APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1997, p. 3151–3157 Vol. 63, No. 8
0099-2240/97/$04.00+0

Characterization of the Gene Encoding an Extracellular Laccase of *Myceliophthora thermophila* and Analysis of the Recombinant Enzyme Expressed in *Aspergillus oryzae*

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Received 31 January 1997/Accepted 20 May 1997

A genomic DNA segment encoding an extracellular laccase was isolated from the thermophilic fungus *Myceliophthora thermophila*, and the nucleotide sequence of this gene was determined. The deduced amino acid sequence of *M. thermophila* laccase (MtL) shows homology to laccases from diverse fungal genera. A vector containing the *M. thermophila* laccase coding region, under transcriptional control of an *Aspergillus oryzae* α-amylase gene promoter and terminator, was constructed for heterologous expression in *A. oryzae*. The recombinant laccase expressed in *A. oryzae* was purified to electrophoretic homogeneity by anion-exchange chromatography. Amino-terminal sequence data suggests that MtL is synthesized as a preproenzyme. The molecular mass was estimated to be approximately 100 to 140 kDa by gel filtration on Sephacryl S-300 and to be 85 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Carbohydrate analysis revealed that MtL contains 40 to 60% glycosylation. The laccase shows an absorbance spectrum that is typical of blue copper oxidases, with maxima at 276 and 589 nm, and contains 3.9 copper atoms per subunit. With syringaldazine as a substrate, MtL has optimal activity at pH 6.5 and retains nearly 100% of its activity when incubated at 60°C for 20 min. This is the first report of the cloning and heterologous expression of a thermostable laccase.

Laccases (EC 1.10.3.1) are multicopper enzymes that catalyze the oxidation of a variety of phenolic compounds, with concomitant reduction of O_2 to H_2O. These polyphenol oxidases are widely distributed among plant (9, 10, 41, 51) and fungal (8, 14) species; however, their biological significance is unclear. Among the filamentous fungi, approximately 30 laccases have been identified in various organisms. These laccases may be involved in conidial pigmentation (4, 22), lignin degradation (2, 17, 34, 39, 56, 57, 63), pathogenicity (5), and formation of fruiting bodies (38). Interest in laccases has been fueled by their potential uses in detoxification of environmental pollutants (13, 15, 16, 26, 46, 54, 61), prevention of wine decoloration (37), paper processing (50), enzymatic conversion of chemical intermediates (1), and production of useful chemicals from lignin (57).

For any of these potential applications to become a reality, an inexpensive source of laccase must be obtained. In most fungi, laccases are produced at levels that are too low for commercial purposes. Cloning of the laccase genes followed by heterologous expression may provide higher enzyme yields. A number of genes encoding fungal laccases are cloned, including those from basidiomycetes such as *Trametes* (*Coriolus*) *versicolor* (32, 33), *Trametes villosa* (75), *Coriolus hirsutus* (35), *Rhizoctonia solani* (69), *Agaricus bisporus* (49), *Phlebia radiata* (56), basidiomycete PM1 (23), and ascomycetes *Cryphonectria parasitica* (19), *Aspergillus nidulans* (4), *Podospora anserina* (28), and *Neurospora crassa* (29). Collectively, the amino acid sequences deduced from these genes suggest that the overall structure of fungal laccases is similar to that of ascorbate oxidase from *Zucchini* (43).

The laccase genes from *C. hirsutus* and *P. radiata* have been expressed in *Saccharomyces cerevisiae* (35) and *Trichoderma reesei* (55), respectively. The yeast *GAL10* promoter was used to direct the expression of enzymatically active *C. hirsutus*, with yields of approximately 5 mg per liter (74). The *P. radiata* laccase was secreted at a level of about 20 mg per liter by using the promoter and terminator regions of the *T. reesei cbh1* gene (55). Clearly, higher enzyme titers are required for commercial enzyme production. Several *Aspergillus* species, including *Aspergillus oryzae*, are well-established as good expression systems for the heterologous production of industrial enzymes (7, 20, 24). We hypothesized that *A. oryzae* might also be well suited as a host for laccase expression and secretion.

It is well documented that thermophilic fungi may comprise a rich source of thermostable industrial enzymes (53). Furthermore, thermal tolerance is an attractive feature for many biotechnological applications of enzymes. The thermophilic fungus *Myceliophthora thermophila* (telomorph = *Thielavia heterothallica*) was described previously as a producer of cellulase and xylanase enzymes with pronounced thermal resistance (48, 53, 58–60, 76). *M. thermophila* was first described by Apinis (3) and given the name *Sporotrichum thermophile*. Its taxonomic position was reassigned to the genus *Chrysosporium* (68) and later to its current genus (65). Our objectives were to determine if *M. thermophila* produced a thermostable extracellular laccase, clone the gene encoding it, express this gene in *A. oryzae*, and biochemically characterize the resulting enzyme.

MATERIALS AND METHODS

Fungal strains and plasmids. Genomic DNA was isolated from *M. thermophila* CBS 117.65. *Escherichia coli* JM101 (45) was used for construction and routine propagation of laccase expression vectors. The fungal host for laccase expression was a uridine-requiring (*pyrG*) mutant of the α-amylase-deficient *A. oryzae* strain HowB104.

The vector pMWR3 was constructed by inserting the *A. oryzae* α-amylase promoter and terminator elements from pTAKA17 (11, 21) into pUC18 (72). In this vector, there are a *Swa*I site at the end of the promoter and an *Nsi*I site at the beginning of the terminator for directional cloning. The cloning vehicle

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pUC519 (6a) was derived by inserting a small linker containing *Nsi*I, *Cla*I, *Xho*I, and *Bgl*II restriction sites between the adjacent *Bam*HI and *Xba*I sites of pUC119 (66).

Materials. The chemicals, buffers, and substrates used were commercial products of at least reagent grade. Endo/*N*-glycosidase F and pyroglutamate aminopeptidase were purchased from Boehringer Mannheim (Indianapolis, Ind.). Chromatography was done by using fast protein liquid chromatography system (Pharmacia LKB, Uppsala, Sweden) or a conventional open low-pressure system. Spectroscopic assays were conducted with either a UV160 UV-visible light spectrophotometer (Shimadzu, Inc., Columbia, Md.) or a microplate reader (Molecular Devices, Menlo Park, Calif.).

DNA extraction and hybridization analysis. Total cellular DNA was extracted from *M. thermophila* cells by the procedure described by Timberlake and Barnard (64). Genomic DNA samples were analyzed by Southern hybridization (25) under conditions of mild stringency (i.e., 5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA; pH 7.7], 35% formamide, 0.3% sodium dodecyl sulfate [SDS]). The laccase-specific probe fragment (approximately 1.5 kb) comprised the 5′ portion of the *N. crassa lcc-1* gene. The purified probe fragment was radiolabeled by nick translation (42) with [α-^{32}P]dCTP (Amersham) and added to the hybridization buffer at an activity of approximately 10^6 cpm per ml. The mixture was incubated overnight at 45°C. Following incubation, the membrane filters were washed once in 0.2× SSPE with 0.1% SDS at 45°C and then twice in 0.2× SSPE (no SDS) at the same temperature. The filters dried on paper towels for 15 min and then were wrapped in Saran Wrap and exposed to X-ray film overnight at −70°C with intensifying screens.

DNA libraries and identification of laccase clones. A genomic DNA library was constructed in λ-EMBL4 (62). Briefly, DNA was partially digested with *Sau*3AI and size fractionated on low-melting-point agarose gels. DNA fragments migrating between 9 and 23 kb were excised and eluted from the gel by using β-agarase (New England Biolabs, Beverly, Mass.). The eluted DNA fragments were ligated with *Bam*HI-cleaved and dephosphorylated λ-EMBL4 vector arms, and the ligation mixtures were packaged by using commercial packaging extracts (Stratagene, La Jolla, Calif.). The packaged DNA library was plated and amplified on *E. coli* K802 cells (42). Approximately 20,000 plaques were screened by plaque hybridization (25) with the radiolabeled *N. crassa* laccase gene fragment under the conditions described above. Plaques which gave hybridization signals with the probe were purified twice on *E. coli* K802 cells, and DNA from three of these phage was purified by using a Qiagen Lambda kit (Qiagen, Inc., Chatsworth, Calif.).

Analysis of laccase genes. Restriction mapping was completed by standard methods (40). DNA sequencing was done with a model 373A automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) by using the primer walking technique with dye-terminator chemistry (30). The final nucleotide sequence was determined on both strands. Oligonucleotides were synthesized on an Applied Biosystems model 394 DNA/RNA synthesizer.

Construction of laccase expression vectors. Construction of the laccase expression vector pRaMB5 is outlined in Fig. 1. The promoter directing transcription of the laccase gene segment was obtained from the *A. oryzae* α-amylase (TAKA-amylase) gene (21). The α-amylase polyadenylation/transcription terminator region from pTAKA17 was also used in construction of this vector (21).

Cotransformation of *A. oryzae*. Methods for cotransformation of *A. oryzae* were described by Christensen et al. (21). Equal amounts (approximately 5 μg each) of laccase vector and one of the following plasmids were used: p*pyrG* (Fungal Genetics Stock Center, Kansas City, Kans.), which contains the *A. nidulans pyrG* gene (47), or pSO2, which harbors the *A. oryzae pyrG* gene. Prototrophic (Pyr^+) transformants were selected on *Aspergillus* minimal medium (52), and the transformants were screened for the ability to produce laccase on minimal medium containing 1 mM 2,2′-azinobis(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS). Cells that secreted active laccase oxidized the ABTS, producing a green halo surrounding the colony.

Analysis of laccase-producing transformants. Transformants that produced laccase activity on agar plates were purified twice through conidiospores, and spore suspensions in sterile 0.01% Tween 80 were made from each. The density of spores in each suspension was estimated spectrophotometrically by absorption at 595 nm. Approximately 0.5 absorbance units of spores was used to inoculate 25 ml of shake flask medium in 125-ml plastic flasks. The shake flask medium contained the following (per liter): 1 g of $CaCl_2 \cdot 2H_2O$, 2 g of yeast extract, 1 g of $MgSO_4$, 2 g of citric acid, 5 g of KH_2PO_4, 1 g of urea, 2 g of $(NH_4)_2SO_4$, 20 g of maltodextrin, and 0.5 ml of trace elements solution (36). The cultures were incubated at 37°C with vigorous aeration (approximately 200 rpm) for 4 to 5 days. Culture broths were harvested by centrifugation, and the amount of laccase activity in the supernatant was determined. Transformants producing the highest levels of the recombinant *M. thermophila* laccase (r-MtL) in shake flask cultures were also grown in laboratory fermentors.

Laccase assays. The syringaldazine oxidase activity of r-MtL was determined by using 19 μM syringaldazine and monitoring the absorbance change at 530 nm (extinction coefficient = 65 mM^{-1} cm^{-1} [6]). One syringaldazine oxidation unit (SOU) was defined as the amount of enzyme that oxidizes 1 μmol of substrate per min in 1 ml at 20°C. ABTS oxidation assays were done by using 1 mM ABTS and monitoring the absorbance change at 418 or 405 nm (extinction coefficient = 36 or 35 mM^{-1} cm^{-1}, respectively [18]). Britton and Robinson (B&R) buffers, made by mixing 0.1 M boric acid–0.1 M acetic acid–0.1 M phosphoric acid with 0.5 M NaOH to the desired pH, were used to determine the pH activity profile of r-MtL. Thermostability analysis of r-MtL was performed by using 0.8 to 1.2 μM samples preincubated in B&R buffer (pH 6) at various temperatures. The samples were assayed for syringaldazine oxidase activity after a 430-fold dilution at room temperature.

Purification of native MtL from *M. thermophila* culture broth. *M. thermophila* was grown for 5 days at 42°C in medium which contained the following: 1% glucose, 4% dextrin, 2% ammonium citrate, 0.1% $MgSO_4 \cdot 7H_2O$, 1% KH_2PO_4, 0.1% $CaCl_2$, 0.01% $FeSO_4 \cdot 7H_2O$, 0.05% $CuSO_4 \cdot 5H_2O$, 0.01% pluronic antifoam, and 0.5% PWH salts [containing (per liter) 0.3 g of $ZnCl_2$, 0.6 g of $FeSO_4 \cdot 7H_2O$, 0.25 g of $CuSO_4$, 0.35 g of $MnSO_4 \cdot H_2O$, 0.2 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 6 g of tetrasodium-EDTA]. The mycelia were removed by filtration, and the culture broth was washed, filtered, adjusted to pH 7, and applied to a Q-Sepharose (Hiload 26/10; Pharmacia) column that was preequilibrated with 0.1 M phosphate buffer, pH 7. The laccase concentration of the crude broth was approximately 5 mg per liter. The laccase activity eluted with a gradient of 0 to 1 M NaCl. An 11-fold purification and recovery yield of 56% were achieved.

Purification of r-MtL from *A. oryzae* culture broth. A washed, concentrated broth sample (pH 7.6; conductivity = 0.8 mS) was loaded onto a Q-Sepharose XK26 column (120 ml; Pharmacia) preequilibrated with 10 mM Tris, pH 7.5. MtL has an intense blue color (corresponding to an absorbance peak at 600 nm) that is typical of multicopper oxidases (44). One group of blue fractions was collected after the column was loaded and washed. A second group eluted with a linear gradient of 0 to 2 M NaCl. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that this preparation was essentially pure laccase. A purification of 121-fold and recovery of 67% were achieved. The purified r-MtL showed no activity loss over a 5-week-long storage frozen in Q-Sepharose elution buffer at −20°C.

Analyses of amino acid composition, carbohydrate content, N-terminal and C-terminal sequences, copper content, and native molecular mass. N-terminal sequencing was done with an Applied Biosystems model 476A protein sequencer. The sequencing reagents were from Perkin-Elmer/Applied Biosystems Division (Foster City, Calif.). A 1090L high-pressure liquid chromatography system (Hewlett-Packard Co., Wilmington, Del.) equipped with diode array detection at 215 and 280 nm and 3D Chemstation software was used for the separation of CNBr- and protease-generated enzyme fragments. Separations were done on a Vydac C_4 or C_{18} reverse-phase column (Vydac, Hesperia, Calif.). C-terminal sequencing was done by J. M. Bailey of Hewlett-Packard Co. Total amino acid analysis, from which the extinction coefficient of r-MtL was determined, was done with a Hewlett-Packard 1090 AminoQuant instrument.

Hydrolyses of protein-bound carbohydrate for monosaccharide compositional analysis were done in duplicate. Lyophilized samples were hydrolyzed in evacuated sealed glass tubes with 100 μl of 2 M trifluoroacetic acid (TFA) for 1 and 4 h at 100°C. Monosaccharides were separated by high-performance anion-exchange chromatography using a CarboPac PA1 column (Dionex Corporation, Sunnyvale, Calif.), eluted with 16 mM NaOH, and detected by pulsed amperometric detection. Due to the different stability and release of the monosaccharides in 2 M TFA, the amounts of glucosamine and mannose were determined after 4 h of hydrolysis, whereas the amount of galactose was determined after 1 h of hydrolysis. Deglycosylation was also achieved by using endo/*N*-glycosidase F (Boehringer Mannheim) according to the manufacturer's instructions, and the carbohydrate content of r-MtL was estimated from the mobility difference in SDS-PAGE. Enzymatic removal of the N-terminal pyroglutamate residue was done with pyroglutamate aminopeptidase (Boehringer Mannheim) in accordance with the manufacturer's instructions. About 80 μg of r-MtL was treated with 4 μg of peptidase with or without 1 M urea or 0.1 M guanidine HCl and then transferred to a polyvinylidene difluoride membrane for sequencing. About 20 pmol of peptidase-treated protein was obtained and sequenced.

SDS-PAGE and native isoelectric focusing (IEF) analysis were done on commercial apparatus (Novex, San Diego, Calif., and Bio-Rad Laboratories, Hercules, Calif.). Proteins were stained with Coomassie brilliant blue. Gel filtration analyses were done on a Sephacryl S-300 (Pharmacia) column, and the native molecular mass was estimated by using blue dextran (2,000 kDa), bovine immunoglobulin G (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and horse heart myoglobin (17 kDa) to calibrate the column.

The copper content was determined by the photometric titration method of Felsenfeld (27) and by atomic absorption spectroscopy.

The extinction coefficient for r-MtL was calculated on the basis of amino acid analysis, and the molecular mass was deduced from the DNA sequence.

Nucleotide sequence accession number. The nucleotide sequence of the *lcc1* coding region was determined and deposited in the GenSeq database under accession no. T10922.

RESULTS

Cloning and characterization of sequence of the laccase gene from *M. thermophila*. Genomic DNA was prepared from *N. crassa* and *M. thermophila*, digested with *Bam*HI, fractionated by agarose gel electrophoresis, blotted, and probed under conditions of mild stringency with a radiolabeled fragment

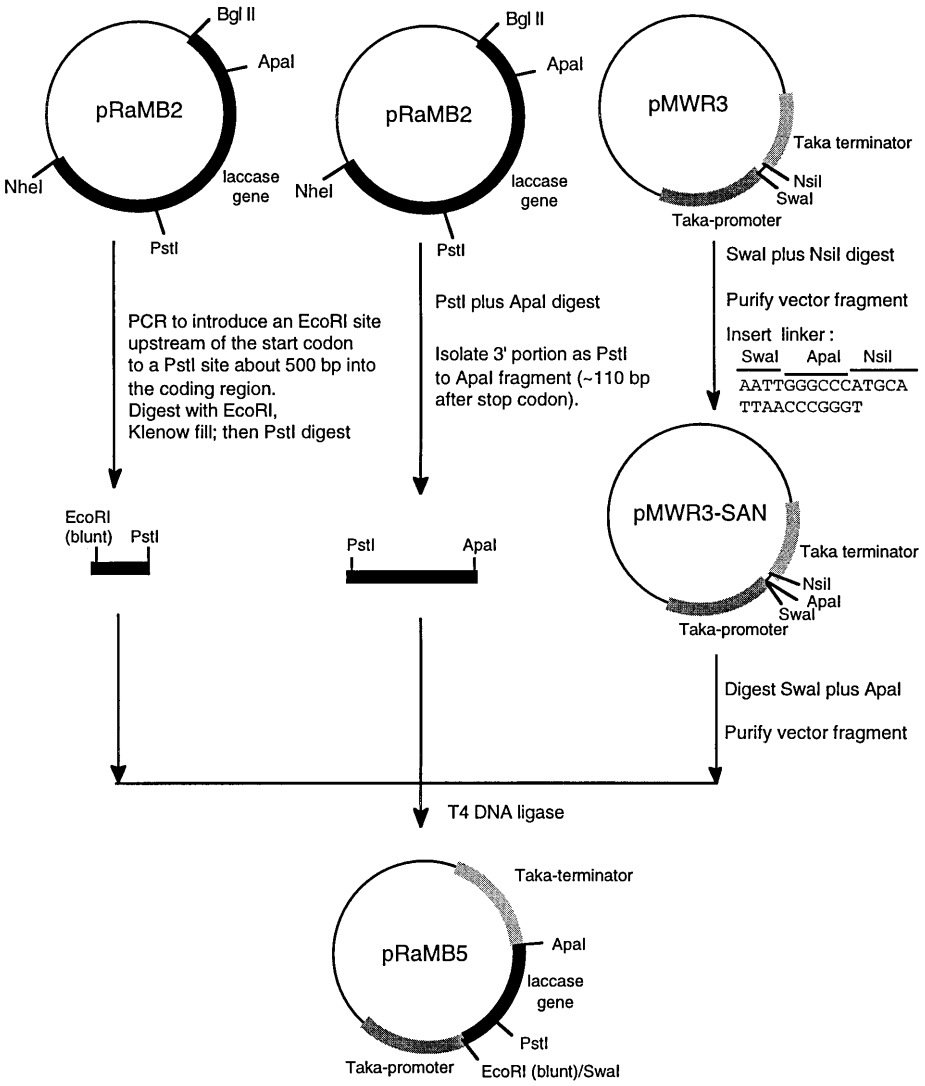

FIG. 1. Scheme for construction of the vector pRaMB5 for *Myceliophthora* laccase expression in *Aspergillus*. First, plasmid pMWR3 was modified by inserting a small linker that contained an *Apa*I site between the *Swa*I and *Nsi*I sites, creating a plasmid called pMWR3-SAN. Second, *Pfu* polymerase-directed PCR (Stratagene) was used to amplify a short DNA segment encoding the 5′ portion of MtL, from the start codon to an internal *Pst*I site (approximately 0.5 kb). The forward primer for this PCR was designed to create an *Eco*RI site just upstream of the start codon (Fig. 2). Next, the amplified fragment was digested with *Eco*RI and *Pst*I and subcloned into an M13mp18 sequencing vector, and its nucleotide sequence was verified. This fragment was subsequently excised by cleavage with *Eco*RI and *Pst*I (during this step, the *Eco*RI site was made blunt by treatment with deoxynucleoside triphosphates and DNA polymerase I [Klenow fragment]) and purified by agarose gel electrophoresis. The 3′ portion of the *lcc1* coding region was excised from pRaMB2 as a 2-kb *Pst*I-*Apa*I fragment (this segment also contains approximately 110 bp from the 3′ untranslated region). Lastly, these two fragments were combined with *Swa*I- and *Apa*I-cleaved pMWR3-SAN in a three-part ligation reaction to generate the laccase expression vector pRaMB5.

encoding a portion of the *N. crassa* laccase gene. A single laccase-specific DNA fragment was detected in both genomic digests. We then screened approximately 20,000 plaques from an *M. thermophila* genomic DNA library. Eight plaques that hybridized strongly to the probe were identified. DNA was isolated from three of these plaques, cleaved with *Eco*RI, and analyzed by agarose gel electrophoresis and Southern hybridization. All three clones contained a 7.5-kb *Eco*RI fragment which hybridized to the laccase-specific probe. One fragment was subcloned into pBR322 (12) to generate plasmid pRaMB1. The entire *M. thermophila* laccase gene (*lcc1*) coding region was contained within a 3.2-kb *Nhe*I-*Bgl*II segment that was subcloned into pUC119 (66) to give plasmid pRaMB2. The nucleotide sequence of this segment was determined on both strands by the primer walking method (30).

The positions of six introns (85, 84, 102, 72, 147, and 95 nucleotides in length) within the *lcc1* coding region were determined by comparing the deduced amino acid sequence of MtL to that of *N. crassa* laccase and by applying the consensus rules for intron features in filamentous fungi (31). Additionally, the amino acid sequences of several internal peptide fragments from recombinant MtL were determined, and the correct reading frame for the *lcc1* gene as well as the positions of the second, third, and sixth introns was verified. The 1,860 nucleotides of coding sequence are 65.5% G+C, with a strong bias (90%) for codons ending in G or C.

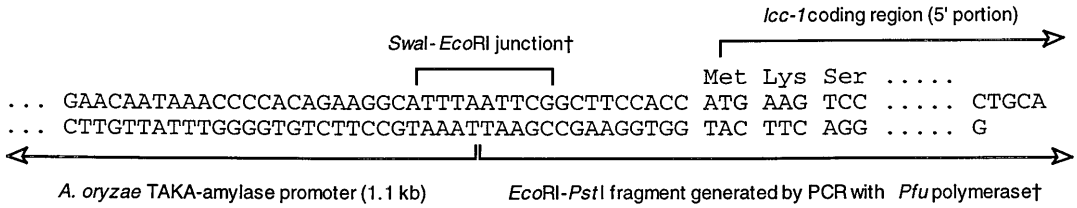

FIG. 2. Scheme for joining an *A. oryzae* α-amylase promoter to the MtL coding region in the expression vector pRaMB5. †, *Eco*RI site that was made blunt by treatment with DNA polymerase I (Klenow fragment) and deoxynucleoside triphosphates.

The deduced amino acid sequence of MtL shares identity with laccases of the following species: *Podospora anserina* (65%), *N. crassa* (60%), *C. parasitica* (53%), *Agaricus bisporus* (24%), *P. radiata* (22%), *Pleurotus ostreatus* (22%), *R. solani* (20%), *T. versicolor* (22%), *T. villosa* (22%), and *A. nidulans* (15%). Similarity is highest in the regions that correspond to the four histidines and one cysteine that form the trinuclear copper cluster (23, 44, 49). There are 11 potential sites (Asn-X-Ser/Thr) for N-linked glycosylation in the deduced amino acid sequence of MtL.

The first 22 amino acids in the deduced primary structure of MtL appear to comprise a canonical signal peptide with a predicted cleavage following an Ala residue (67). The purified extracellular forms of both native MtL and r-MtL are blocked with N-terminal pyroglutamate residues. Enzymatic removal of these residues followed by amino acid sequencing suggests that mature MtL begins with a Gln residue. Thus, MtL is apparently synthesized as a 619-residue preproenzyme having a 22-residue signal peptide and a propeptide of 25 residues.

Expression of *Myceliophthora* laccase. The expression vector pRaMB5 (Fig. 1 and 2) was used to generate *A. oryzae* cotransformants which produce r-MtL that were detected by incorporation of ABTS into selective media. As determined by using the *pyrG* gene from *A. nidulans* or *A. oryzae* as the selectable marker, the frequencies of laccase-producing cotransformants among Pyr^+ colonies were 59% with *A. nidulans pyrG* as the selected marker and 31% with *A. oryzae pyrG* as the marker. Several cotransformants that produced intense color reactions on ABTS plates were grown in shake flask cultures to quantitate the amount of r-MtL produced. The amount of extracellular laccase activity produced ranged from 0.49 to 0.85 SOU/ml (Table 1). On the basis of the specific activity of 45 SOU/mg (see below), the level of r-MtL secreted in these shake flask cultures ranged from 11 to 19 mg per liter. Preliminary SDS-PAGE analyses of culture broth samples showed a prominent laccase band at approximately 85 kDa, which is similar to the size of the native enzyme purified from *M. thermophila*.

TABLE 1. MtL expression among selected *A. oryzae* transformants[a]

Transformant	Transforming DNAs[b]	SOU/ml in shake flask
Control	None	0.00
RaMB5.15	pRaMB5 + p*pyrG*	0.85
RaMB5.30	pRaMB5 + p*pyrG*	0.71
RaMB5.33	pRaMB5 + p*pyrG*	0.60
RaMB5.108	pRaMB5 + pSO2	0.68
RaMB5.111	pRaMB5 + pSO2	0.70
RaMB5.121	pRaMB5 + pSO2	0.49
RaMB5.142	pRaMB5 + pSO2	0.54

[a] *A. oryzae* HowB104 *pyrG* was the host strain.
[b] Plasmids p*pyrG* and pSO2 contain the *pyrG* genes of *A. nidulans* and *A. oryzae*, respectively.

Biochemical characterization of r-MtL produced in *A. oryzae*. Q-Sepharose chromatography yielded two active fractions that contained essentially pure laccase (one passed through the column, and another was eluted by a 0 to 2 M NaCl gradient). Purified r-MtL has a molecular mass of 100 to 140 kDa as determined by S-300 gel filtration (data not shown) and a molecular mass of 75 to 95 kDa as determined by SDS-PAGE (Fig. 3). Under nondenaturing conditions, both r-MtL fractions had a pI of 4.2. Treatment of the purified enzyme with *N*-glycosidase resulted in a decrease in the apparent molecular mass to approximately 73 kDa (Fig. 3). The increased mobility on SDS-PAGE after deglycosylation suggested that N-linked carbohydrates accounted for approximately 14% of the total mass of each subunit. Total-carbohydrate analysis showed that the laccase fractions that passed through the Q-Sepharose column (preequilibrated with 10 mM Tris, pH 7.5) contained 26 mol of glucosamine, 67 mol of galactose, 9 mol of glucose, and 138 mol of mannose per mol of enzyme. The laccase fractions that eluted from the Q-Sepharose with NaCl had 23 mol of glucosamine, 38 mol of galactose, 4 mol of glucose and 85 mol of mannose per mol of enzyme.

Attempts to directly sequence the N terminus of r-MtL from samples either in desalted solution or on polyvinylidene difluoride membranes were unsuccessful. Treatment of r-MtL with pyroglutamate aminopeptidase yielded a protein with a deblocked N terminus, beginning 48 residues after the putative translation start (Met). Sequencing of internal peptides generated by CNBr cleavage confirmed the DNA sequence and several intron and exon assignments. Direct C-terminal sequencing indicated that r-MtL had a C terminus of -Gly-Leu.

The UV-visible absorbance spectrum of r-MtL shows absorption maxima at 276 and 589 nm. The ratio of the absorbance at 280 nm to the absorbance at 600 nm was 35. This is higher than reported for *T. villosa* (75) and *R. solani* (69) laccases, suggesting that MtL contains more tryptophan, phe-

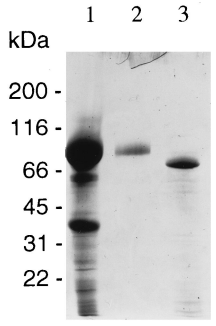

FIG. 3. SDS-PAGE profile for the purification of r-MtL. Lane 1, concentrated *A. oryzae* culture broth to be loaded onto Q-Sepharose; lane 2, purified r-MtL; lane 3, r-MtL treated with endo-/*N*-glycosidase F. The gel was stained with Coomassie brilliant blue.

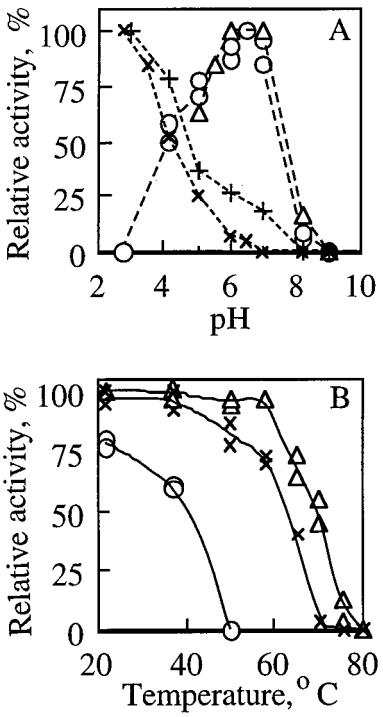

FIG. 4. Dependence of r-MtL activity on pH and temperature. (A) Laccase activity as a function of pH (normalized to the optimum activity value) of native (nonrecombinant) MtL with ABTS as a substrate (+), r-MtL with ABTS as a substrate (×), native MtL with syringaldazine as a substrate (△), and r-MtL with syringaldazine as a substrate (○). (B) Thermostability of r-MtL. Enzyme samples (0.8 to 1.2 μM) were preincubated in B&R buffer (pH 6) for 0.3 (△), 1.0 (×), and 17 (○) h at various temperatures, diluted 430-fold, and assayed for residual activity in B&R buffer (pH 6) at 20°C with syringaldazine as the substrate. The activities were normalized to the initial activity at 20°C before the preincubation.

nylalanine, and cysteine. This suggestion was confirmed by comparing the amino acid compositions deduced from the corresponding gene sequences. Photometric titration and atomic absorption spectroscopy indicated a stoichiometry of 3.9 copper atoms per enzyme subunit.

r-MtL had an optimal pH of 6.5 with syringaldazine as the substrate (Fig. 4A). At the optimum pH, the specific activity of r-MtL with syringaldazine used as a substrate was 45 SOU per mg. With ABTS as a substrate, r-MtL showed maximum activity at the lowest pH studied (pH 2.7). The difference in optimum pH values for ABTS and syringaldazine substrates is consistent with the hypothesis that electron transfer kinetics are more important than substrate binding in determining the pH activity profile of laccase (71). Thermostability analysis shown in Fig. 4B indicated that the upper temperature limit for retaining full activity after a 20-min preincubation was 60°C.

Purification and characterization of native MtL from *M. thermophila*. Q-Sepharose chromatography yielded an 11-fold purification of laccase from *M. thermophila*. Purified MtL migrated as a diffuse band in SDS-PAGE with a molecular mass of 80 kDa, and on IEF gels it had an isoelectric point of 4.2. MtL showed a UV-visible spectrum with an absorbance maximum of 280 and two smaller shoulders at 330 and 600 nm. The weak absorbance maximum at 600 nm indicated the presence of apo-MtL (copper depleted) in the preparation, possibly resulting from instability during purification. The pH activity profile of native MtL on syringaldazine is also shown in Fig. 4A. At optimum pH, MtL had an activity of 15 SOU per mg. N-terminal sequencing analysis indicated that the amino terminus was also blocked.

DISCUSSION

We cloned a genomic DNA segment encoding an extracellular laccase from the thermophilic fungus *M. thermophila*. On the basis of a comparison of its deduced amino acid sequence, MtL shows identity with laccases from diverse fungal genera. However, the greatest degree of sequence identity (53 to 65%) is between MtL and the laccases of related species in the order Sphaeriales, such as *P. anserina* (28), *N. crassa* (29), and *C. parasitica* (19). Laccases from basidiomycetes show more-limited sequence identity (20 to 24%) to MtL. Interestingly, the conidial laccase (*yA* gene product) of *A. nidulans* (4) shows the lowest degree of similarity, suggesting a possible evolutionary or functional difference between conidial and secreted laccases.

At the genomic level, the *lcc1* gene of *M. thermophila* also has the highest homology with laccase genes from *P. anserina*, *N. crassa*, and *C. parasitica*; however, their architecture is very different. For example, *N. crassa lcc1* contains a single intron, *lac2* from *P. anserina* has three introns, *lcc1* from *M. thermophila* laccase has six intervening sequences, and the *C. parasitica* laccase gene has 12 introns. The position of the first intron is conserved among laccase genes from *M. thermophila*, *N. crassa*, and *P. anserina*. Additionally, introns II and III in *P. anserina lac2* align with the third and fourth introns of *M. thermophila lcc1*. The positions of five intervening sequences in *M. thermophila lcc1* are conserved in the *C. parasitica* laccase gene. Therefore, we postulate that the laccase genes from these four species were derived from a common ancestral form but diverged during evolution. The comparatively low level of sequence similarity between the laccase genes of basidiomycetes and ascomycetes probably reflects the large phylogenetic distance between these fungal classes.

The primary structures of the laccase gene products from *Neurospora*, *Podospora*, and *Myceliophthora* predict similar mechanisms of posttranslational processing. On the basis of the rules of von Heijne (67), the predicted signal peptide cleavage site for MtL lies after the first 22 amino acids. However, direct sequencing of the amino terminus of native and recombinant forms of MtL suggested that the first residue of the mature enzyme is Gln_{49}. Therefore, residues 23 through 48 probably comprise a propeptide whose proteolytic removal occurs during maturation of MtL, leaving Gln_{49} as the first amino acid residue of the mature enzyme, which may subsequently cyclize to pyroglutamate, yielding a blocked N terminus. It was reported that *N. crassa* and *P. anserina* laccases are processed similarly at their amino-terminal ends (28, 29). In addition, *N. crassa* laccase is also reportedly processed at its C terminus, resulting in the proteolytic removal of 13 residues (29). The processing site is contained within the sequence Asp-Ser-Gly-Leu ↓ Arg_{558} (where ↓ designates the cleavage site). Strikingly similar sequences exist near the C termini of MtL (Asp-Ser-Gly-Leu-Lys_{560}) and *P. anserina* laccase (Asp-Ser-Gly-Leu-Lys_{559}). C-terminal sequencing showed that the C terminus of MtL was Gly-Leu, indicating that the enzyme was processed (Asp-Ser-Gly-Leu ↓ Lys_{560}) similarly to *N. crassa* laccase and 13 residues were removed. C-terminal processing of *P. anserina* laccase was also postulated (28). It is particularly interesting that the *C. parasitica* laccase has Asp-Ser-Gly-Val as its C terminus, and thus no processing may be needed. The importance of C-terminal processing for the catalytic activity of these enzymes is unknown, and the protease involved in this cleavage has not been identified.

By ion-exchange chromatography, r-MtL from *A. oryzae* fermentor broth was separated into multiple isoforms with different elution properties. However, no significant difference among these isoforms was seen in terms of SDS-PAGE, native PAGE, native IEF, S-300 gel filtration, UV-visible spectrum, specific activity towards syringaldazine, and unblocked-N-terminus sequencing measurements. On the basis of total-carbohydrate analyses, it appears likely that the different elution patterns of various r-MtL isoforms from Q-Sepharose arose from differential glycosylation with galactose and mannose. Total-carbohydrate analyses also gave an estimate of 33 to 60% total glycosylation, of which 14% is estimated to be N linked, on the basis of the mobility change on SDS-PAGE after *N*-glycosidase treatment. The r-MtL differed from native MtL in two other respects. First, the molecular mass of native MtL (80 kDa) was less than that of r-MtL (85 kDa), presumably reflecting differences in glycosylation. Second, the specific activity of native MtL (15 SOU/mg) was lower than that of r-MtL (45 SOU/mg). Since native MtL also had a lower absorbance at 600 nm, the decreased specific activity is probably due to a percentage of holoenzyme lower than that of r-MtL. Since the type II copper is easily depleted (73), extra copper ions were added to the culture medium of *A. oryzae* transformants expressing r-MtL. This appears to have yielded a purified r-MtL preparation with a specific activity higher than that of native MtL which was isolated from cultures not supplemented with additional copper.

The ascomycete fungus *M. thermophila* produces a constellation of thermostable cellulases (48, 53, 58–60) and at least one thermotolerant xylanase (76). Whether this organism might be a good source of thermostable laccase was a subject of this investigation. The observation that r-MtL retains virtually 100% activity after 20-min incubation at 60°C seems to validate our approach. In addition, Xu et al. (71) disclose that MtL not only is more thermostable than laccases from the basidiomycetes *T. villosa* and *R. solani*, but also demonstrates a pronounced thermal activation such that preincubation at elevated temperatures gives higher activity.

The yield of r-MtL from *A. oryzae* cotransformants grown in shake flasks was modest (11 to 19 mg per liter). However, these yields are consistent with those obtained by heterologous expression of basidiomycete laccases in other hosts (35, 55). In addition, it seems likely that the heterologous expression of laccases in *A. oryzae* will benefit from the successful history of industrial scale-up, strain development, and process methods for other *Aspergillus* enzyme products.

ACKNOWLEDGMENTS

We thank Ejner B. Jensen (Novo Nordisk A/S), Alan V. Klotz, Glenn E. Nedwin, Jeffrey Shuster, Jill A. Wahleithner, and Debbie S. Yaver (Novo Nordisk Biotech, Inc.) for critical reading of the manuscript and for helpful suggestions throughout the course of this work. We are grateful to W. Shin and E. I. Solomon of Stanford University for copper atomic absorption spectroscopy of MtL, J. M. Bailey of Hewlett-Packard for C-terminal sequencing, and S. Osborn of Zymogenetics, Inc., for help with carbohydrate analysis. We also thank Howard Brody for the *A. oryzae* host strain, Debbie S. Yaver for the *N. crassa lcc-1* gene fragment, Michael Rey for pMWR3, and Suzie Otani for pSO2.

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