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Characterization of iron superoxide dismutase cDNAs from plants obtained by genetic complementation in *Escherichia coli*

(*Arabidopsis thaliana*/electroporation/*Nicotiana plumbaginifolia*/*sodA sodB* mutant)

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ABSTRACT **The inability of superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1)-deficient mutants of *Escherichia coli* to grow aerobically on minimal medium can be restored by functional complementation with a heterologous SOD-encoding sequence. Based upon this property, a phenotypic selection system has been developed for the isolation of clones containing eukaryotic SOD cDNAs. cDNA expression libraries from both *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* were transformed into a SOD-deficient *E. coli* strain by electroporation, and clones containing functional SODs were selected by growth on minimal medium. Analysis of these clones revealed the identity of cDNAs encoding the iron form of superoxide dismutase (FeSOD)—the first SODs of this type to be cloned from eukaryotes. The presence of this enzyme in these two divergent plant species challenges previous ideas that FeSOD is found in only a few plant families. In addition, these results show the potential for shotgun cloning of eukaryotic genes by complementation of bacterial mutants, particularly when it is combined with a highly efficient transformation method, such as electroporation.**

Superoxide dismutases (SODs; superoxide:superoxide oxidoreductase, EC 1.15.1.1) form part of an organism's defense against highly reactive oxygen species by removing the superoxide anion (O_2^-). Their importance has been demonstrated by the isolation of SOD-deficient mutants of *Escherichia coli* (1), yeast (2, 3), and *Drosophila* (4), which are all hypersensitive to conditions in which the formation of toxic oxygen species is enhanced.

According to their metal cofactor, three different classes of superoxide dismutases can be distinguished: copper/zinc (Cu/ZnSOD), iron (FeSOD), and manganese (MnSOD). The FeSOD and MnSOD are very similar in their primary, secondary, and tertiary structures (5). The Cu/ZnSOD, however, shows no structural relationship to the other two types and therefore is considered to represent an evolutionarily distinct type of SOD. This is reflected in the phylogenetic distribution pattern: prokaryotes and protists contain MnSOD, FeSOD, or both, and, except for a few bacterial species (6), Cu/ZnSOD is absent. Cu/ZnSOD has been found in most eukaryotic organisms examined. In higher plants, it is usually present in the cytosol and in chloroplasts. Eukaryotes also contain a mitochondrial MnSOD, whereas FeSOD has been reported within the chloroplasts of some plant species (for review, see ref. 7).

Several prokaryotic FeSODs have been characterized. FeSOD amino acid sequences are known from *E. coli* (8), *Pseudomonas ovalis* (9), *Photobacterium leiognathi* (10), and *Anacystis nidulans* (11), but only the FeSOD gene from *E. coli* has been isolated and sequenced (12).

Most plant species appear to lack FeSOD activity (13). However, its presence in a few phylogenetically diverse plant families (13–16) raises questions as to the evolutionary basis of its distribution. The identification of FeSOD in plants has been carried out chiefly by spectrophotometric SOD activity assays or by *in situ* stains on nondenaturing gels—methods that have limited sensitivity and resolution. Also, enzymes that are not expressed constitutively can be easily overlooked. It has been shown that the FeSOD present in tomato chloroplasts can be strongly induced during some unspecified stress conditions, suggesting that the importance of FeSOD in plants has been greatly underestimated (14, 17). To date, no molecular data have been forthcoming and, because of the lack of a specific probe, it has never been possible to screen plant species for the presence of an FeSOD gene or its transcript.

Analysis of SOD profiles showed the presence of a chloroplastic FeSOD in both *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*. We attempted to isolate corresponding FeSOD cDNAs by shotgun cloning, based on the principle that a SOD-deficient *E. coli* mutant (1) can regain its ability to grow aerobically on minimal medium when complemented by a heterologous *sod* gene (18–20). Hence, by introducing plasmid-based cDNA expression libraries from *N. plumbaginifolia* and *Ar. thaliana* into this mutant, we were able to isolate cDNAs encoding functionally active FeSOD enzymes from both species,‡ thus showing the general applicability of the method. The availability of these clones should allow the clarification of distribution, importance, and function of FeSOD in plants.

MATERIALS AND METHODS

Protein Extraction and SOD Activity Assays. Proteins were extracted from greenhouse-grown *N. plumbaginifolia* var. P2 and *Ar. thaliana* (L.) Heynh. var. C24 (maintained at 25°C under a 16/8-hr light/dark cycle) as described (21). Chloroplast and mitochondrial fractions were prepared from fresh leaf material by using Percoll gradients as described (21).

Proteins were extracted from *E. coli* by osmotic shock as described by Koshland and Botstein (22) with the addition of two freeze/thaw steps.

Protein samples were separated on native 10% polyacrylamide gels run at 4°C, and SOD activity stains and inhibitor studies were performed directly on the gels as described (21).

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Abbreviation: SOD, superoxide dismutase.

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‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M55909 (*N. plumbaginifolia* FeSOD cDNA) and M55910 (*A. thaliana* FeSOD cDNA)].

Optimal results were obtained when the gels were run at 50 mA for 5–6 hr.

Recombinant DNA Techniques. The construction of the cDNA libraries of *N. plumbaginifolia* and *Ar. thaliana* has been described in detail (23, 24). Standard procedures were used for recombinant DNA manipulations (25). The DNA sequences were determined on both strands by the dideoxy chain-termination method (26).

Electroporation of the cDNA Library and Selection for SOD-Positive Clones. The *E. coli sodA sodB* mutant (QC774) was a gift of D. Touati (Université de Paris, France) and has been described (1). Preparation of mutant *E. coli* cells for electroporation was carried out as described in the Bio-Rad manual for bacterial electrotransformation. The electrotransformation was performed as described in the same manual with some minor changes. One microgram of the cDNA library was mixed with 10^9 *sodA sodB E. coli* cells and electroporated by using the Bio-Rad gene-pulse apparatus and pulse controller. Before plating out on minimal medium, the rich medium was removed from the cells by centrifuging at 15,000 *g* washing the cells with water. Finally, cells were resuspended in water, and 10^8 cells were plated out on minimal medium (27) supplemented with 100 μg of triacillin per ml. Colonies formed after 3 days of growth at 37°C were taken for further analysis.

RESULTS AND DISCUSSION

Profile of SOD Enzymes and Their Subcellular Localization in *N. plumbaginifolia*. By using the staining technique of Beauchamp and Fridovich (28) directly on nondenaturing polyacrylamide protein gels, we have demonstrated (21) the presence of two major bands of SOD activity in protein extracts derived from leaves of *N. plumbaginifolia*. Inhibitor studies with H_2O_2 and KCN, commonly used to distinguish between the different classes of SOD, identified these as a MnSOD (resistant to both H_2O_2 and KCN) and a Cu/ZnSOD (sensitive to both H_2O_2 and KCN).

Subsequent studies with an improved protocol revealed that the band initially identified as a Cu/ZnSOD in fact represents a doublet of two SOD enzymes, one of which copurifies with chloroplast preparations and the other of which does not appear to be associated with any organelle (Fig. 1*A*). The nonorganellar SOD enzyme indeed behaves like a Cu/ZnSOD in inhibitor studies, but the chloroplastic enzyme appears to be an FeSOD (resistant to KCN and sensitive to H_2O_2) (Fig. 1*A*).

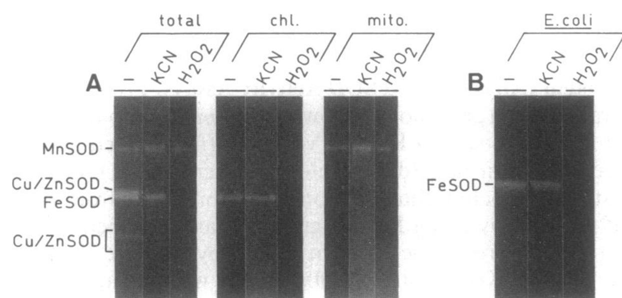

FIG. 1. (*A*) Profile of SOD enzymes in *N. plumbaginifolia*. Protein samples (150 μg of total protein or 75 μg of organellar protein) were separated on native protein gels and stained for SOD activity immediately (lanes –) or after incubation in 2 mM KCN (lanes KCN) or 5 mM H_2O_2 (lanes H_2O_2). The subcellular localization of the SOD isozymes was determined by analyzing chloroplastic (lanes chl.) and mitochondrial (lanes mito.) fractions in addition to total extracts. *N. plumbaginifolia* contains one mitochondrial MnSOD, one chloroplastic FeSOD, and three cytosolic Cu/ZnSODs as indicated. (*B*) Expression of *N. plumbaginifolia* FeSOD in the SOD-deficient *E. coli* mutant and its behavior in the presence of H_2O_2 or KCN. The position of the FeSOD activity is indicated.

In addition, we identified two quickly migrating SOD enzymes that are most likely cytosolic Cu/ZnSODs (Fig. 1*A*). It is possible, however, that one or more of these Cu/ZnSODs is loosely associated with chloroplasts and is lost during our chloroplast purification scheme.

Development of a Method for Isolating Eukaryotic FeSOD Clones. Since there are no obvious DNA probes or antibodies available to screen a cDNA library for FeSOD sequences, we developed an alternative strategy based upon the complementation of a SOD-deficient *E. coli* mutant. *E. coli* normally contains MnSOD (encoded by the *sodA* gene) and FeSOD (the product of the *sodB* gene). In the *sodA sodB* mutant, both genes have been inactivated by insertional mutagenesis, resulting in a complete inability to grow aerobically on minimal medium (1) and in a higher spontaneous mutation rate compared to wild-type cells (29). The *sodA sodB* mutant can regain wild-type characteristics when complemented with a eukaryotic SOD, as has been demonstrated with the human Cu/ZnSOD (18) and with the MnSOD from *N. plumbaginifolia* (19).

Hence, we attempted to transform this *E. coli* mutant with a cDNA expression library and to obtain clones that express an active SOD enzyme by selecting for colony formation on minimal medium. Crucial to the success of such an approach is (*i*) the availability of a highly efficient transformation method and (*ii*) a low frequency of phenotypic reversion. Since conventional $CaCl_2$ or RbCl methods give very low transformation efficiencies for this mutant (data not shown), we decided to use electroporation as an alternative transformation method. By using pLKSODm (19), which is a plasmid designed to express the mature MnSOD protein from *N. plumbaginifolia*, we were able to determine the efficiency of electrotransformation as being between 0.5% and 1.5% when cells were plated out on minimal medium. The reversion frequency was determined by electroporation of plasmid pGem-2 (Promega) into the SOD-deficient *E. coli* strain and by scoring the number of colonies that grew on minimal medium containing triacillin: in a typical experiment, such colonies appeared with a frequency of about 7×10^{-8}.

Isolation of cDNAs Encoding the FeSOD Enzyme of *N. plumbaginifolia* and *Ar. thaliana*. A cDNA expression library derived from mRNA of a *N. plumbaginifolia* heterotrophic cell suspension culture was constructed in plasmid pUC18 (30). Since the cDNAs are inserted into the *lacZ* gene of pUC18, they can be expressed under control of the *lacZ* promoter. One microgram of this library was electroporated in the *sodA sodB* mutant, and 10^8 cells were plated on minimal medium containing 100 μg of triacillin per ml. Sixty-seven colonies appeared after 3 days of growth at 37°C, from which plasmid DNA was prepared and used to retransform the *sodA sodB* mutant. Of these, 51 plasmids were able to restore growth on minimal medium. Restriction enzyme analysis of these plasmids allowed us to distinguish several classes of clones. Protein extracts of representative clones from each class were separated on nondenaturing protein gels and stained for SOD activity. We found 34 cDNAs similar to the MnSOD previously isolated (21) that differed from each other with respect to the 5′ and 3′ ends of the cDNAs. The remaining 17 clones synthesized a SOD enzyme with characteristic properties of an FeSOD (resistant to KCN and sensitive to H_2O_2) (Fig. 1*B*). Sequencing of the cDNA inserts revealed that they were all identical and that the protein was synthesized as a fusion protein initiating at the β-galactosidase ATG of pUC18, which is adjacent to the cloning site (Fig. 2*A*). This would explain why the protein migrates somewhat more slowly in nondenaturing gels in comparison with the endogenous *N. plumbaginifolia* FeSOD (Fig. 1*B*).

FIG. 2. (*A*) Sequence of an FeSOD cDNA from *N. plumbaginifolia*. The nucleotide sequence of a cDNA insert designated pSOD2 is shown beginning at the initiation codon of the *lacZ* gene and includes the flanking G/C homopolymer tails added during the cloning procedure. The predicted amino acid sequence corresponding to the fusion protein, which shows SOD activity in *E. coli*, is written below the nucleotide sequence in the one-letter code. The stop codon is indicated with an asterisk. The first amino acid identifiable from the cDNA insert is marked with an arrow (lysine, position 26). The cDNA insert is 947 nucleotides. (*B*) Sequence of an FeSOD cDNA from *Ar. thaliana*. The sequence of a cDNA insert designated pSOD10 starting from the first discernible nucleotide flanking the G/C homopolymer tail is shown. The amino acid that presumably is the first of the mature protein is indicated with an arrow (asparagine; position 24). The stop codon is marked with an asterisk. The cDNA insert is 969 nucleotides.

The first discernible amino acid encoded by the cDNA is a lysine (residue 26; Fig. 2*A*). Based upon comparison with bacterial FeSOD sequences, this is probably the first amino acid of the mature protein (see Fig. 3). Although fractionation studies demonstrated that the FeSOD is located within the chloroplasts (Fig. 1*A*), we were not able to identify amino-terminal targeting sequences because none of the cDNA clones extended sufficiently upstream from this lysine residue.

In *Ar. thaliana* we also were able to detect chloroplastic FeSOD activity (data not shown). To clone this, an *Arabidopsis* cDNA library in pUC18 (24) was transformed into the *E. coli sodA sodB* mutant, and a complementation assay was performed as described above. All of the SOD-containing clones we isolated by this method were FeSOD clones with dissimilar 5′ and 3′ ends. Most of these cDNAs begin at an asparagine, which presumably is the first amino acid of the mature protein (residue 24; Fig. 2*B*). However, some cDNAs contain 23 additional amino acids at the 5′ end (Fig. 2*B*), which could encode part of a chloroplast transit sequence. The inability to clone MnSOD from the *Arabidopsis* cDNA library can be explained by the low level of MnSOD expression in green material (data not shown).

For unknown reasons, we failed to clone a Cu/ZnSOD either from *N. plumbaginifolia* or *Ar. thaliana* by complementation, even though full-length cDNA clones subsequently have been isolated by conventional procedures (unpublished data). When selection was performed in the presence of 10 μM $CuSO_4$, which was found to increase the activity of human Cu/ZnSOD in *E. coli* (18), this also did not yield any plant Cu/ZnSOD clones. In our view, the most plausible explanation is that the Cu/ZnSOD is not active as a fusion protein linked to the amino terminus of β-galactosidase.

Comparison of the Plant FeSOD Sequences with Those of MnSOD and Prokaryotic FeSODs. Because of their common origin and analogous function, FeSODs and MnSODs are very similar in structure: the metal ligands are conserved between both enzymes as are other structurally or functionally important residues (Fig. 3). Nonetheless, MnSOD and FeSOD sequences are distinguishable, except for some bacterial SODs that are active with both Mn and Fe metal cofactors (32). Parker and Blake (5) have made a list of residues that they considered to be primary and secondary candidates for distinguishing MnSOD and FeSOD sequences, based upon the analysis of several sequences. In their opinion, the two chief residues for distinguishing Fe-

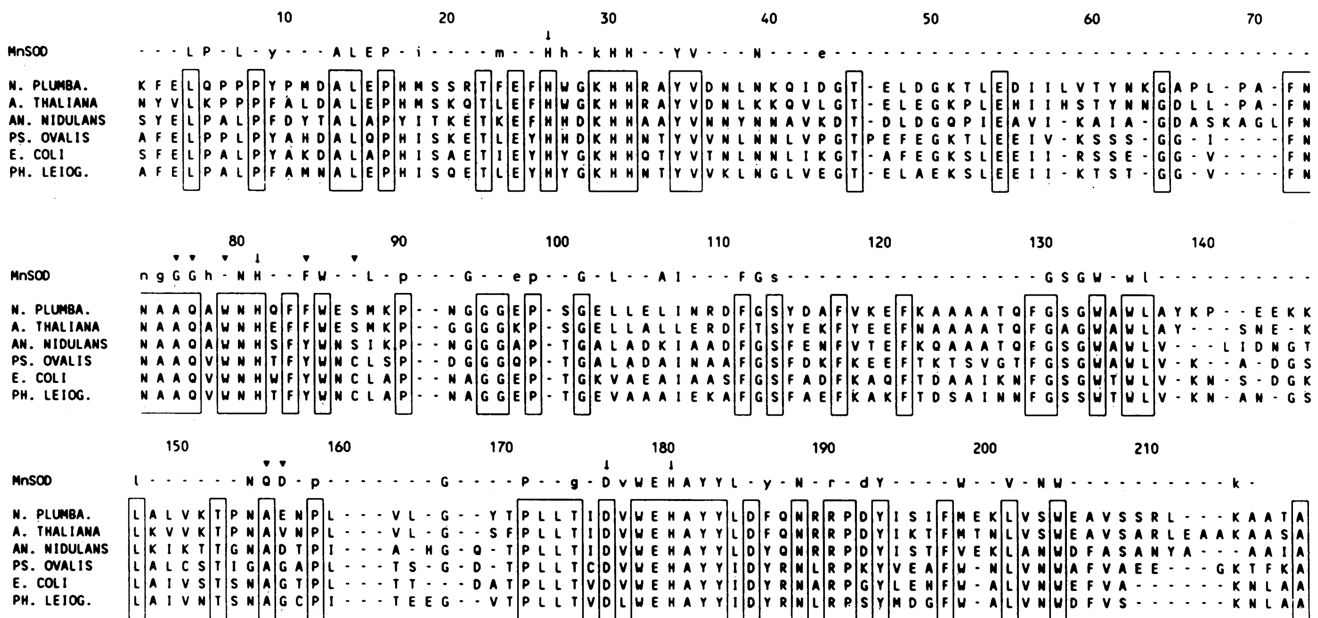

FIG. 3. Comparison between the FeSOD sequences of *N. plumbaginifolia*, *Ar. thaliana*, *An. nidulans* (11), *Ps. ovalis* (9), *E. coli* (8), *Ph. leiognathi* (10), and a MnSOD consensus sequence. The MnSOD consensus sequence is based upon the sequences of *E. coli*, *Thermus thermophilus*, *Bacillus stearothermophilus*, *Saccharomyces cerevisiae*, mouse, human (ref. 5 and references therein), maize (31), and *N. plumbaginifolia* (21). Only the residues that are present in all of the sequences (uppercase letters) or all but one sequence (lowercase letters) are indicated. Residues that are conserved in all of the FeSOD sequences are boxed. In the FeSOD sequences, gaps introduced to obtain maximal homology are indicated by horizontal lines. The metal ligands are conserved between FeSOD and MnSOD and are marked with tailed arrows. The primary candidates for distinguishing FeSOD from MnSOD (according to ref. 5) are indicated with arrowheads.

SOD from MnSOD are residues 77 and 155 (Fig. 3). Tyrosine-34 is believed to interact with glutamine-77 in FeSOD and with glutamine-155 in MnSOD and is thought to be important for catalytic activity (33). Residues 77 and 155 are closely positioned in the tertiary structure of both MnSOD and FeSOD. As a consequence, the MnSOD has small residues at positions 76 and 77 adjacent to glutamine-155, and, conversely, FeSOD has a small residue at position 155. The residues present at these positions within the two plant sequences clearly show the characteristics of FeSOD. Residues 84 (tyrosine), 87 (cysteine), and 156 (glycine) were also proposed to be primary candidates for distinguishing MnSOD from FeSOD (5), but recent sequence data from *An. nidulans* (11) and now from *N. plumbaginifolia* and *Ar. thaliana* are not consistent with this hypothesis (Fig. 3). We propose that, apart from residues 76, 77, and 155, only residue 79 can be of use for distinguishing between FeSOD and MnSOD. In FeSOD, tryptophan-79 is believed to confer H_2O_2 sensitivity (34) and therefore can be considered as specific for FeSOD.

Besides these specific residues, MnSODs and FeSODs should be distinguishable by their overall homologies. In general terms, this appears to be true (Table 1). For example, the plant FeSODs show higher homology to known FeSOD sequences (48–55%) than to MnSODs (38–47%). This difference becomes more pronounced when only eukaryotic SOD sequences are considered—e.g., the MnSOD from *N. plumbaginifolia* is 80% homologous to the maize MnSOD and 59% to the human MnSOD, whereas it has less than 40% conservation with the plant FeSOD sequences. This reveals that in plants, FeSOD has not simply arisen from MnSOD by replacement of the metal cofactor.

Independent gene transfers from prokaryotes also have been suggested to account for the irregular distribution of FeSOD in plants (13). However, the similarity of the plant FeSODs with each other is at least 20% higher than with the prokaryotic FeSODs, thus questioning this hypothesis. We rather believe that FeSOD genes were originally encoded by the chloroplast and at a later stage were transferred to the

Table 1. Pairwise homologies (expressed as percentages) between the different FeSOD sequences together with MnSOD sequences from *E. coli*, *B. stearothermophilus*, human (ref. 5 and references therein), *N. plumbaginifolia* (21), and maize (31)

		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
1. *N. plumba.*	Fe	X	76	55	52	54	51	44	47	43	39	39
2. *Ar. thal.*	Fe		X	53	49	48	49	45	42	40	34	36
3. *An. nidul.*	Fe			X	53	54	51	46	50	40	38	40
4. *Ps. ovalis*	Fe				X	69	68	45	54	44	35	34
5. *E. coli*	Fe					X	74	43	51	42	37	37
6. *Ph. leiog.*	Fe						X	39	51	39	35	34
7. *E. coli*	Mn							X	62	45	41	41
8. *B. stearo.*	Mn								X	51	45	43
9. Human	Mn									X	59	57
10. *N. plumba.*	Mn										X	80
11. Maize	Mn											X

N. plumba., *N. plumbaginifolia*; *Ar. thal.*, *Ar. thaliana*; *An. nidul.*, *An. nidulans*; *Ph. leiog.*, *Ph. leiognathi*; *B. stearo.*, *B. stearothermophilus*.

nucleus, thus accounting for the absence of FeSOD in animal cells. In this respect, it would be interesting to study the FeSOD from *Crithidia fasciculata*, a protozoan, in more detail (35). Because of its presence in a nonphotosynthetic organism, the crithidial SOD could provide more insights into the evolutionary origin of FeSODs.

According to the endosymbiotic theory, cyanobacteria are closely related to the ancestors of modern chloroplasts, which implies that the plant genes of chloroplastic origin should correspond better with cyanobacterial than with bacterial genes. For the FeSOD, this relationship cannot be convincingly deduced from overall homologies, but detailed analysis reveals some conserved regions specific for plant and cyanobacterial sequences that are not present in bacterial sequences. From the current data, we can conclude that nonbacterial FeSODs have the following features in common: an Ala-Ala-Ala-Thr-Gln sequence (residues 124–128), a Gln-Asn-Arg-Arg-Pro-Asp-Tyr-Ile sequence (residues 187–194), and an Ala-Ala-Xaa-Ala sequence at the carboxyl terminus.

Based on SOD activity assays, FeSOD has been reported to be absent from most plant species (13), suggesting that its presence is not obligatory. Although the requirement for an FeSOD is clear in plants that lack any other source of chloroplast SOD, such as *Nuphar luteum* (36), the advantage of possessing FeSOD in addition to a chloroplastic Cu/ZnSOD, as is the case for *Brassica campestris* (37) and *Lycopersicon esculentum* (14, 38), is less obvious. However, the presence of FeSOD in the two plants examined in this paper suggests that it may be ubiquitous in plants but is not expressed constitutively and hence escapes detection. In support of this hypothesis, recent studies of the expression of FeSOD in *N. plumbaginifolia* have revealed that it is highly inducible by environmental conditions (E.W.T.T and C.B., unpublished results). The availability of molecular probes for FeSOD together with the development of a shotgun cloning approach for the isolation of new SOD sequences should allow a much clearer picture of phylogenetic distribution to be obtained.

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