnp1*. From the

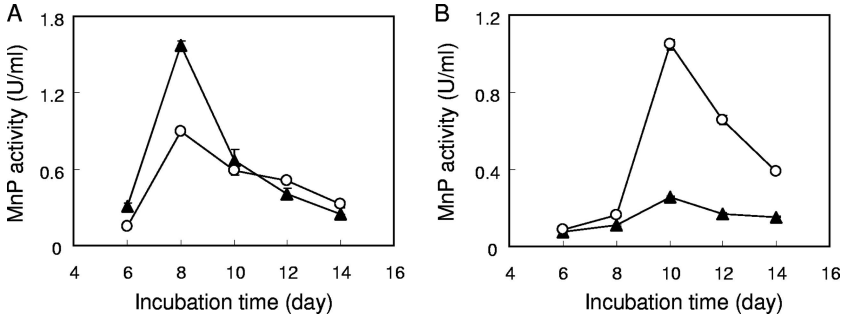


FIG. 1. Extracellular MnP activities of *P. chrysosporium* (A) and *Phlebia* sp. strain MG-60 (B) in LNM (black triangles) and LN-SSM (open circles) under shaking conditions. Values are means ± standard deviations of two duplicates.

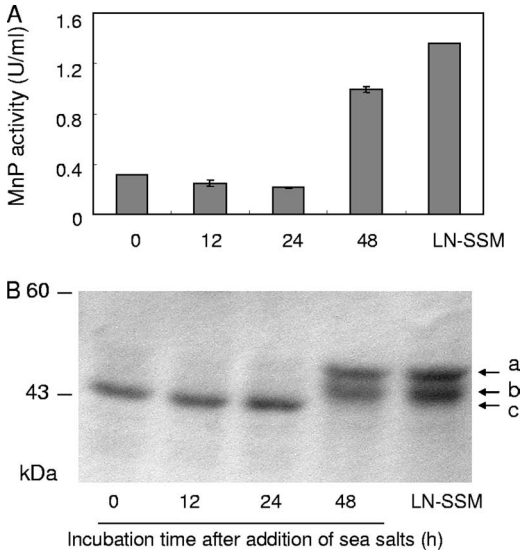


FIG. 2. Induction of extracellular MnP activity after the addition of sea salts (A) and SDS-PAGE of concentrated extracellular culture fluid (B). The lanes of time zero and LN-SSM correspond to the cultures incubated for 10 days with LNM and LN-SSM, respectively. Values are means ± standard deviations of two duplicates.

phylogenetic grouping (Fig. 3), MG-MnP1 appears to be more closely related to the versatile peroxidase group, whereas MG-MnP2 and MG-MnP3 are clustered in the classical manganese peroxidase group.

**Identification of MnP-encoding genes.** Protein digestion was performed using trypsin, and a peptide mass map was obtained using matrix-assisted laser desorption ionization–time of flight mass spectrometry. The peptide mass map was compared with the predicted amino acid sequences from MG*mnp* genes. In the case of the 45-kDa protein that is expressed mainly in LNM, 48% sequence coverage (10/15 masses matched) was achieved with MG-MnP1. Additionally, 47-kDa and 50-kDa proteins showed 38% (8/16 masses matched) and 30% (4/11 masses matched) sequence coverages with MG-MnP3 and MG-MnP2, respectively. These results indicated that the 45-, 47-, and 50-kDa proteins are encoded by the MG*mnp1*, MG*mnp3*, and MG*mnp2* genes, respectively. Furthermore, the results showed that MG-MnP3 and MG-MnP2 were found mainly under hypersaline conditions.

**Effect of sea salts and NaCl on transcription of MG*mnp* genes.** Higher levels of MnP production in LN-SSM were observed under static culture conditions (Fig. 4A) as well as in the shaking culture (Fig. 1B). The main component of sea salts is NaCl, and consistent with its major role, MnP production was also increased in the culture containing 2% (wt/vol) NaCl in LN-NaClM (Fig. 4A). SDS-PAGE analysis showed that MG-MnP2 and MG-MnP3 were also produced mainly in the LN-SSM static culture (Fig. 4B) as well as in the shaking culture (Fig. 2B). Under the LNM condition, the transcription of each gene was observed on days 4, 5, 6, and 7 (Fig. 5). However, in the medium containing sea salts (LN-SSM) or NaCl (LN-NaClM), MG*mnp2* and MG*mnp3* transcripts increased substantially on days 6 and 7, while the MG*mnp1* transcript levels remained low.

**Effect of $Mn^{2+}$ on transcription of MG*mnp* genes.** Extracellular MnP activities under $MnSO_4$-sufficient and -free culture conditions were compared. Although the highest MnP activity

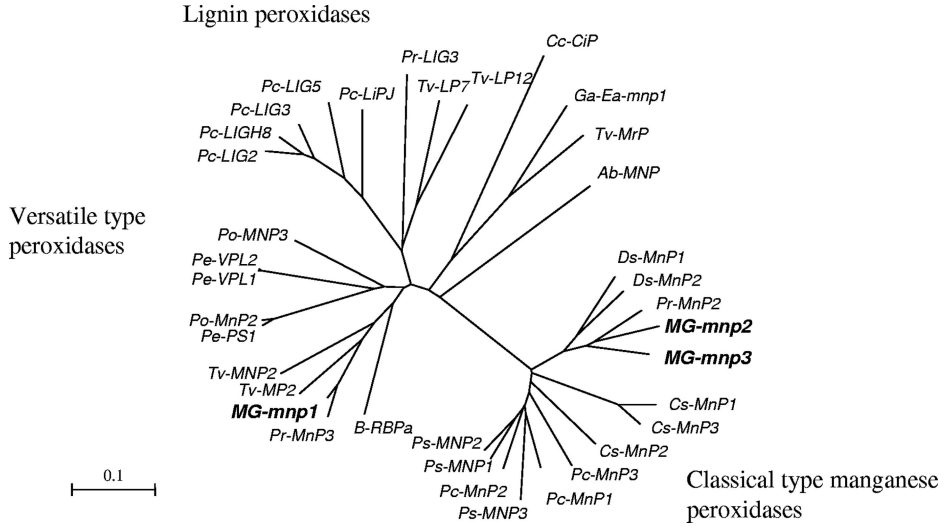


FIG. 3. Neighbor-joining tree of the evolutionary relations of deduced amino acid sequences of selected class II fungal secretory heme peroxidases. Ab, *Agaricus bisporus*; Ba, *Bjerkandera adusta*; B, *Bjerkandera* sp.; Cc, *Coprinus cinereus*; Cs, *Ceriporiopsis subvermispora*; Ds, *Dichomitus squalens*; Ga, *Ganoderma applanatum*; Pc, *Phanerochaete chrysosporium*; Pe, *Pleurotus eryngii*; Po, *Pleurotus ostreatus*; Pr, *P. radiata*; Ps, *Phanerochaete sordida*; Tv, *Trametes versicolor*. Sequence accession numbers were retrieved from the DDBJ Nucleotide Sequence Databank and are as follows: AJ699058 (Ab-MNP), AY217015 (B-RBPa), E03952/E51135 (Ba-LiP), Q12575 (Cc-CiP), AF013257 (Cs-MnP1), AF036254 (Cs-MnP2), AF161585 (Cs-MnP3), AF157474 (Ds-MnP1), AF157475 (Ds-MnP2), AB035734 (Ga-Ea.mnp1), M74229 (Pc-LIG2), X51590 (Pc-LIG3), M18794 (Pc-LIG5), M37701 (Pc-LIGH8), AF140062 (Pc-LiPJ), M60672 (Pc-MnP1), L29039 (Pc-MnP2), U70998 (Pc-MnP3), AF007223 (Pe-VPL1), AF007222 (Pe-VPL2), AF175710 (Pe-PS1), AJ243977 (Po-MnP2), AB011546 (Po-MNP3), X14446 (Pr-LIG3), AJ315701 (Pr-MnP2), AJ310930 (Pr-MnP3), AB078604 (Ps-MNP1), AB078605 (Ps-MNP2), AB078606 (Ps-MNP3), Z31011 (Tv-LP12), Z30667 (Tv-LP7), Z54279 (Tv-MNP2), Z30668 (Tv-MP2), AF008585 (Tv-MrP), AB360587 (MG-MnP1), AB360588 (MG-MnP2), and AB360589 (MG-MnP3).

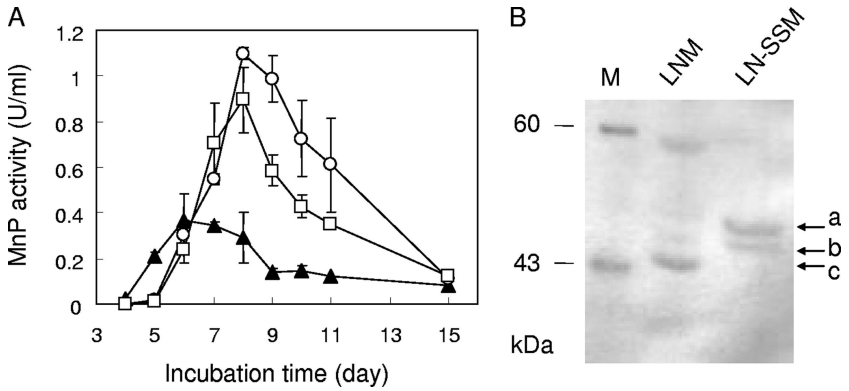


FIG. 4. Extracellular MnP activities of *Phlebia* sp. strain MG-60 in LNM (black triangles), LN-SSM (open circles), and LN-NaClM (open squares) under static conditions (A) and SDS-PAGE of concentrated extracellular culture fluid at day 8 (B). Lane LNM refers to the 6-day-incubation culture in LNM, and lane LN-SSM refers to the 8-day-incubation culture in LN-SSM (B). Values are means ± standard deviations of triplicate cultures. Lane M, molecular mass marker.

was observed on day 6 using $MnSO_4$-sufficient medium, a lack of detectable extracellular MnP activity was observed in the absence of $MnSO_4$ (data not shown). In the case of $MnSO_4$-free LN-SSM, no MnP activity was observed in these static cultures. The highest level of *mnp* mRNA for each gene was obtained on day 5 in $MnSO_4$-sufficient LNM, whereas the transcript was undetectable in $MnSO_4$-free LNM (Fig. 6). On the other hand, mRNA of MG*mnp2* was observed on day 7 in $MnSO_4$-free LN-SSM irrespective of the lack of detectable extracellular MnP activity. Transcripts of MG*mnp1* or MG*mnp3* were not observed in $MnSO_4$-free LN-SSM (Fig. 6). In another experiment in which the culture was preincubated for 5 days in

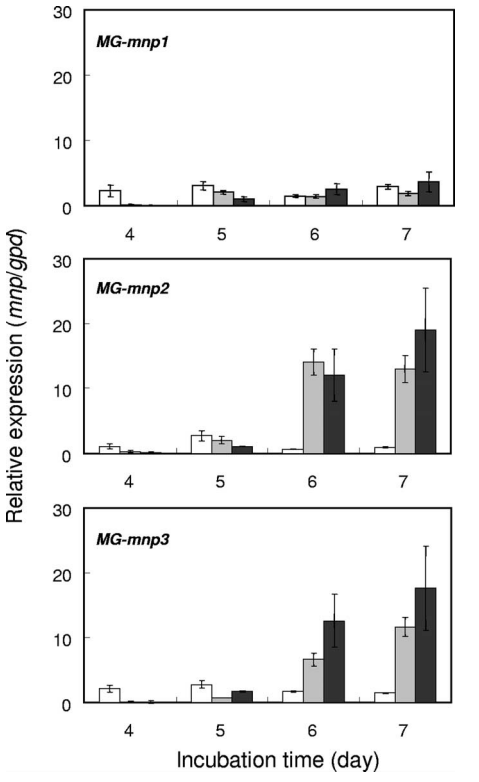


FIG. 5. Relative transcription levels of MG*mnp1*, MG*mnp2*, and MG*mnp3* in LNM (white bars), LN-SSM (light gray bars), and LN-NaClM (dark gray bars) under static culture conditions. Gene expression was determined by real-time RT-PCR and normalized to the *gpd* expression. Error bars show the standard deviations for triplicate samples.

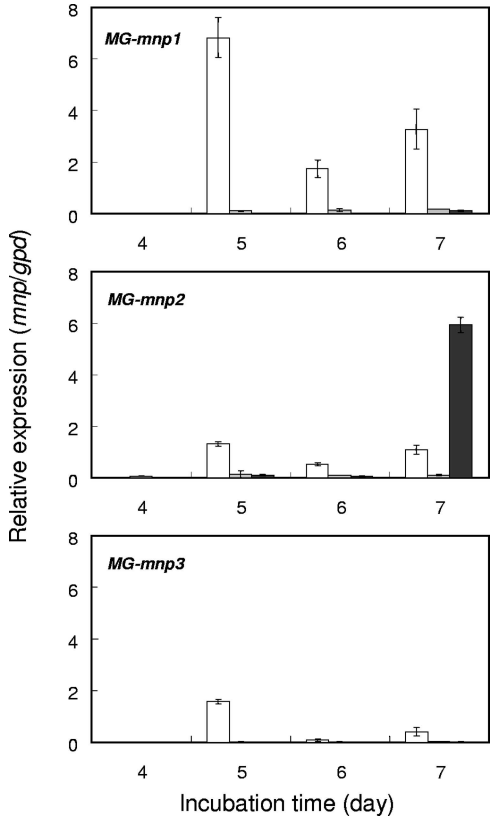


FIG. 6. Relative transcription levels of MG*mnp1*, MG*mnp2*, and MG*mnp3* in LNM (white bars), $MnSO_4$-free LNM (light gray bars), and $MnSO_4$-free LN-SSM (dark gray bars) under static culture conditions. Gene expression was determined by real-rime RT-PCR and normalized to *gpd* expression. Error bars show the standard deviations for triplicate samples.

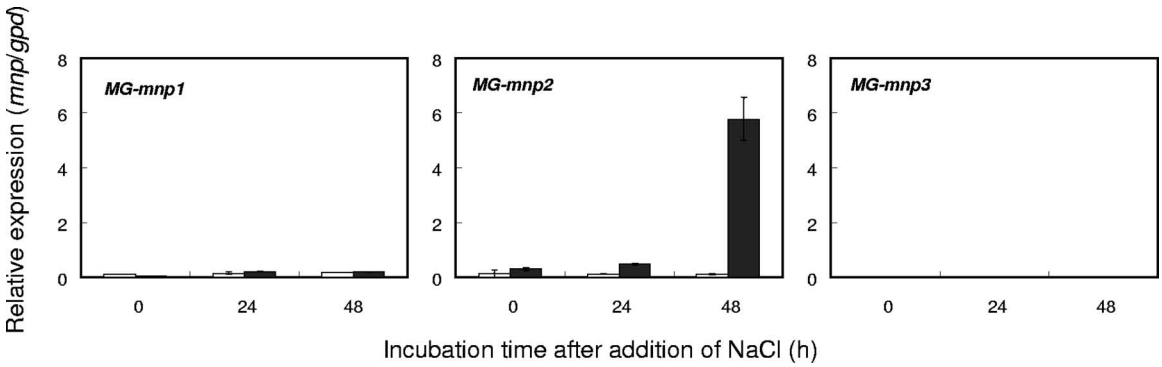


FIG. 7. Induction of MG*mnp2* transcription in $MnSO_4$-free LNM by the addition of NaCl. The fungus was cultured for 5 days in $MnSO_4$-deficient LNM (10 ml), and NaCl at a final concentration of 2% (wt/vol) was then added to the culture. Gene expression was determined by real-time PCR and normalized to the *gpd* expression. Error bars show the standard deviations for triplicate samples.

$MnSO_4$-free LNM and NaCl as 2% (wt/vol) of the final concentration was then added to the culture, the transcript of mRNA of MG*mnp2* was also induced 48 h after the addition of NaCl and $MnSO_4$-free LN-SSM (Fig. 7). These results indicate that the transcription of MG*mnp2* was responsive to NaCl but independent of $MnSO_4$.

## DISCUSSION

The mineralization of $^{14}C$-labeled lignin to $^{14}CO_2$ by marine fungi was reported previously (25, 31). Isolated from decaying sea grass from a coral lagoon off the west coast of India, the white rot fungus *Flavodon flavus* produced lignin peroxidase, MnP, and laccase in nitrogen-limited medium prepared with distilled water or with synthetic seawater using Instant Ocean salts (Instant Ocean, Mentor, OH). However, enzyme production in LN medium containing Instant Ocean salts was reduced by about 50% relative to that in LN medium (26). In the present study, the growth of *T. versicolor* was severely inhibited and MnP production by *P. chrysosporium* was reduced in LN-SSM. These results indicate that the seawater condition is unsuitable for growth and enzyme production by these lignin-degrading fungi. When MG-60 was incubated in PDA medium containing several concentrations of sea salts, the growth of this fungus was observed even at a 5% (wt/vol) sea salt concentration (15). Furthermore, the growth of MG-60 in PDA medium containing 1.5% (wt/vol) sea salts was faster than that in normal PDA medium, while the growths of *P. chrysosporium* and *T. versicolor* were inhibited using the same concentration of sea salts (unpublished data). MG-60 had better tolerance to a hypersaline environment, and MnP production by this fungus was strongly enhanced (Fig. 1). Therefore, it seems that *Phlebia* sp. strain MG-60 has a special mechanism for the expression of MnP under hypersaline conditions.

In the present study, the results show that saline stress up-regulated the specific MnP isozymes MG*mnp2* and MG*mnp3* of *Phlebia* sp. strain MG-60 at the transcriptional level. To our knowledge, this is a unique system among the white rot fungi characterized so far. Figure 2 clearly shows that MnP production was enhanced in response to the addition of sea salts. Moreover, MG-MnP2 and MG-MnP3 were de novo-synthesized MnP isoforms under saline conditions. These characteristics were similarly observed under either shaking or static culture conditions, suggesting that the production of MG-MnP1, MG-MnP2, and MG-MnP3 isozymes was differentially regulated by hypersaline conditions.

Genetic analysis revealed three MnP-encoding genes of *Phlebia* sp. strain MG-60, MG*mnp1*, MG*mnp2*, and MG*mnp3*. All *mnp* genes were closely related to the *mnp* genes described previously for *P. radiata* strain 79 (ATCC 64658) (8). Recently, phylogenetic analysis based on the internal transcribed spacer (ITS) region (containing 5.8S ribosomal DNA, ITS1, and ITS2) was carried out (11). The ITS sequence of MG-60 (DDBJ accession number AB210077) showed only 90% identity to *P. radiata* strains (ATCC 64658 [accession number DQ056859], HHB-5324-sp [accession number AB084619], JLL-15608-sp [accession number AY219366], and 345B [accession number AY089740]), while the sequences of *P. radiata* strains shared identities of approximately 99 to 100% to each other. These results indicate that *Phlebia* sp. strain MG-60 is not *Phlebia radiata*.

MG*mnp2* and MG*mnp3* were classified as being typical MnP group I representatives (Fig. 3) (19). The calculated molecular masses of MG-MnP1, MG-MnP2, and MG-MnP3 were 32 kDa, 36 kDa, and 36 kDa, respectively, although actual masses determined by SDS-PAGE were 44 kDa, 50 kDa, and 47 kDa. This difference may be caused by glycosylation, a common feature of MnP isozymes. Such differences were also reported for MnP from *P. chrysosporium* (21, 24). According to the general eukaryotic rule for N glycosylation (Asn-X-Ser/Thr) (13), two and three asparagines potentially involved in glycan linkage were found in MG-MnP2 (N132 and N218) and MG-MnP3 (N132, N218, and N348), respectively, whereas only one (N103) was found in MG-MnP1. Glycans generally modify protein conformation, leading to stabilization toward thermal denaturation or protease attack (20).

The production of three MnP isozymes by *Phlebia* sp. strain MG-60 was regulated by $Mn^{2+}$ at the mRNA level (Fig. 6). Typically, lignin degradation and peroxidase production occur as a secondary metabolic event triggered by nutrient nitrogen and/or carbon limitation. In several fungi, a sufficient amount of $Mn^{2+}$ in the medium is required for MnP expression at the mRNA level (19). Differential regulation of *mnp* genes by manganese in *Pleurotus ostreatus* (4), *C. subvermispora* (18, 32), and *T. versicolor* (6, 10, 12) was previously described. Addi-

tionally, there is a report of the existence of Mn-responsive *cis*-acting sequences found in the upstream region of *P. chrysosporium mnp1* (17). In the present study, real-time RT-PCR analysis shows that the accumulation of MG*mnp3* transcripts in sea salts/NaCl medium was dependent on $Mn^{2+}$ regulation. However, it should be noted that the MG*mnp2* transcripts were observed under $MnSO_4$-free conditions (Fig. 6 and 7), indicating that $Mn^{2+}$ is not essential for the expression of MG*mnp2*.

Higher levels of MG*mnp2* and MG*mnp3* mRNAs were obtained with simultaneous induction by $Mn^{2+}$ and sea salts than by sea salts alone: the maximum transcription ratios (*mnp*/*gpd*) of MG*mnp2* and MG*mnp3* in LNM were approximately 2 (Fig. 5), whereas those in $MnSO_4$-free LN-SSM were approximately 5 and 0 (Fig. 6) and those in LN-SSM were approximately 15 and 21 (Fig. 5), respectively. These results imply that sea salts and $Mn^{2+}$ activate the transcription of MG*mnp2* and MG*mnp3* synergistically, while sea salt-driven activation of MG*mnp3* transcription follows in an $Mn^{2+}$-dependent transcriptional manner. The accumulation of MG*mnp2* transcripts occurred within 48 h of the addition of NaCl. This induction time seems longer than those of other known stress-driven *mnp* inductions or osmotic responses in yeast. In previous studies, several stresses on *mnp* gene expression by *P. chrysosporium* were examined. The accumulation of *mnp* transcripts by heat shock (3), hydrogen peroxide, oxygen, and chemical agents (14) was reported. In these studies, the accumulation of *mnp* transcripts occurs rapidly at 0.25 h after treatment with stress agent. Osmotic stress responses in *Saccharomyces cerevisiae* have been well studied. A high-osmolarity glycerol pathway mediates the response to hyperosmotic stress (2). The activation of the high-osmolarity glycerol pathway leads to a massive and rapid transcriptional response in which the expression of several hundred genes was induced within 5 to 10 min (23, 28). Little is known about the influence of saline stress on gene expression on basidiomycetes, and the observed delay in the induction of MG*mnp2* remains to be investigated.

There was a clear correlation between *mnp* mRNA levels, MnP proteins, and MnP activity under $Mn^{2+}$-sufficient conditions. On the other hand, there was no clear correlation between *mnp* mRNA levels and MnP activity under Mn-free conditions. MnP activity was undetectable even after the addition of NaCl in the absence of $Mn^{2+}$, although higher transcripts of the MG*mnp2* gene were observed. Based on this result, besides the transcriptional regulational function of $Mn^{2+}$, $Mn^{2+}$ also seems to be involved in posttranscriptional regulation. A similar effect was reported previously for oxidative stress induction of MnP in the case of *P. chrysosporium* (14) and for metal induction of MnP in the case of *Ceriporiopsis subvermispora* (18). Those authors observed that hydrogen peroxide and some metals, $Cu^{2+}$, $Zn^{2+}$, $Ag^{2+}$, and $Cd^{2+}$, induce *mnp* mRNA accumulation but not the production of extracellular MnP activity in medium where $MnSO_4$ is absent.

This is the first report of the isolation and characterization of *mnp* genes from marine fungus and the first observation that hypersaline conditions regulate *mnp* gene expression. A marine habitat requires adaptation to cation toxicity and to osmotic stress. *Phlebia* sp. strain MG-60 might be evolutionarily adapted to saline-rich conditions for the sufficient expression of its lignin degradation ability. Further investigations of this marine fungus may provide insight into physiological adaptations to saline stress and into lignin degradation in severe environments.

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## REFERENCES

1. **Bao, W., Y. Fukushima, K. A. Jensen, Jr., M. A. Moen, and K. E. Hammel.** 1994. Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. FEBS Lett. **354:**297–300.
2. **Brewster, J. L., T. Devaloir, N. D. Dwyer, E. Winter, and M. C. Gustin.** 1993. An osmosensing signal transduction pathway in yeast. Science **259:**1760–1763.
3. **Brown, J. A., D. Li, M. Alic, and M. H. Gold.** 1993. Heat shock induction of manganese peroxidase gene transcription in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**4295–4299.
4. **Cohen, R., Y. Hadar, and O. Yarden.** 2001. Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by $Mn^{2+}$. Environ. Microbiol. **3:**312–322.
5. **Cohen, R., M. R. Suzuki, and K. E. Hammel.** 2004. Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete *Gloeophyllum trabeum*. Appl. Environ. Microbiol. **70:**324–331.
6. **Collins, P. J., M. M. O'Brien, and A. D. W. Dobson.** 1999. Cloning and characterization of a cDNA encoding a novel extracellular peroxidase from *Trametes versicolor*. Appl. Environ. Microbiol. **65:**1343–1347.
7. **Gold, M., and M. Alic.** 1993. Molecular biology of the lignin degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57:**605–622.
8. **Hilden, K., A. T. Martinez, A. Hatakka, and T. Lundell.** 2005. The two manganese peroxidases Pr-MnP2 and Pr-MnP3 of *Phlebia radiata*, a lignin-degrading basidiomycete, are phylogenetically and structurally divergent. Fungal Genet. Biol. **42:**403–419.
9. **Hyde, K. D., E. B. G. Jones, E. Leano, S. B. Pointing, A. D. Poonyth, and L. L. P. Vrijmoed.** 1998. Role of fungi in marine ecosystems. Biodivers. Conserv. **7:**1147–1161.
10. **Johansson, T., P. O. Nyman, and D. Cullen.** 2002. Differential regulation of *mnp2*, a new manganese peroxidase-encoding gene from the ligninolytic fungus *Trametes versicolor* PRL 572. Appl. Environ. Microbiol. **68:**2077–2080.
11. **Kamei, I., H. Suhara, and R. Kondo.** 2005. Phylogenetical approach to isolation of white-rot fungi capable of degrading polychlorinated dibenzo-*p*-dioxin. Appl. Microbiol. Biotechnol. **69:**358–366.
12. **Kim, Y., S. Yeo, J. Kum, H. G. Song, and H. T. Choi.** 2005. Cloning of a manganese peroxidase cDNA gene repressed by manganese in *Trametes versicolor*. J. Microbiol. **43:**569–571.
13. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54:**631–664.
14. **Li, D., M. Alic, J. A. Brown, and M. H. Gold.** 1995. Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. Appl. Environ. Microbiol. **61:**341–345.
15. **Li, X., R. Kondo, and K. Sakai.** 2002. Studies on hypersaline-tolerant white-rot fungi. I. Screening of lignin-degrading fungi in hypersaline conditions. J. Wood Sci. **48:**147–152.
16. **Li, X., R. Kondo, and K. Sakai.** 2003. Studies on hypersaline-tolerant white-rot fungi. IV. Effects of $Mn^{2+}$ and $NH^{4+}$ on manganese peroxidase production and Poly R-478 decolorization by the marine isolate *Phlebia* sp MG-60 under saline conditions. J. Wood Sci. **49:**355–360.
17. **Ma, B., M. B. Mayfield, B. J. Godfrey, and M. H. Gold.** 2004. Novel promoter sequence required for manganese regulation of manganese peroxidase isozyme 1 gene expression in *Phanerochaete chrysosporium*. Eukaryot. Cell **3:**579–588.
18. **Manubens, A., M. Avila, P. Canessa, and R. Vicuna.** 2003. Differential regulation of genes encoding manganese peroxidase (MnP) in the basidiomycete *Ceriporiopsis subvermispora*. Curr. Genet. **43:**433–438.
19. **Martinez, A. T.** 2002. Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme Microb. Technol. **30:**425–444.
20. **Olden, K., B. A. Bernard, M. J. Humphries, T. K. Yeo, K. T. Yeo, S. L. White, S. A. Newton, H. C. Bauer, and J. B. Parent.** 1985. Function of glycoprotein glycans. Trends Biochem. Sci. **10:**78–82.
21. **Pease, E. A., A. Andrawis, and M. Tien.** 1989. Manganese-dependent peroxidase from *Phanerochaete chrysosporium*—primary structure deduced from cDNA sequence. J. Biol. Chem. **264:**13531–13535.
22. **Pointing, S. B., L. L. P. Vrijmoed, and E. B. G. Jones.** 1998. A qualitative assessment of lignocellulose degrading enzyme activity in marine fungi. Bot. Mar. **41:**293–298.

23. **Posas, F., J. R. Chambers, J. A. Heyman, J. P. Hoeffler, E. de Nadal, and J. Arino.** 2000. The transcriptional response of yeast to saline stress. J. Biol. Chem. **275:**17249–17255.
24. **Pribnow, D., M. B. Mayfield, V. J. Nipper, J. A. Brown, and M. H. Gold.** 1989. Characterization of a cDNA encoding a manganese peroxidase, from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. **264:**5036–5040.
25. **Raghukumar, C., D. Chandramohan, F. C. Michel, and C. A. Reddy.** 1996. Degradation of lignin and decolorization of paper mill bleach plant effluent (BPE) by marine fungi. Biotechnol. Lett. **18:**105–106.
26. **Raghukumar, C., T. M. D'Souza, R. G. Thorn, and C. A. Reddy.** 1999. Lignin-modifying enzymes of *Flavodon flavus*, a basidiomycete isolated from a coastal marine environment. Appl. Environ. Microbiol. **65:**2103–2111.
27. **Raghukumar, C., S. Raghukumar, A. Chinnaraj, D. Chandramohan, T. M. D'Souza, and C. A. Reddy.** 1994. Laccase and other lignocellulose modifying enzymes of marine fungi isolated from the coast of India. Bot. Mar. **37:**515–523.
28. **Rep, M., M. Krantz, J. M. Thevelein, and S. Hohmann.** 2000. The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock—Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J. Biol. Chem. **275:**8290–8300.
29. **Rohrmann, S., and H. P. Molitoris.** 1992. Screening for wood-degrading enzymes in marine fungi. Can. J. Bot. Rev. Can. Bot. **70:**2116–2123.
30. **Shimizu, M., N. Yuda, T. Nakamura, H. Tanaka, and H. Wariishi.** 2005. Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. Proteomics **5:**3919–3931.
31. **Sutherland, J. B., D. L. Crawford, and M. K. Speedie.** 1982. Decomposition of C-14-labeled maple and spruce lignin by marine fungi. Mycologia **74:**511–513.
32. **Tello, M., G. Corsini, L. F. Larrondo, L. Salas, S. Lobos, and R. Vicuna.** 2000. Characterization of three new manganese peroxidase genes from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochim. Biophys. Acta **1490:**137–144.
33. **Tien, M., and T. K. Kirk.** 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161B:**238–249.
34. **Wariishi, H., K. Valli, and M. H. Gold.** 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*—kinetic mechanism and role of chelators. J. Biol. Chem. **267:**23688–23695.
35. **Wariishi, H., K. Valli, V. Renganathan, and M. H. Gold.** 1989. Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium*. J. Biol. Chem. **264:**14185–14192.
36. **Wen, L., L. L. P. Vrijmoed, and E. B. G. Jones.** 2005. Screening of marine fungi for lignocellulose-degrading enzyme activities. Bot. Mar. **48:**376–386.