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The manganese superoxide dismutase from the penicillin producer *Penicillium chrysogenum*

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Abstract The antioxidant enzyme superoxide dismutase has been studied in order to define mechanisms for the influence of oxygen on penicillin production. Manganese-containing SOD activity was purified from penicillin-producing cultures of the filamentous fungus *Penicillium chrysogenum* and reverse genetics was used to identify full-length cDNA and genomic clones. Sequence analysis revealed a 630-bp ORF containing three exons and two introns with fungal consensus splice-site junctions. The deduced amino-acid sequence (210 amino acids; 23.13 kDa) includes conserved residues required for enzymatic activity and metal binding, and shares significant similarity with Mn- and Fe-containing superoxide dismutases. The *sod* gene is present as a single copy in the genome of different *P. chrysogenum* strains and its expression level is not correlated with penicillin-G productivity.

Key words Superoxide radical · Oxygen · Oxidative damage · Stress

Introduction

Penicillium chrysogenum is the microorganism used industrially for the production of the clinically and economically important antibiotic penicillin (Díez et al. 1997). Penicillin production by this aerobic filamentous fungus is achieved by fermentation in shaken submerged cultures. Oxygen availability has been proposed to regulate penicillin production and, consequently, fermentation is carried out maintaining a high level of dissolved oxygen. Unfor-

tunately, oxygen supply is usually a limited resource in aerobic fermentations because of its poor solubility in aqueous solutions (Peng and Chen 1994). The penicillin biosynthetic enzyme isopenicillin N synthase requires molecular oxygen and is stimulated in vitro by increasing oxygen availability (Ramos et al. 1985). Similarly, fermentation of the cephalosporin-producing fungus *Acremonium chrysogenum* under low-oxygen conditions severely reduces the overall rate of cephalosporin biosynthesis, possibly affecting the biosynthetic oxidation reactions of the pathway: α -aminoadipyl-cysteinyl-valine tripeptide (ACV) cyclization, thiazolidine ring expansion and deacetoxycephalosporin (DAOC) hydroxylation, leading to the accumulation of penicillin N in a stirred tank reactor (Herold et al. 1988). Moreover, an oxygen supply around 50% saturation is critical, and no cephalosporin production is obtained under this value (Scheidegger et al. 1984).

On the other hand, reactive oxygen species, produced as a normal by-product of aerobic metabolism, become toxic or lethal when they damage nucleic acids, proteins and membrane lipids (Docampo and Moreno 1984). In order to resist these potentially damaging oxygen species, aerobic organisms have developed a protection system where the antioxidant enzymes superoxide dismutase (EC 1.15.1.1, abbreviated SOD), glutathione peroxidase and catalase play an essential role. SOD activity reduces the superoxide radical O_2^- into hydrogen peroxide and molecular oxygen (Fridovich 1975; Carliz and Touati 1986; Farr et al. 1986). Eukaryotic SODs may be classified in three groups: (1) manganese-containing SOD (MnSOD) synthesized in the cytosol and imported post-translationally into the mitochondrial matrix, (2) cytosolic copper/zinc-containing SOD (Cu/ZnSOD), and (3) extracellular SOD secreted by many types of cells (Weisiger and Fridovich 1973; Marklund 1982; Mantonavi and Dejana 1989). MnSODs described in eukaryotes are homologous to iron-dependent bacterial SODs (Fridovich 1975; Mantonavi and Dejana 1989), whereas Cu/ZnSODs, although catalyzing the same reaction, have a completely different structure.

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Mutations generating a defective SOD cause hypersensitivity to oxygen-induced damage in bacteria, yeast and *Drosophila melanogaster* (Van Loon et al. 1986; Mantovani and Dejana 1989; Rosen 1993), as well as neuro-degenerative human disorders (Balzan et al. 1995). Nevertheless, the expression of FeSOD from *Escherichia coli* or MnSOD from maize in SOD-deficient strains of *Saccharomyces cerevisiae* protects the cells against oxidative stress (Larsen 1993; Zhu and Scandalios 1995) and its over-expression increases the mean life-span in *Caenorhabditis elegans* and *D. melanogaster* (Reveillaud et al. 1991; Larsen 1993).

MnSOD and Cu/ZnSOD eukaryotic enzymes are encoded by different genes (Weisiger and Fridovich 1973) which share a narrow sequence similarity (Barra et al. 1984) and are expressed at different levels in response to diverse forms of oxidative stress (Visner et al. 1990). Genes encoding MnSOD enzymes have been identified in yeast (Ditlow et al. 1982; Marres et al. 1985) and many higher eukaryotes (Wan et al. 1994; Henkle-Dührsen et al. 1995; Jones et al. 1995), whereas only recently have they been reported from the filamentous fungi *Aspergillus fumigatus* (Crameri et al. 1996) and *Ganoderma microsporum* (Pan et al. 1997). Likewise, a large number of bacterial (iron-containing SOD (FeSOD) genes have been described (Campbell and Laudenbach 1995; Lah et al. 1995; Tsois et al. 1995).

The aerobic metabolism of *P. chrysogenum* requires high expression of free-radical scavenging enzymes such as SOD and catalase. Attempts to clarify the mechanisms of oxygen influence on penicillin production led us to investigate these antioxidant enzymes. The present paper describes the purification of the MnSOD from *P. chrysogenum* (PcMnSOD) and the structure and expression of the gene encoding it (*sod*).

Materials and methods

Microorganisms and vectors used. *P. chrysogenum* AS-P-78 (Barredo et al. 1988), was employed as the source of DNA. *E. coli* DH5 α (Hanahan 1983) was the recipient strain for high-frequency plasmid transformation (10^7 – 10^8 transformants/ μ g of DNA). *E. coli* SURE (Stratagene) and *E. coli* LE392 (Sambrook et al. 1989) were the hosts for λ ZAP and λ GEM12 phage derivatives. *E. coli* WK6 (Kramer et al. 1984) was used as the host strain for single-stranded DNA production with phagemid derivatives. The phage vector λ ZAP (Stratagene) was employed for the construction of a cDNA library of *P. chrysogenum* AS-P-78. The genomic library of this strain was made using λ GEM12 phage vector (Promega). pBluescript I KS (+) and pBC KS (+) phagemids (Stratagene) were used for routine subcloning and sequencing. Phage M13K07 (Mead and Kemper 1987) was used as a phagemid helper for the infection of strain WK6 in order to obtain single-stranded DNA.

SOD purification and sequencing. Cell-free extracts were prepared from 72–110-h-old mycelium of *P. chrysogenum* grown in batch in penicillin-producing conditions. Cells from 200 ml of culture broth were collected by filtration, washed twice with sterile saline solution, and suspended in 100 ml of 500 mM potassium phosphate buffer pH 8.2 containing 4 mM EDTA and 1 mM phenylmethylsulphonyl fluoride. The mycelium was disrupted with 100 g of glass-beads (Ballotini, 0.25–0.5 mm diameter) using a glass-bead mill appara-

tus (Dynamill) during six periods of 1 min with intervals of 1 min between each period, and the lysate was cleared by centrifugation at 17 000 *g* for 20 min at 4°C. The SOD enzymatic assay (Beauchamp and Fridovich 1971) and protein concentration (Bradford 1976) were determined by the described methods. The cleared lysate was precipitated with ammonium sulphate and the SOD-active fraction (25–45% saturation) was dissolved in 100 ml of PG buffer (10 mM potassium-phosphate buffer pH 8.5 containing 1 mM ascorbic acid, 4 mM EDTA, 5 mM Mg₂Cl and 20% w/v glycerol) with 20% ammonium sulphate. Phenyl-Sepharose chromatography was performed through a CL-4B column of 7×2 cm equilibrated with the same buffer. The column was first washed with 150 ml of PG buffer followed by 100 ml of the same buffer without glycerol. SOD activity, recovered in the last fraction, was precipitated with 65% ammonium sulphate and the pellet was dissolved in 10 ml of ET buffer (10 mM ethanolamine pH 9.0, 1 mM ascorbic acid, 4 mM EDTA and 5 mM Mg₂Cl) with 20% glycerol (w/v). This preparation was desalted through a Sephadex G-25 (PD-10) column (Pharmacia Biotech) equilibrated with ET buffer. Fractions containing maximal activity from five Sephadex G-25 (PD-10) batches were mixed and applied to a DEAE-Sepharose column (7×2 cm) equilibrated with ET buffer. At pH 9.0, most of the protein was retained in the column, whereas SOD activity was eluted. Active fractions were precipitated again with 65% ammonium sulphate, dissolved in 1 ml of ET buffer and applied to a Sephacryl S-200 superfine column (65×2 cm). Aliquots of 2 ml were collected and SOD activity was detected between fractions 112 to 118, with the highest activity (106 U) in fraction 117. One unit of purified enzyme is capable of causing 50% inhibition to the standard xanthine-xanthine oxidase system. Samples from the different purification steps were analyzed by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the Miniprotein II electrophoresis system (Bio-Rad). Isoelectric focusing (IEF) gels were employed as previously reported (Dunbar et al. 1990). The protein recovered from a piece of SDS-PAGE was sequenced by Eurosequence by (Groningen, The Netherlands). Amino-acid sequences of three internal peptides were determined and used to design degenerated oligonucleotides.

Construction of a *P. chrysogenum* cDNA library. Total RNA was purified from batch fermentations of *P. chrysogenum* AS-P-78 in penicillin-G production conditions (Barredo et al. 1988) at 48, 72 and 96 h as described by Ausubel et al. (1987). Poly A⁺ mRNA was purified from total RNA using the "Poly (A) Quick mRNA purification Kit" (Stratagene) and library construction was carried out with the "ZAP-cDNA synthesis Kit" (Stratagene). The library was packaged with the kit "Gigapack II Gold" (Stratagene), infected in *E. coli* SURE and plated to obtain about 10⁸ pfus. After the screening of the library, the cDNA inserts from purified recombinant phages were directly recovered in pBluescript I SK (–) by in vivo excision.

Construction of a *P. chrysogenum* genomic library. Total DNA was purified as previously described (Specht et al. 1982) from 48-h-old mycelium of *P. chrysogenum* AS-P-78 grown in batch fermentations. Fragments of 17–22 kb were purified from *Sau*3AI partially digested DNA and ligated to λ GEM12 phage vector (Promega) according to standard procedures (Sambrook et al. 1989). Ligation products were packaged in vitro with the kit "Gigapack II Gold" (Stratagene), infected in *E. coli* LE392 and plated to obtain about 5×10⁴ pfus. Recombinant phages were amplified in liquid medium to purify their DNA (Sambrook et al. 1989).

Identification of PCR fragments encoding PcMnSOD. A PCR reaction was set up with the "Opti-Prime™ PCR Optimization Kit" (Stratagene). Two primer pairs were used: # 4 (5' CCCTCSAGGCCS-AGSAGCTCGCGCCGAAGGC 3') versus reverse (5' GGAAACAGCTATGACCATG 3'), or #12 (5' GCICCGCDATRTTCRTTIGC 3') versus reverse (5' GGAAACAGCTATGACCATG 3'). Optimized amplification reactions (50 μ l) contained about 20 ng of DNA from the *P. chrysogenum* AS-P-78 cDNA library, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 25 mM KCl, 100 pmoles of primer # 4 or # 12, 200 pmoles of "reverse primer", 1.25 U of *Taq* DNA polymerase

(Boehringer Mannheim) and 200 mM of each dNTP. A Perkin-Elmer DNA Thermal Cycler 480 was used for amplification with a program of 35 cycles [94 °C, 30 s (2 min for the first cycle); 51 °C, 60 s; 72 °C, 90 s (extending 3 s per cycle)]. The PCR-amplified products were purified from agarose gel by the "Qiaex II gel extraction kit" (Qiagen) and cloned in pBluescript I KS (+).

Identification of cDNA and genomic clones encoding PcMnSOD. The 440-bp PCR-amplification product of primer # 4 versus "reverse primer" was cloned in pBluescript I KS (+) giving rise to pALP612. A 180-bp *Hind*III fragment bearing the primer # 4 annealing region was purified from this construction and used as a hybridization probe for screening of the cDNA library. About 60 positive phage clones were identified and ten of them were in vivo excised for further analysis. The restriction maps of these plasmids were determined and the largest insert (about 1 kb) was sequenced. The same probe and hybridization conditions were used for the screening of the λ GEM12 genomic library. In this case, about 30 positive clones were identified and ten of them were mapped, selecting phage fALP185 for further analysis.

Nucleic acid hybridization and sequencing. Total RNA for Northern blotting was purified with a RNeasy kit (Qiagen). Southern and Northern blots were carried out according standard procedures (Sambrook et al. 1989). Sequencing clones were constructed with the kit "Erase a base" (Promega) and sequenced by the dideoxynucleotide method (Sanger et al. 1977) using Sequenase 2.0 (Amersham).

Nucleotide sequence accession number. Genomic and cDNA nucleotide sequences encoding PcMnSOD have been deposited in GenBank under the accession nos. AF026790 and AF026523 respectively.

Results

PcMnSOD purification, internal sequence determination and primer design

SOD enzymatic activity from cell-free extracts of *P. chrysogenum* AS-P-78 was detected and purified as described in Materials and Methods. The homogeneity of the purified protein was ascertained by the presence of a single band with a molecular mass around 21 kDa in SDS-PAGE analysis and confirmed by the existence of only one band with a pI between 8.0 and 8.5 in IEF analysis.

The N-terminal sequence of PcMnSOD could not be determined, suggesting N-terminal blocking. Therefore, samples of purified enzyme were subjected to digestion either with endoproteinase Lys-C or with trypsin after pyridylethylation. A successful digestion was observed with trypsin and a portion of the digested sample was fractionated by preparative HPLC. Three fractions, corresponding to internal peptides of the purified enzyme, were subjected to 23 Edman degradation cycles using an automated sequencer (Applied Biosystems 477A) generating the sequences: (1) AFGAELLGLNGSGXGXLV, (2) NLTPPGTPANDIAGAPALR and (3) AGYVEGIWKI. From sequence number 1 the degenerate oligonucleotide # 4 (5' CCCTCSAGGCCSAGSAGCTCGGCGCCAAGGC 3'), and from sequence number 2 the degenerate oligonucleotides # 6 (5' GGGGCGCCGGCGATGTCGTTGGCNGGGGTGCC 3'), # 12 (5'GCICCIIGCDATRTCRT-

TIGC 3') and # 13 (5' AAYGAYATHGCNGGIGCICC 3'), were synthesized by Isogen bioscience bv, Maarssen, The Netherlands.

Cloning and identification of the 3'-end of the cDNA encoding PcMnSOD

In order to identify the *sod* gene, cDNA and genomic libraries were constructed. *P. chrysogenum* mRNA, isolated from a mycelium under penicillin-G production conditions, was used to construct an expression library in phage vector λ ZAP. The complete cDNA library was amplified and used as a template for PCR-amplification reactions.

PCR reactions were conducted using pairs of degenerate primers designed from the internal peptide sequences. However, primer degeneracy hindered the identification of specific fragments above the background. Consequently, a different amplification strategy was adopted, combining each degenerate SOD primer with the reverse "sequencing" primer located on the vector.

DNA from the cDNA library was employed as a template in PCR reactions using primer pairs # 4 versus the reverse and # 12 versus the reverse. In the first case a major band of about 555 bp appeared, while the second reaction gave a band of about 440 bp. The 120-bp region between the reverse primer and 5'-end of the cDNAs was removed from both amplification products by *Eco*RI digestion. The resulting fragments of 435 bp and 320 bp were cloned into *Eco*RI-*Eco*RV-digested pBluescript I KS (+), yielding plasmids pALP612 and pALP614 respectively. The inserts present in both plasmids were sequenced. Internal amino-acid sequences 1 and 2, corresponding to oligonucleotides # 4 and # 12, were searched for in the three potential reading frames with the program Findpro (DNASTAR), locating both amino-acid sequences in pALP612 but only sequence 2 in pALP614. The search of the SwissProt and PIR databases, with the polypeptide deduced from the DNA sequence, revealed significant similarity with Mn and Fe SODs.

Characterization of the cDNA encoding PcMnSOD

A 180-bp *Hind*III fragment obtained from pALP612 was used as a probe for screening the cDNA library. About 60 positive clones were detected and ten were further characterized. The plasmids included in the ten selected recombinant phages were in vivo excised as pBluescript I SK (-) recombinant plasmids and named from pALP649 to pALP658. Restriction-enzyme mapping of their inserts, showed that pALP650 carried the largest insert, with the longest 5' upstream region of the gene.

The insert of pALP650 (Fig. 1) was sequenced in both strands and the nucleotide sequence obtained (771 bp) was analyzed with the program Geneplot (DNASTAR) using the codon-preference algorithm. The identified ORF coded for a 210 amino-acid protein. The cDNA included 5' and

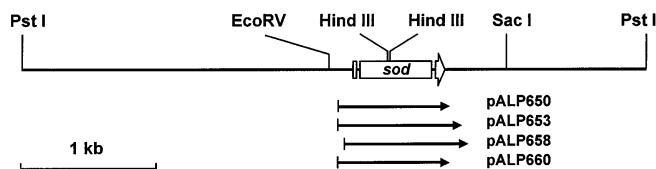


Fig. 1 Genomic DNA region of *P. chrysogenum* including the *sod* gene. The depicted 4.5-PstI DNA fragment was subcloned in pBlue-script I KS (+) from the genomic recombinant phage fALP185, giving rise to plasmids pALP668 and pALP669. Two introns are represented by discontinuities in the box. Four cDNA clones including the *sod* gene are illustrated as arrows beneath the *sod* coding region

3' non-translated regions. The 3' non-translated region was located between positions 672 and 771, excluding the polyA tail, and it lacked the polyadenylation consensus sequence 5' AATAA 3'. A pyrimidine-rich region usually appears immediately upstream of the transcription start site in highly expressed fungal genes (Davis and Hynes 1991). A pyrimidine stretch was detected in the 5' non-translated region, matching with the starting point of most of the cDNA clones, suggesting a possible transcription start site (Fig. 2).

Analysis of the deduced amino-acid sequence of PcMnSOD

The 210 amino-acid protein deduced from the cDNA sequence had a predicted molecular mass of 23.13 kDa. Comparison of the protein with SwissProt and PIR databases using the program Proscan (DNASTAR) gave significant scores with MnSODs (*A. fumigatus*, *G. microsporum*, *D. melanogaster*, *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Bos taurus*, *Pisum sativum*, *Oryza sativa*, *C. elegans*, *Nicotiana glauca*, *Hevea brasiliensis*, etc.) and FeSODs (*Tetrahymena pyriformis*, *Methylobacterium* sp., *Bordetella pertussis*, *Methanobacterium thermoautotrophicum*, *Pseudomonas ovalis*, *Bacteroides fragilis*, *E. coli*, *Porphyromonas gingivalis*, etc.). Four residues known to be involved in metal binding in Fe or Mn SODs (Carlioz et al. 1988; Borgstahl et al. 1992) were found in the PcMnSOD amino-acid sequence (His-30, His-78, Asp-166 and His-170). The consensus sequence DXWEH located between the above mentioned Asp-166 and His-170 was also conserved (DMWEH) and a single potential N-glycosylation site (NXS/T), present in all homologous enzymes except in *S. cerevisiae* MnSOD, was also found at His-78 (NHS) in PcMnSOD (see Fig. 2 and 3). Most of the eukaryotic MnSODs include a putative mitochondrial transit peptide. Nevertheless, PcMnSOD lacks this signal peptide as do bacterial [i.e. *E. coli* MnSOD (Takeda and Avila 1986)] or some eukaryotic MnSODs [i.e. the fungi *A. fumigatus* (Cramer et al. 1996) and *G. microsporum* (Pan et al. 1997)] as well as chlorophycean *Chlamydomonas reinhardtii* (Kitayama et al. 1995). Although mitochondrial transit peptides from MnSODs are not very similar, targeting sequences are generally rich in posi-

AAGAGCAACTGGCGATAACGTCATCACTCAACTCATCCCAATTCCTT	50
CGCACTATCCGGTGGATATCCACCTGGTGGCCCCGAGGTCTTGAAGA	100
TGCTTACTAGCAGCTCACTCAAGGAGGGGTATCCAGAGGACCTTGGTGG	150
TTTATCTCTTGCCTACACCTTCCAATCTTCCAATCTTCTTCTCACTCTT	200
CTCTGCCCCTTGACGCACACTAACAACCACAATCACACTATGACTTCTCAA	250
m t s q	
ACACACACACTGCCTCTCTCCCTATGCCTACGATgtaagcctcatcgg	300
t h t l p p l p y a y d	
attgagactcctcataatctggcgccatgaagctaacaatgaacagCGGT	350
a l	
TGGAGCCCCGTCTCTCAAGCAGATAATGGAGCTGCATCATCAGAAGCAC	400
e p v i s k q i m e l [H] h q k h	
CACCAAACTTACATCAACAATCTGAACGACGCTCTCCGCCCAAGCTTC	450
h q t y i n n l n a a l s a q a s	
TGCAACAGCATCAAAATGATGTCCCACTTGTATTCATGCAACAAAAGC	500
a t a s n d v p t l i s l q q k l	
TTGCTTTAACGGTGGAGGCCATCAATCACTCTCTGTTCTGGAAGAAC	550
r f n g g g h i N [H] S l f w k n	
CTGACTCCCCCGGTACACCTGCGAATGACATCGCTGGTCTCCGCTCT	600
l t p p g t p a n d i a g a p a l	
GCGCAAGCCATCGTCTCCCGTGGGGCTCGCATGAAGCGTTTGTCAAGG	650
r e a i v s r w g s h e a f v k a	
CTTTTGGCCCGAGCTGCTCGGTCTTCAAGGGAGTGGCTGGGGATGGCTG	700
f g a e l l g l q g s g w g w l	
GTGAGCAAGGTTGGTCCCAAGGACGACTTGAGATCGTTACGACTAAGGA	750
v s k g g a k g r l e i v t t k d	
CCAGGACCTGTGTTAATGCACCTGATGTGCTGTCTTGGTGTGGATATGT	800
q d p v n a p d v p v f g v D [M] W	
GGGAGCATGCTACTACCTCCAGgtgagctcggtctcatgtatctttgtg	850
E [H] a y y l q	
tgtattttctgctaattgcgttacgtgcagTACCTGAACAACAAGGCTGG	900
y l n n k a g	
CTATGTTGAGGGGATTGGAAGATCATTCAGTGGGCTGAAGCTGAGAAGC	950
y v e g i w k i i h w a e a e k r	
GTTACACTGCTGGCGTTGAGAACCCTGAAGCTGTGAGGTGTTTCATCGA	1000
y t a g v e n p l k l .	
ACCGGGGTTTCGCTTTGATTAGCTGAATGAGCCTCCCACTAAACAAATGT	1050
AGTATTTCACACTCATGCAAAATGATTGATGAATC	1085

Fig. 2 Nucleotide sequence of the *sod* gene of *P. chrysogenum*. Intron/exon boundaries, the signal for lariat formation and potential regulatory sequences identified in the promoter region (pyrimidine stretch and CCAAT boxes) are underlined. Introns are in lower case. The deduced amino-acid sequence appears in a lower case single-letter code and the location of three internal peptides chemically sequenced from purified PcMnSOD is underlined. Conserved metal-binding residues are boxed in uppercase. The potential glycosylation sequence NXS/T and the consensus sequence for MnSODs (DXWEH) are in uppercase and double underlined

tively charged and hydroxylated amino acids and lack acidic amino acids (Roise et al. 1988; Hartl and Neupert 1990).

The PcMnSOD-deduced amino-acid sequence was aligned by the Clustal method with 15 FeSODs and 46 MnSODs (data not shown). Eight residues were absolutely conserved in all these sequences: His-30, Tyr-38, His-78, Asp-166, Trp-168, Glu-169, His-170 and Tyr-173. An alignment of PcMnSOD with six representative MnSODs is shown in Fig. 3. A Lipman-Pearson and Needleman-Wunsch comparison with the program A align (DNASTAR) showed the highest similarity index with *A. fumigatus* MnSOD, showing a 66.2% identity in 201 overlap-

Ec	M.....	SYTLPSLPYAYDALEPHFDKQTMEIHHKHHQTY	35
Sc	MFAKTAANLTKGGSLSTARRT	KVTLPDLKWDGALPEYISGQINELHYTHKHHQTY	60
Pc	MTSQ.....	THTLPPPLPYAYDALEPYISQIMELHHKHHQTY	38
Sf	M.SQ.....	QYTLPPPLPYAYDALEPYISQIMELHHKHHQTY	37
Gm	MA.....	HVLPDLPPYAYNALEPFISQIMELHHKHHQTY	35
Ov	M...NLIIIGVAGRLLVGKYNCLNTRQLKHVL	PDLPPYDYGALPEILSAEIMQVHHGKHAAAY	58
Hs	MLSRVCGTSRQLPPVLYG.LGS.RQKHS	PDLPPYDYGALPEHINAQMLHSHKHHAAAY	58

Ec	VNNANALESLEPFANLPVEELITKLDQLPADKKTVLRNNAAGGHANHSFWKGLK....	90
Sc	VNGFNTAVDQFELSLLAKEPSPANARKMIAIQNKIFHGGGFTNHCLFENLAPESQG	120
Pc	INNLAALSAQA.....SATASNDVPTLISLQKLRFNNGGGINHSLFWKNLTTPPGTP	91
Sf	VNGLNALEAQK.....KAAEATDVPKLVSVQQAIFKNGGGHINHSFWKNLAPKESG	90
Gm	VNSLNAEEQAYA.....KASTPKER...IALQSALKFNGGGHINHSFWKNLAPAKSE	85
Ov	VNALNQAEKVK.....EALAKGDTQAAVAGTKLMNFNTGGHINHTLFWGLTAVKNS	111
Hs	VNNLVTEEKYQ.....EALAKGDTVTAIALQPAKLFNNGGGINHSIEWTNLSP.NGG	110

Ec	...KGTTLQGD...LKAATERDFGSDVNFKAIEFEKAASRFSSGAWLVKGD...KLEAVV	142
Sc	GGE.....PPTGALAKAIDEQFSGDELIKLTNTLAGVQSSGAFIVKNLSNGKLDVV	175
Pc	A...NDIAGAPALREAIIVSRWGSHEAFVKAFAELLGQSSGWLVSFGGAKGRLEIV	147
Sf	G...GKIDQAPVLKAAIEQRWGSFDFKDAFNTLLGIQSSGWLVSFGGAKGRLEIV	145
Gm	GKNGGALADGP.LKSAIEQNWGSVDNFIKEFNATTAIQSSGWLVLNLPATK.RLEIT	143
Ov	GEPNSE.....LMTAIIKDFGSLTMDIKLNAKTIAIQSSGWLVDYDKE.MKRLQLA	163
Hs	GEPKGE.....LLEATIKLDFGSDFKFKEKLTAAASVGVSQSSGWLGNFKE.RGHEQLA	162

Ec	STANQDSPLMGEAIGASGF.PIMGLDVWEHAYYEKFQNRPPDYIKEFNNVNVWDEAAAR	201
Sc	QTYNQD.....TVTGP...LVPLVAIDAEHAYYLOQYQNKADYFKAIWNVNVWKEASRR	227
Pc	TTKQDQ.....PVNAPD...VPVFGVDMWEHAYYLOYLNNKAGYVEGIWKIHWAEAEKR	199
Sf	TTHQDQ.....PVTGAA...PVFGVDMWEHAYYLOYLNDKASYAKGIWNVNVWAEENR	196
Gm	TTANQD.....PLLSH...VPIIGVDIWEHAYYLOYLNVKADYLAATIVINFKAEARR	194
Ov	CCPNQD.....LLEPTTGLIPLFCIDWEHAYYLOYKNLRDPFKATIKWIANWKIISDR	217
Hs	ACPNDQ.....PLQGTGLIPLLGIDVWEHAYYLOYKNVRPDYKAIWNVNVWENVTER	216

Ec	FAA.....KK	206
Sc	FDAG.....KI	233
Pc	YTAGVENP...LKL	210
Sf	YIAGDKGGHPFMKL	210
Gm	LIEATK	200
Ov	YIKARG	223
Hs	YMACKK	222

Fig. 3 Alignment of amino-acid sequences of MnSODs. Sequences from *Escherichia coli* (Ec) (GenBank accession number X03951), *Saccharomyces cerevisiae* (Sc) (GenBank accession number X02156), *Penicillium chrysogenum* (Pc) (GenBank accession number U53561), *Aspergillus fumigatus* (Sf) (GenBank accession number U56403), *Ganoderma microsporum* (Gm) (GenBank accession number U56403), *Onchocerca volvulus* (Ov) (GenBank accession number X82171) and *Homo sapiens* (Hs) (GenBank accession number X07834) were compared. The amino-acid position in each sequence is shown on the right. The amino-acid sequences of internal peptides determined in the purified protein are underlined. Shadowed boxes represent identical residues located in the same position in all seven proteins. Gaps were introduced into the sequences to maximize similarities and compensate for differences in chain length. The leader sequence, a characteristic feature of eukaryotic MnSODs, does not exist in fungal MnSODs (*P. chrysogenum*, *A. fumigatus* and *G. microsporum*) or in the prokaryote *E. coli*. The beginning of the mature proteins from the leader-bearing sequences is indicated with a vertical line. Residues involved in metal binding are marked with an asterisk. Note that the potential glycosylation site NHS/T, including His-78 in *PcMnSOD*, is present in all of them except *S. cerevisiae*.

ping residues. Other striking features were shared by both fungal enzymes: *A. fumigatus* MnSOD also lacks the mitochondrial targeting signal and matches the *PcMnSOD* C-terminal sequence LKL, which resembles the SKL consensus peroxisomal targeting sequence (PTS1) (Gould et al. 1989). The sequence LPPL, resembling the polypeptide LPDL conserved at the N-terminal region in most of eukaryotic MnSODs, is also present in *PcMnSOD* (Fig. 3). A phylogenetic tree of MnSODs was constructed with Clustal alignment data to show the evolutionary relationship between them (Fig. 4).

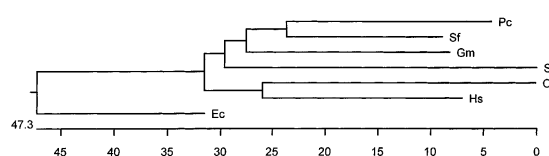


Fig. 4 Phylogenetic tree of MnSODs. Sequences from *Escherichia coli* (Ec), *Saccharomyces cerevisiae* (Sc), *Penicillium chrysogenum* (Pc), *Aspergillus fumigatus* (Sf), *Ganoderma microsporum* (Gm), *Onchocerca volvulus* (Ov) and *Homo sapiens* (Hs) were analysed by the Clustal method with the PAM250 residue-weight table. Multiple alignment parameters were: gap penalty = 15 and gap length penalty = 15. Pairwise alignment parameters were: ktuple = 1, gap penalty = 3, window = 5 and diagonals = 10. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.

Genomic structure analysis of *sod*

The genomic *P. chrysogenum* library was screened with the same probe used for screening of the cDNA library. After three purification cycles, ten recombinant phages were characterized by restriction mapping and Southern blotting. The phage fALP185 contained the *sod* gene in a 4.5-kb *Pst*I fragment. This fragment was subcloned in both orientations in pBluescript I KS (+) giving rise to the plasmids pALP668 and pALP669. The restriction map of this *Pst*I fragment is shown in Fig. 1.

A region of 1085 bp including the *sod* gene was sequenced and analyzed with the codon-preference algorithm of the program Geneplot (DNASTAR). The gene contains three exons with two intervening sequences. The exons encode for 16, 159 and 35 amino acids respectively and the introns are 60- and 57-bp long. A comparison between the cDNA and the genomic exons revealed complete identity. The fungal consensus sequences described for the 5' (g/GTAHGT) and 3' (MYAG/g) intron/exon boundaries and the splice signal for lariat formation (WRCTAC) (Ballance 1986), are present in both introns.

All of the isolated recombinant phages containing the *sod* gene had an equivalent restriction pattern, thus corresponding to the same genomic locus. To establish the number of copies of the *sod* gene in the genome of *P. chrysogenum*, Southern blots of genomic DNA digested with the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I and *Xho*I were hybridized with an equimolar mix of *sod* and *pyrG* (Cantoral et al. 1988) probes; *pyrG*, the orotidine 5'-monophosphate decarboxylase encoding gene, is a single-copy gene. The intensity of the bands corresponding to the *sod* and *pyrG* genes was equivalent. These results indicate that the gene coding for *PcMnSOD* was single-copy as in the murine (Jones et al. 1995) and *Onchocerca volvulus* (Henkle-Dührsen et al. 1995) *sod* genes.

Southern hybridization of *Eco*RI digestions of total DNA from seven *P. chrysogenum* strains with different penicillin productivity with the above-mentioned probes, showed the same hybridization pattern and signal intensity for all of them, indicating neither gene amplification nor rearrangement related to penicillin over-production (Fig. 5A).

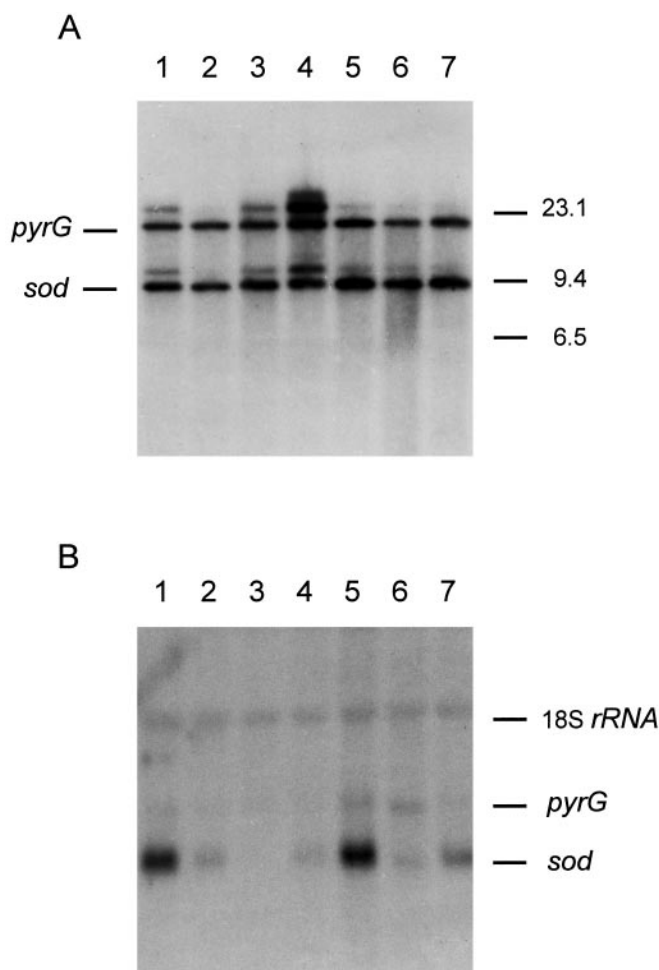


Fig. 5A, B Copy number and expression level of *sod* gene in different *P. chrysogenum* strains. **A** Southern hybridization of *Eco*RI-digested DNA from seven *P. chrysogenum* strains with penicillin productivity increasing from left to right: lane 1 Wisconsin 54-1255, 2, AS-P-78, 3–7 industrial strains. The 17-kb and 8.5-kb bands correspond to the *pyrG* and *sod* genes using 883-bp *Eco*RI-*Xho*I and 856-bp *Eco*RI-*Sac*I DNA fragments as probes respectively. **B** Northern hybridization of total RNA obtained from the above-mentioned seven strains with the same *pyrG* and *sod* probes. Unspecific hybridization with 18 S *rRNA* (about 1.8 kb) denotes gel loading. The sizes of the transcripts detected were around 1.1 kb for *pyrG* and 0.8 kb for *sod*.

Analysis of the 5' flanking sequences of the *sod* gene

The promoter region of the *sod* gene was examined for potential regulatory elements, including heme and stress-related regulatory sites. A pyrimidine-rich region was detected between positions 184 and 210 (Fig. 2). This region appears in highly expressed genes and is located immediately upstream of the transcription start site (Davis and Hynes 1991). Furthermore, a CCAAT box is present in the promoter region of about 30% of the known eukaryotic genes located between 50 and 200 bp upstream of the transcription start site (Davis and Hynes 1991; Papaianopoulos et al. 1996). Three potential CCAAT-like boxes are located in positions 40, 172 and 180. No consensus TATA box, nor either of the potential stress-re-

sponse elements (STRE), CCCCT and TAGGGA, described in the *SOD2* gene from *S. cerevisiae* (Pinkham et al. 1997), were identified. Neither were consensus binding sites for heme-sensitive repression (HSR) (Pinkham et al. 1997) detected.

MnSOD expression

The level of expression of the *sod* gene was studied by Northern hybridization with total RNA extracted from a penicillin-producing mycelium at different fermentation times. In standard complex fermentation medium (Barredo et al. 1988) the level of expression was maximal at 48 h after seed production, decreasing thereafter. Fermentation in the absence of phenylacetic acid, the penicillin-G side-chain precursor, decreased *sod* expression, whereas when fermentation was carried out in the absence of oil the addition of phenylacetic acid did not affect the level of *sod* gene transcription which remained low, but steady, during the fermentation. Concomitant monitoring of the penicillin biosynthetic gene *penDE*, encoding acyl-CoA: 6-aminopenicillanic acid acyltransferase, showed a different expression pattern which was also influenced by the presence of oil and phenylacetic acid. Simultaneous addition or absence of both supplements was required for maximal *penDE* expression. Otherwise, phenylacetic acid without oil, or the reverse case oil without phenylacetic acid, produced a poor *penDE* expression that decreased to almost zero around 96 h.

Sod and *pyrG* gene expression was also analyzed by Northern blots, with total RNA from 48-h cultures, in seven *P. chrysogenum* strains with different penicillin productivity. Significant variation in the level of *sod* gene expression was observed, although these differences were not correlated with the penicillin productivity of the strains (Fig. 5B).

Discussion

SODs are the first line of defense against toxic intracellular radicals produced during normal cellular metabolism or under oxidative stress. MnSODs are inducible enzymes located in the eukaryotic mitochondrial matrix that scavenge superoxide anions and, typically, are active as homotetramers. Crystallographic studies with human MnSOD and *E. coli* FeSOD have shown that metal-binding sites of these two enzymes are located at three histidine residues and one aspartate residue corresponding to His-30, His-78, Asp-166 and His-170 respectively of PcMnSOD. These residues, as well as the sequence DXWEH including Asp-166 and His-170, are conserved in all MnSODs so far described. A potential N-glycosylation site, NXS/T, located at His-78 in PcMnSOD is also conserved in all other eukaryotic species except *S. cerevisiae*. The MnSOD phylogenetic tree (Fig. 4) shows the expected grouping, with the closest relationship between the ascomycetes

A. fumigatus and *P. chrysogenum* and the basidiomycete *G. microsporum*.

Except for the *S. cerevisiae* gene, the presence of introns in eukaryotic genes encoding MnSOD is a common characteristic. The yeast *sod* gene is encoded by a single uninterrupted exon, whereas *sod* genes from rat or the human parasite *O. volvulus* have four introns. The *sod* gene has two introns, one near the 5', the other near the 3', end of the gene. The existence of introns is a common situation in several *P. chrysogenum* genes: *penDE* (Barredo et al. 1989b), *pyrG* (Cantoral et al. 1988), *acuA* (Martínez-Blanco et al. 1993), *pacC* (Suárez and Peñalva 1996), etc.; but uninterrupted genes have also been found: *pcbAB* (Díez et al. 1990), *pcbC* (Barredo et al. 1989a), *trpC* (Peñalva and Sánchez 1987), etc.

The *sod* gene is present in *P. chrysogenum* in a single copy per haploid genome as deduced in a Southern-hybridization comparison of the single-copy *pyrG* gene and the multi-copy *pcbC* gene encoding isopenicillin N synthase (Barredo et al. 1989c). This result agrees with the situation in *O. volvulus*, human and murine *sod* genes, but rat and mice genes are present as two copies per haploid genome. The 5' flanking region shows several potential regulatory tracts, including pyrimidine-rich regions present in highly expressed genes and CCAAT boxes, but it lacks the TATA box and several regulatory elements described in the *SOD2* gene of *S. cerevisiae*, encoding MnSODs as potential stress response elements (STRE) and consensus heme-sensitive repression binding sites (HSR).

Oil addition to the fermentation medium was required for growth and penicillin production in the presence of phenylacetic acid, because without oil phenylacetic acid was not consumed and completely prevented cell growth and penicillin production. The protecting effect of the oil addition could be explained by fatty acid-induced peroxisomal biogenesis for the β -oxidation of the fat, which in turn could aid in the consumption of phenylacetic acid. During this oxidative metabolism the generation of superoxide radicals probably induces *sod* gene expression. Several factors regulate MnSOD gene expression both in prokaryotes and eukaryotes. Environmental conditions such as O_2^- , iron chelators, compounds that increase intracellular O_2^- flux, and strong oxidants able to change cell redox potential, are all known to regulate *sodA* gene expression in *E. coli* (Hassan and Sun 1992). In certain eukaryotic cells containing both Cu/ZnSOD and MnSOD activities, it is this last enzyme which responds first to oxygen toxicity threats (Henkle-Dührsen et al. 1995).

The protective role that MnSOD, together with other antioxidant and detoxification enzymes, plays in aerated cultures of *P. chrysogenum* and its relation to oxygen need and penicillin production is currently under investigation in several strains with different penicillin productivity.

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