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Molecular cloning, characterization and regulation by cadmium of a superoxide dismutase from the ectomycorrhizal fungus *Paxillus involutus*

Christophe Jacob¹, Mikaël Courbot¹, Annick Brun¹, Howard M. Steinman², Jean-Pierre Jacquot¹, Bernard Botton¹ and Michel Chalot¹

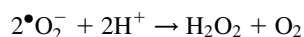
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The gene encoding a superoxide dismutase (PiSOD) was cloned by suppressive subtractive hybridization from cDNA library of the ectomycorrhizal fungus, *Paxillus involutus*, grown under cadmium-stress conditions. The encoded protein was presumed to be localized in the peroxisomes because it contained a C-terminal peroxisomal localization peptide (SKL) and lacked an N-terminal mitochondrial transit peptide. Complementation of an *Escherichia coli* SOD null strain that is unable to grow in the presence of paraquat or cadmium indicated that cloned *Pisod* encoded a functional superoxide dismutase. Sensitivity of PiSOD activity to H₂O₂ but not KCN, and sequence homologies

to other SODs strongly suggest that it is a manganese-containing superoxide dismutase. Monitoring PiSOD transcript, immunoreactive polypeptide and superoxide dismutase activity following cadmium stress suggests that the principal level of control is post-translational. This is, to our knowledge, the first insight in the characterization of molecular events that take place in an ectomycorrhizal fungus during exposure to heavy metals.

Keywords: cadmium; complementation; expression analysis; *Paxillus involutus*; superoxide dismutase.

A fraction of the molecular oxygen generated in respiring cells is subjected to sequential univalent reduction to superoxide radicals ($\bullet\text{O}_2^-$), hydrogen peroxide (H₂O₂), and hydroxyl radicals ($\bullet\text{OH}$). Unless effectively removed, these highly reactive oxygen species (ROS) cause damage to almost all known biological molecules, including DNA, membrane lipids, and other vital cellular components. Superoxide dismutase (SOD; superoxide: superoxide oxidoreductase, EC 1.15.1.1) functions as a defense mechanism against high concentrations of ROS by catalysing the conversion of superoxide radicals ($\bullet\text{O}_2^-$) to molecular oxygen and hydrogen peroxide (H₂O₂) [1]:



SODs have been categorized into two major families on the basis of metal cofactors at the active site and their evolutionary homology: copper and zinc-containing SOD (Cu/ZnSOD), and manganese or iron-containing SOD (Mn or FeSOD) [2]. Recently, a novel type of SOD containing nickel as a cofactor has been found in several *Streptomyces*

spp. [3–5]. Cu/ZnSOD is found almost exclusively in eukaryotic species, where it is often present as several isoforms. One of these is always present in the cytosol, and a chloroplastic isoform is frequently found in plant cells [6]. Some bacterial species contain a periplasmic Cu/ZnSOD [7]. FeSOD is present in prokaryotes and in some plants. MnSOD is widely distributed among prokaryotic and eukaryotic organisms; in eukaryotes it is found in mitochondria and peroxisomes [8–10].

Although ROS species are produced as by-products of normal cell metabolism [11], their levels are enhanced by stresses induced by exposure to chemical or adverse environmental conditions [1,12]. Tolerance to these environmental stresses correlates with increasing capacities of detoxifying enzymes, including SODs [13–17]. In some eukaryotic cells containing both Cu/ZnSOD and MnSOD activities, MnSOD is induced in response to oxygen toxicity threats [18]. SOD overproduction was found to be associated with enhanced tolerance to oxidative stress [19].

The heavy metal cadmium is a nonessential element for metabolic processes, which can be toxic at very low concentrations. It has been demonstrated that cadmium accumulation is involved in the generation of activated oxygen species in a sensitive clone of *Holcus lanatus* [20], in the induction of oxidative stress in *Saccharomyces cerevisiae* [21] and in germinating seedlings of mung bean [22]. Moreover, one possible mechanism, via which elevated concentrations of heavy metals may damage plant tissues, is the stimulation of free radical production and lipid peroxidation by induced oxidative stress [23–25].

P. involutus is an abundant species in many forest ecosystems and has been one of the most widely found

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Abbreviations: EST, expressed sequence tag; IPTG, isopropyl thio- β -D-galactopyranoside; ROS, reactive oxygen species; SOD, superoxide dismutase.

Enzyme: superoxide dismutase (EC 1.15.1.1).

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species on industrial wastes polluted by heavy metals [26]. The response of mycorrhizal fungi to toxic metals is of importance in view of their interest in the reclamation of polluted sites and their importance in tree growth [27]. We have recently demonstrated that *P. involutus* could tightly bind cadmium onto its cell walls or accumulate it in the vacuolar compartment, these two mechanisms being considered as essential in the metal-detoxification processes [28]. However cadmium was also found in the cytoplasm [28], where it could induce an oxidative stress, as already demonstrated for *S. cerevisiae* [21].

We set out to search for genes that might be involved in metal tolerance in *P. involutus*. The acquisition of several expressed sequence tags (ESTs) in the laboratory facilitates the screening of a cDNA library to isolate a full-length cDNA clone from *P. involutus* encoding a SOD. This *P. involutus* SOD cDNA can efficiently complement the SOD deficiency of *E. coli* OX326A and rescue the transformed *sod* mutant from oxidative stress generated by paraquat or cadmium. This report also investigated the relationship between cadmium stress and regulation of this SOD by analysing transcript level, polypeptide content and enzyme activation.

EXPERIMENTAL PROCEDURES

Organisms and culture media

The ectomycorrhizal fungus used was *P. involutus* (Batsch) Fr. (ATCC 200175) strain which was originally isolated from a fruitbody associated with 15- to 30-year-old *Betula pendula* trees growing on coal waste in Midlothian, Scotland. It was grown on cellophane covered agar medium containing modified Melin–Norkrans medium [29]. The medium contained (mg·L⁻¹): KH₂PO₄ (500), (NH₄)₂HPO₄ (250), CaCl₂ (50), NaCl (25), MgSO₄·7H₂O (150), thiamine hydrochloride (0.1), and FeCl₃·6H₂O (1). A glucose concentration of 10 g·L⁻¹ was used.

The *E. coli* strains BL21 (DE3) (Novagen) containing the pSBET plasmid [30], Δ *sodA* Δ *sodB* OX326A [31] and Y1090r⁻ (Clontech) were maintained on Luria–Bertani (10 g·L⁻¹ bactotryptone, 5 g·L⁻¹ yeast extract and 10 g·L⁻¹ NaCl) agar medium supplemented with either 50 µg·mL⁻¹ kanamycin (BL21 and OX326A strains) or 50 µg·mL⁻¹ ampicillin (Y1090r⁻ strain).

RNA isolation

Fungal colonies were fixed in liquid nitrogen. Total RNA isolation was performed with the RNeasy Plant Mini kit (Qiagen) from approximately 100 mg of frozen mycelium. According to the manufacturer's recommendations, a buffer containing guanidium hydrochloride was used instead of a buffer containing guanidium isothiocyanate to avoid solidification of samples due to secondary metabolites in mycelia of filamentous fungi. An average of 800 ng total RNA per mg frozen material was isolated and stored in diethyl pyrocarbonate-treated water at -70 °C until further use.

cDNA library construction and screening

A cDNA library was prepared using 2 µg of total RNA isolated from a 12-h cadmium-stressed mycelium using the

SMART PCR cDNA library construction kit (Clontech). cDNAs were cloned into λ gt11 and phage DNA was packaged using the Gigapack Gold III Plus (Stratagene) and plated using *E. coli* strain Y1090r⁻ (Clontech). A total of 1.5 × 10⁵ p.f.u. was screened using standard techniques [32]. Membranes were then prehybridized for 2 h at 42 °C in buffer A consisting of 50% (v/v) formamide, 5 × NaCl/Cit (0.75 M NaCl, 0.075 M sodium citrate), 5 × Denhardt's solution (0.1% Ficoll/0.1% poly(vinylpyrrolidone)/0.1% bovine serum albumin), 0.1% SDS, 20 mM Tris/HCl pH 7.6, and 400 µg·mL⁻¹ of denatured sonicated salmon sperm DNA. The probe was made of a partial length cDNA (accession no. AW064502) isolated using a suppressive subtractive hybridization procedure [33] and a cadmium-treated mycelium as starting material (C. Jacob, unpublished results). The probe was generated by PCR amplification of the clone with the primer pair T3/T7 and labelled by the random priming procedure [34,35] using the NonaPrimerTM kit (Appligene-Oncor) with [α -³²P]dCTP (110 Tbq·mmol⁻¹) as the labelled nucleotide. Prehybridized membranes were hybridized with the denatured labelled probe in the buffer described above for 16 h at 42 °C. Hybridized membranes were subjected to three washes in 2 × NaCl/Cit/0.5% SDS at room temperature for 30 min, followed by one wash in 1 × NaCl/Cit/0.1% SDS at 65 °C for 45 min. The membranes were then dried and exposed to X-ray films.

DNA and protein sequencing and sequence analysis

The cDNA inserts from isolated plaques were amplified by PCR using the reverse (RP) and forward (FP) primers designed from the λ gt11 vector (Table 1). In addition to standard components, each 25 µL reaction contained 1 µL of template (heat-denatured phage suspension), and 0.2 µM of each primer. Thermal cycling conditions were: 94 °C for 1 min; 30 cycles at (94 °C for 30 s; 72 °C for 2 min); and a final extension at 72 °C for 3 min. The reaction mixture was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.). Sequencing reactions were performed with the RP and FP primers and the PrismTM Ready Reaction AmpliTaq (R) FS kit (PerkinElmer). Data collection was done with an ABI 310 Genetic Analyser. The full length cDNA was named *Pisod*. N-terminal sequencing of the electroblotted protein was performed on a Beckman Instrument LF 3000 sequencer. Phenylthiohydantoin-amino-acids were identified by chromatography on a micro-PTH ODS Spherogel column (2 × 150 mm) using the system Gold analytical microbore HPLC (Beckman Instruments). Homology searches in databases were performed by using the BLAST program [36] of the National Center for Biotechnology

Table 1. Names and sequences of oligonucleotides used in this work.

Oligonucleotide	Sequence
FP	5'-GGTACCTGCAGGACACCAGACCAACTGG-3'
RP	5'-GGTACCTCGAGGGTGGCGACGACTCCTGG-3'
5'- <i>sod</i>	5'-CCCCCATGGCTGGCCAACACACTC-3'
3'- <i>sod</i>	5'-CCCCGGATCCTTACAGCTTGGAGCCAGA-3'

Information and BEAUTY postprocessing program provided by the Human Genome Sequencing Center [37,38].

Northern blot analysis

Total RNAs (7.5 µg) were separated on 1.5% agarose/formaldehyde gels, blotted to positive nylon membranes (Applicgene-Oncor) by capillary elution according to standard procedures [32] and fixed by UV irradiation for 2.5 min. Membranes were then prehybridized for 2 h at 37 °C in buffer A, the composition of which is described above. After the prehybridization step, the radioactive probe was denatured and added to the prehybridization buffer. After 20 h, membranes were subjected to three washes in 2 × NaCl/Cit/0.5% SDS at room temperature for 30 min, followed by one wash in 1 × NaCl/Cit/0.1% SDS at 55 °C for 20 min. After drying, membranes were exposed to X-ray films.

Cloning and functional complementation in *E. coli* OX326A *sod* deficient cells

PCR was performed on cDNA clone to amplify only the coding region. In addition to standard components, each 25 µL reaction contained 1 µL of template (heat-denatured phage suspension for *Pisod*), and 0.2 µM of each primer (5'-*sod* and 3'-*sod*). Thermal cycling conditions were: 92 °C for 2 min; 35 cycles at (92 °C for 1 min; 68 °C for 1 min; 72 °C for 1 min); and a final extension at 72 °C for 3 min. The reaction mixture was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.), double-digested with *Bam*HI and *Nco*I, and ligated to *Bam*HI-*Nco*I digested pTrc99A (Amersham Pharmacia Biotech Inc.) in the presence of 1 U of T4 DNA Ligase (Boehringer Mannheim Corp.). Positive clones were named pTrcsod. *E. coli* OX326A cells were transformed with pTrc99A or pTrcsod and grown in Luria-Bertani medium with antibiotics supplemented with 100 µM MnCl₂. When an A₆₀₀ of 0.5 was reached, 1 mM IPTG was added to induce cultures and growth was continued for 6 h. To test the sensitivity of the transformants to paraquat (methyl viologen, Sigma), equal numbers of cells were streaked onto Luria-Bertani plates supplemented with antibiotics, 1 mM IPTG, 50 or 0 µM paraquat and incubated at 37 °C overnight. These cells were also used in an experiment in which paraquat was replaced by cadmium at a concentration of 75 µM. Uninduced controls (0 mM IPTG) were performed separately.

Cloning and expression of recombinant PiSOD in *E. coli* BL21(DE3)

Cloning was performed as described above except that the double-digested fragment was inserted in pET3d (Novagen). Positive clones were named pETSod. *E. coli* BL21 (DE3) cells containing the pSBET plasmid [30] were transformed with pET3d or pETSod and grown in Luria-Bertani medium with antibiotics supplemented with 100 µM MnCl₂. When an A₆₀₀ of 0.5 was reached, 0.4 mM IPTG was added to induce cultures and growth was continued for 5 h. The total cell and the insoluble cytoplasmic proteins were extracted according to the manufacturer's instructions (Novagen). The protein content

of the total cell extract was determined by a modification of the Bradford method [39]. The protein content of the insoluble fraction was determined by using the BCA-200 Protein Assay Kit (Pierce Chemical) using BSA as a standard.

Denaturing and nondenaturing PAGE

For native PAGE, proteins were extracted from complemented *E. coli* OX326A by centrifuging 100-mL samples at 5000 g for 15 min and sonicating the pellet resuspended in 10 mL buffer containing 50 mM potassium phosphate and 1 mM EDTA, pH 7.8. For SOD enzymatic activity staining, protein samples were separated on 8% native PAGE stained for superoxide dismutase using nitroblue tetrazolium [7,39], after electrophoresis performed in a Mini-Protean II unit at 25 mA for about 1 h. SDS/PAGE (12.5%) [41] was performed in a Mini-Protean II unit at 18 mA for about 2 h, and gels were stained with Coomassie Brilliant Blue R250 according to standard procedures [32]. Molecular mass estimations of the subunits were carried out by SDS/PAGE using standards of known molecular mass (phosphorylase *b*, 94 kDa; BSA 67 kDa; ovalbumin 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa).

Size estimation by gel filtration

The molecular mass of PiSOD was estimated by loading 200 µL of sample (proteins from complemented *E. coli* OX326A cells) onto a Superose 12 (HR 10/30, 10 × 300 mm) gel permeation FPLC column (LKB Pharmacia) and eluted with 50 mM phosphate buffer, pH 8.2, and 150 mM KCl at a flow rate of 0.2 mL·min⁻¹ in 0.5-mL fractions. The column was previously calibrated with calibration molecular mass markers (Bio-Rad Laboratories: vitamin B-12, 1.35 kDa; myoglobin, 17 kDa; ovalbumin, 44 kDa; IgG 150 kDa; thyroglobin, 670 kDa). Twenty microliters of each fraction were used for SOD activity assay based on the method of Beauchamp and Fridovich [40] as described by Donahue *et al.* [42], which measures inhibition of the photochemical reduction of nitroblue tetrazolium at 560 nm. The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 0.1 mM EDTA, 13 mM methionine, 75 mM nitroblue tetrazolium and 16.7 mM riboflavin.

Partial purification of SOD from *P. involutus* mycelium

All purification steps were carried out at 4 °C. Approximately 100 mg of mycelium was ground in a chilled mortar and pestle with 10 vol. of 0.1 M Tris/HCl, pH 7.8, containing 5 mM MgSO₄, 10% (v/v) glycerol, 2% (w/v) poly(vinyl pyrrolidone)-40, 50% (w/w) poly(vinyl poly-pyrrolidone), 2 mM EDTA and 0.8% Triton-X100. The homogenate was centrifuged at 16 000 g for 20 min and 500 µL of the resulting supernatant were adjusted to 1 M NaCl, and 1.3 mL of a DEAE-cellulose (Merck Eurolab) suspension [consisting of 6% (w/v) DEAE-cellulose prepared in 0.1 M Tris/HCl, 1 M NaCl, pH 7.8] were added. After gently mixing, the suspension was centrifuged at 16 000 g for 20 min. The supernatant was collected and concentrated using a Microsep column 10 kDa (Pall Filtron

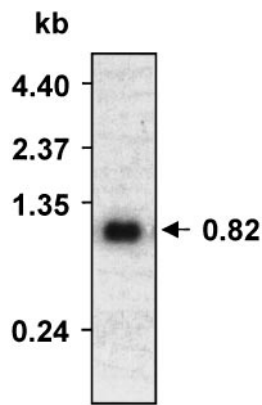


Fig. 2. Northern blot analysis of the *P. involutus sod* mRNA. Mycelia were grown in liquid Melin–Norkrans medium supplemented with 5 p.p.m. cadmium for 12 h. Total RNAs (7.5 μ g) from frozen *P. involutus* mycelium were separated on 1.5% agarose-formaldehyde gel and hybridized to the 32 P-labeled *Pisod* cDNA coding region. RNA size marker are shown to the left.

upstream of polyA tail resembling the eukaryotic consensus sequence for polyadenylation (AATAAA). This polyadenylation signal is found only in some homobasidiomycetes 3' UTRs [47]. Moreover, the size of the 3' UTR is relatively small compared to other eukaryotic cDNAs [48].

Further analysis is consistent with the proposal that the gene encodes a putative MnSOD. The 206-amino-acid sequence deduced from the cDNA sequence (with a predicted molecular mass of 22.7 kDa) was analysed using the Prosite database, which identified it as a MnSOD with the consensus pattern DXWEH[STA][FY]Y (residue Asp163 to Tyr170, Fig. 1) [49], although this pattern cannot discriminate between MnSOD and FeSOD sequences. The protein sequence was aligned with other MnSOD and FeSOD sequences which allowed identification of the putative metal-binding residues as His29, His74, Asp163 and His167. Furthermore, the protein sequence conformed to the signature of Parker and Blake [49] that defines a SOD as a MnSOD with the presence of the following amino acids: Gly69, Gly70, Phe77, Gln150 and Asp151 (Fig. 1). Most of the eukaryotic MnSODs include a putative

mitochondrial transit peptide. However, *Pisod* lacked this signal peptide as do bacterial [50], fungal [51,52], and chlorophycean [53] MnSODs. The presence of a C-terminal peroxisomal targeting sequence (SKL) matching the consensus motif of C-terminal peroxisomal targeting signal (PTS1) indicates a putative peroxisomal localization of PiSOD [54].

Several separate Northern blot analyses gave reproducible results and revealed a single transcript for *Pisod* of approximately 820 nucleotides (Fig. 2), which is in agreement with the predicted size and further supports the proposal that the isolated cDNA is full length.

SOD-deficient bacteria are rescued by PiSOD under oxidative and cadmium stress

The region coding for the predicted mature PiSOD protein was amplified by PCR using the primer pair 5'-sod and 3'-sod (Table 1) and the cDNA clone as template. For convenience in cloning experiments, the *Nco*I restriction site was introduced at the 5' end of the cDNA by modifying the penultimate amino acid serine (codon TCT) into alanine (codon GCT). The functional role of *Pisod* in transformed bacterial cells was investigated under oxidative stress caused by paraquat (methyl viologen) which generates superoxide by redox cycling. The paraquat resistance was analysed by growing *E. coli* OX326A cells transformed either with pTrc99A (Fig. 3A,D) or with pTrcsod bearing the *Pisod* cDNA coding region (Fig. 3B, C, E and F) onto Luria–Bertani agar plates. OX326A cells were grown either in the absence (Fig. 3A,B,C) or presence (Fig. 3D,E,F) of 50 μ M paraquat. In the absence of paraquat, all bacterial cells were able to grow (Fig. 3A,B,C). Paraquat inhibited growth of bacterial cells harboring pTrc99A (not shown) or pTrcsod (Fig. 3E) plasmid, when cells were not induced by IPTG. Upon induction with IPTG, bacterial cells harboring the pTrcsod plasmid were able to grow in the presence of paraquat (Fig. 3F) which was not the case for pTrc99A alone (Fig. 3D). The same experiment was performed using 75 μ M cadmium instead of paraquat. It was found that cadmium also inhibited the growth of bacterial cells harboring the pTrc99A or pTrcsod under noninduced conditions. However, the growth of bacterial cells harboring

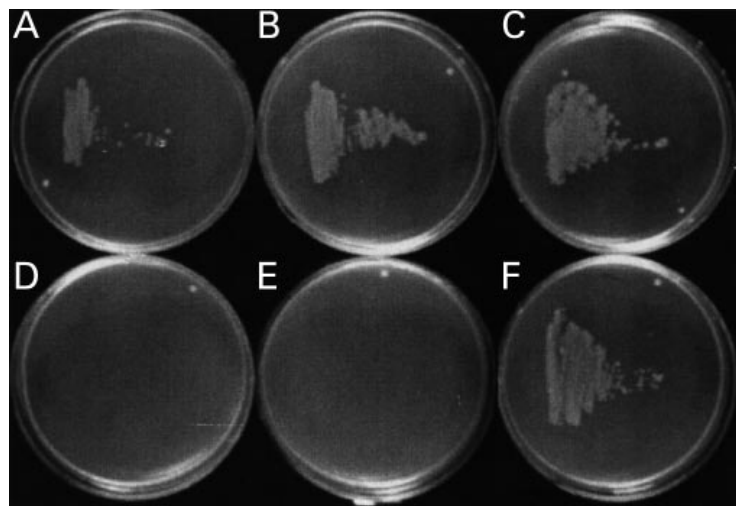


Fig. 3. Complementation of the *sod* mutant OX326A from *E. coli* by the *Pisod* cDNA coding region. Cultures were streaked onto Luria–Bertani medium containing the appropriate antibiotics with (D, E, F) or without (A, B, C) 50 μ M paraquat. (A,D) OX326A cells transformed with pTrc99A plasmid and cultivated with IPTG; b, c, e, f: OX326A cells transformed with pTrcsod plasmid and cultivated with IPTG (c, f) and without IPTG (b, e). Plates were incubated overnight at 37 $^{\circ}$ C.

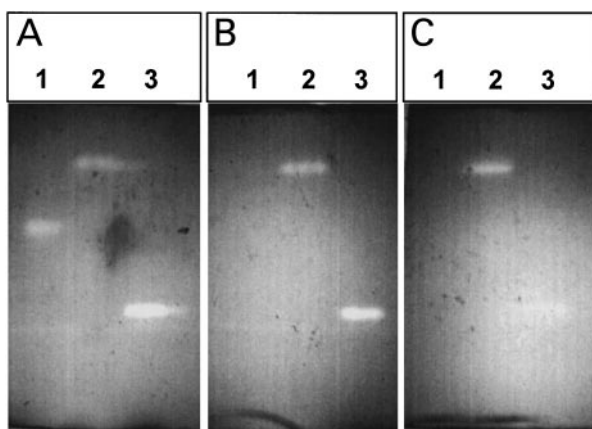


Fig. 4. Native PAGE of SOD proteins stained for SOD activity. SOD proteins were electrophoresed through 8% polyacrylamide gels and stained for SOD activity. SOD inhibitors (none, A; 5 mM potassium cyanide, B; or 5 mM hydrogen peroxide, C) were added during incubation in nitroblue tetrazolium. Sample lanes correspond to bovine Cu/ZnSOD (Sigma, 0.21 unit, lane 1), PiSOD expressed from pTrcsod in *E. coli* OX326A (10 μ g, lane 2), and *E. coli* FeSOD (Sigma, 1.1 units, lane 3) in each panel.

the pTrcsod plasmid was restored under IPTG induction (not shown). These results indicate that the PiSOD can functionally substitute for the *E. coli* SODs and therefore efficiently protect the cells from the detrimental effects of superoxide ions generated by paraquat. In addition, a role for PiSOD in the detoxification processes against cadmium was demonstrated. As PiSOD is the only SOD activity detected in crude extracts of *P. involutus* grown under oxidative or cadmium stress conditions (C. Jacob, unpublished

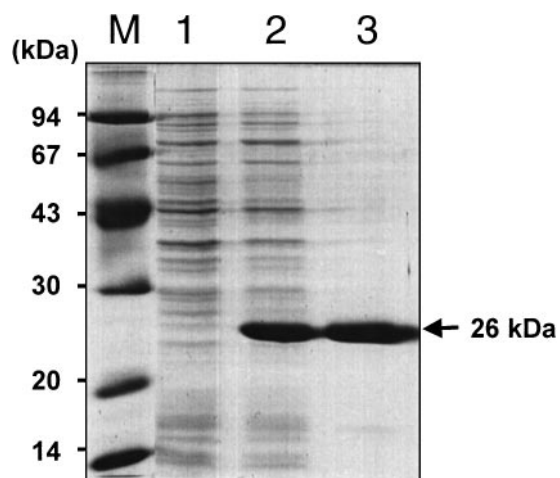


Fig. 5. SDS/PAGE of proteins expressed in *E. coli* BL21 (DE3) containing pSBET transformed with the two plasmids pET 3d and pETsod. Total cell proteins of *E. coli* cells harboring pET 3d (5 μ g, lane 1) or pETsod (5 μ g, lane 2), and insoluble cytoplasmic proteins of *E. coli* cells harboring pETsod (5 μ g, lane 3) were induced with 0.4 mM IPTG and electrophoresed through a 12.5% polyacrylamide gel. The arrow on the right indicates the position of the expressed SOD protein bands. Molecular mass size markers were run in the lane labeled M and sizes (kDa) are indicated on the left.

results), these results with *E. coli* suggested that PiSOD plays similar roles in *P. involutus*.

Analysis using native PAGE and staining for SOD activity revealed that OX326A cells transformed with pTrcsod contained an active SOD when induced by IPTG (Fig. 4, lane 2). A protein extract from the induced culture of OX326A cells transformed with pTrcsod was used for SOD activity staining on native 8% PAGE and compared with commercial FeSOD and Cu/ZnSOD (Fig. 4A). When the gel was incubated in the presence of 5 mM KCN, the Cu/ZnSOD was inhibited (Fig. 4B, lane 1) whereas in the presence of 5 mM H₂O₂, both Cu/ZnSOD (Fig. 4C, lane 1) and FeSOD (Fig. 4C, lane 3) were inhibited. In agreement with Fridovich [7], the insensitivity of the recombinant SOD toward these two inhibitors is consistent with the proposal that the isolated *P. involutus* sequence encodes a manganese-dependent SOD.

Molecular size of the PiSOD

The native molecular mass of PiSOD determined by gel filtration was 53.2 kDa. The subunit molecular mass, as determined by SDS/PAGE, was 26.2 kDa, a value close to the predicted molecular mass (22.7 kDa, 206 amino acids). The molecular masses of 53.2 kDa and 26.2 kDa for the native enzyme and the subunit, respectively, suggested that the enzyme was dimeric. By SDS/PAGE, only one band was detected on gels suggesting the PiSOD is composed of two identical subunits. It has been reported that the Cu/ZnSODs, as well as most prokaryotic MnSODs and FeSODs, are dimeric whereas the MnSODs from mitochondria and certain thermophilic bacteria are tetrameric [1]. However, the mitochondrial MnSOD from *Caenorhabditis elegans* was found to be dimeric [55]. PiSOD has a predicted pI of 6.75 which is similar to the pI of the *C. elegans* MnSODs.

Overexpression of PiSOD in *E. coli*

The region coding for the predicted mature PiSOD protein was amplified by PCR as described above and the product was subcloned into the pET 3d expression vector before transformation into *E. coli* BL21 (DE3) containing the pSBET plasmid [30]. BL21 (DE3) pSBET cells harboring the empty plasmid did not exhibit any major band (Fig. 5, lane 1), whereas a major band at approximately 26 kDa was observed with the pETsod vector (Fig. 5, lane 2). The overexpressed protein was mainly associated with the insoluble protein fraction (Fig. 5, lane 3). The overproduced protein was excised from the gel and sequenced at its N-terminus. The amino-acid sequence of the recombinant SOD agreed with the sequence predicted from the cDNA analysis except that the penultimate amino acid serine was replaced by alanine from introduction of an *Nco*I cloning site, as described above. This result further confirmed that there was no cleavage of any putative N-terminal signal sequence. Moreover, the first amino acid sequenced was alanine and not methionine, presumably because of the small size of the alanine residue, as previously described by Hirel *et al.* [56].

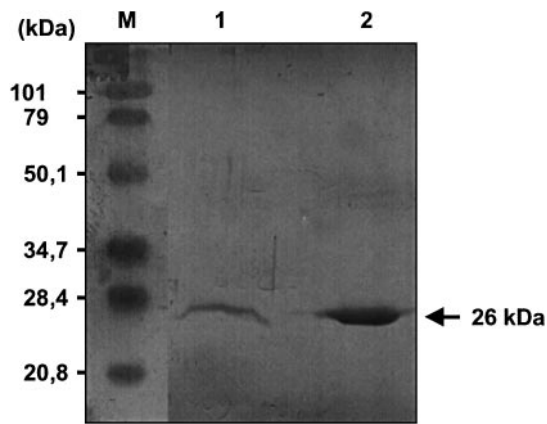


Fig. 6. Immunoblot of PiSOD from *P. involutus*. Samples of 40 μ g crude mycelial extract (lane 1) and 5 μ g insoluble protein fraction extracted from the *E. coli* BL21 (DE3) pETsod cells were subjected to SDS/PAGE (12.5% resolving gel), transferred to an Immobilon PVDF membrane, and probed with antibodies produced against PiSOD. Goat anti-(rabbit IgG) Ig conjugated to alkaline phosphatase was used to develop the blot.

Immunological characterization

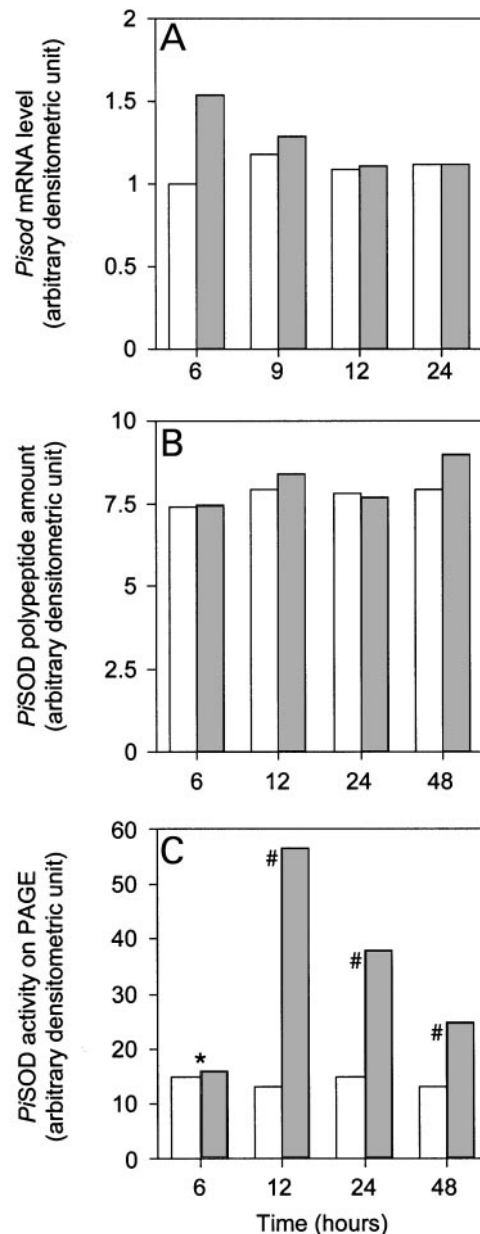
A single polypeptide (molecular mass \approx 26 kDa) was observed when a crude protein extract from *P. involutus* mycelium was submitted to SDS/PAGE, blotted, then probed with SOD antiserum (Fig. 6, lane 1). A strong band with a similar size was found when an insoluble fraction extracted from the BL21 (DE3) pETsod cells was

probed under the same conditions (Fig. 6, lane 2). Under native conditions, a single band was observed with a crude protein extract from *P. involutus* mycelium (not shown). The immunoblots showed the high reactivity and the monospecificity of the immune sera produced against the PiSOD.

Expression analysis of *P. involutus* SOD

P. involutus mycelia were grown in the presence or absence of 5 p.p.m. cadmium and assayed for SOD mRNA, polypeptide content and SOD activity (Fig. 7). The steady state transcript levels of *sod* increased by 45 and 20% in cadmium-treated mycelia, at 6 and 9 h, respectively, compared with control mycelia (Fig. 7A). The early increase in transcript levels allowed us to isolate differentially expressed *Pisod* mRNA by the suppressive subtractive

Fig. 7. Effects of cadmium treatment on transcript level (A), polypeptide quantity (B) and protein activity (C) of the SOD from *P. involutus*. Mycelia were transferred onto Melin–Norkrans medium supplemented (shaded bars) or not (open bars) with 5 p.p.m. cadmium. (A) Total RNAs (7.5 μ g) were isolated from *P. involutus* mycelium, separated by electrophoresis, blotted onto a membrane and hybridized with a cDNA probe encoding SOD as described in experimental procedures. Transcript levels were quantified using a Biorad Model GS-690 Densitometer with MULTI-ANALYST Macintosh image analysis software. The data were normalized to the respective 28S ribosomal RNA. (B) Protein extracts (50 μ g) were subjected to SDS/PAGE and immunoblotting as described in experimental procedures. The quantitative intensity was determined by a Biorad Model GS-690 Densitometer. (C) Protein extracts (25 μ g) were separated by native PAGE and stained for SOD activity as described in the experimental procedures. The quantitative intensity was determined by a Biorad Model GS-690 Densitometer. The results are representative of at least three separate experiments and were reproducible. For each of the densitometric methods (mRNA, immunoblot, and SOD activity) the data shown are from determinations in a range of linear relationship between amount and densitometer response. ANOVA analysis (A,B), no statistical significance for changes in control or cadmium-treated cultures over time or in a comparison of control to cadmium-treated cultures at a specific time. (C), $P = 0.05$ (*) for the increase in cadmium-treated compared to control culture at 6 h, $P < 0.0001$ (#) for the increase in cadmium-treated compared to control cultures at 12–48 h and for increase in cadmium-treated at 12–48 h compared to cadmium-treated culture at 6 h. $P > 0.06$, not statistically significant for changes in control cultures at 12–48 h compared to control culture at 6 h.



hybridization procedure. However these increases at 6 and 9 h were not statistically different from control values (ANOVA $P = 0.11, 0.72$). No significant changes were detectable at stages 12 and 24 h with 5 p.p.m. cadmium ($P = 0.93, 0.99$, Fig. 7A). The amount of SOD polypeptide did not vary significantly in this time period ($P = 0.60\text{--}0.96$, Fig. 7B). The absence of a correlation between mRNA and protein level at 6 and 9 h may be due to differences in efficiency of translation in cadmium-treated mycelia and control mycelia. After 48 h the amounts of total RNA and total polypeptide decreased dramatically in cadmium-treated mycelia. Therefore, it was not possible to follow SOD transcript and polypeptide levels at those later stages. In contrast (Fig. 7C), the basal SOD activity was greatly increased following treatment with cadmium, the maximum being 4.5-fold increase after 12 h with cadmium ($P < 0.0001$ for SOD activity at 12, 24 and 48 h compared to 6 h). In untreated mycelia, SOD activity remained constant (no significant change for SOD activity at 12, 24 and 48 h compared to 6 h).

Induction of SODs in response to metals and oxidative stress has been studied in other species. In the marine dinoflagellate *G. polyedra*, exposure to lead, mercury, copper or cadmium induces MnSOD and to a lesser extent FeSOD [57]. In *A. thaliana*, levels of MnSOD mRNA, polypeptide and enzymatic activity were not affected by different oxidative stresses [58]. However, other SOD isoforms were highly responsive to these stresses in *A. thaliana* [58]. In the ectomycorrhizal fungus *Rhizopogon roseolus*, SOD inhibition assays similar to those performed in this study implicated the presence of a single SOD, an MnSOD [59]. Moreover, MnSOD but not Cu/ZnSOD activity was found in protein extracts from certain flagellate fungi [60]. In crude extracts of *P. involutus* a single SOD isozyme, a putative MnSOD, was found following growth under oxidative stress conditions or under cadmium stress conditions (C. Jacob, unpublished results). Whether the failure to find Cu/ZnSOD, FeSOD or other putative MnSODs in *P. involutus* reflects their absence, or instead, a low specific activity for those SODs is unclear. As the only SOD identified in crude extracts, PiSOD is implicated as essential in the detoxification mechanisms against ROS in the ectomycorrhizal fungus *P. involutus*.

Our results comparing PiSOD polypeptide and PiSOD activity suggest that post-translational mechanisms are involved in activating a pool of pre-existing SOD in response to cadmium stress. One possible mechanism would be a competition between Fe and Mn for the metal-binding site as shown in *E. coli* [61]. In that case, MnSOD polypeptide is inactive when Fe is bound and is active when Mn is bound. In the PiSOD case, addition of oxidants or a change in redox balance may shift the competitive balance in favor of Mn and thus allow synthesis of the active PiSOD. Another possibility is that cadmium influences the synthesis, metal binding or metal delivery activity of a metal chaperone which converts apo PiSOD to active metal-bound PiSOD. Such a metal chaperone is involved in the delivery of copper to Cu/Zn superoxide dismutase in *S. cerevisiae* [62]. Thus far, no eukaryotic chaperones for metals other than copper have been established; however, it is likely that analogous cofactor trafficking pathways exist for metals other than copper [63].

The increasing activity of the PiSOD as well as the fact that it can functionally substitute for the *E. coli* SODs under cadmium stress suggests that this enzyme is involved in the cellular response of *P. involutus* to cadmium. This is to our knowledge the first insight in the characterization of molecular events that take place in an ectomycorrhizal fungus during exposure to heavy metals. Alongside studies on SOD, we are currently investigating other antioxidant and detoxification enzymes such as those involved in biosynthesis of glutathione and chelating agents. The isolation of genes and proteins involved in the response to cadmium-induced stress open new perspectives in the understanding of molecular mechanisms that promote tolerance in mycorrhizal fungi, which are key organisms in remediation programmes [64].

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