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Characterization of the iron-containing superoxide dismutase and its response to stress in cyanobacterium *Spirulina* (*Arthrospira*) *platensis*

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Abstract Superoxide dismutase (SOD) is considered a primary antioxidant which defends against reactive oxygen species that are induced by environmental stress. In this study, we examined changes in SOD activity and expression in the cyanobacterium *Spirulina* (*Arthrospira*) *platensis* under iron and salinity stress; we characterized its induction under these stress conditions and we overexpressed the enzyme in a bacterial host for preliminary characterization. Analysis of SOD isoforms concludes that *S. platensis* was found to regulate only the iron-containing SOD isoform (FeSOD) in response to two types of stress that were tested. The FeSOD expression (on the level of both mRNA and enzyme activity) was induced by the stress conditions of salinity and iron levels. The FeSOD from *S. platensis* was overexpressed in *Escherichia coli* BL21. The recombinant FeSOD protein (about 23 kDa) was purified for characterization. It showed high specific activity and pH stability at 6.0–9.0, and it is relatively thermostable, retaining 45 % of its activity after 30 min at 90 °C. Phylogenetic analysis reveals that *S. platensis* FeSOD is grouped with the FeSODs from other cyanobacterial species and separated from those of the eukaryotic Chlorophyta, suggesting that the FeSOD gene may be used as a molecular marker in physiological, phylogenetic, and taxonomic studies. This study also suggests that the increased activity and expression of SOD may play a role in algal survival under stress conditions.

Keywords FeSOD expression · Iron-containing superoxide dismutase · Phylogeny · pH stability · Reactive oxygen species (ROS) · Recombinant SOD · *Spirulina platensis* · Thermostability

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Introduction

Cyanobacteria are photosynthetic organisms that may be subjected to various kinds of environmental stresses. Although temperature and light are required for optimum growth of any photosynthetic organism, both respiratory and photosynthetic activity are inhibited by extreme conditions (Torzillo and Vonshak 1994). Environmental stresses are known to enhance the production of the harmful reactive oxygen species (ROS). Unless the concentrations of ROS are controlled, they can damage many cellular targets, including proteins, DNA, and lipids (Padmapriya and Anand 2010). Therefore, multiple antioxidant systems have evolved to control ROS generation and levels, and the tolerance of cells to environmental stress conditions is determined by the effectiveness of these systems. Antioxidants may be enzymatic or non-enzymatic; however, the non-enzymatic antioxidants are not considered as efficient detoxifying agents as the enzymatic set (Wolfe-Simon et al. 2005). Enzymatic antioxidants include catalases, peroxidases, and superoxide dismutases, with the latter considered to be the first line of defense against ROS in most organisms (Bhattacharya et al. 2004). Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anions ($O_2^{\cdot -}$) into hydrogen peroxide and molecular oxygen (Fridovich 1995). Harmful effects of the product hydrogen peroxide are

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alleviated by catalases and peroxidases which decompose hydrogen peroxide into water and molecular oxygen (Regelsberger et al. 2002).

Five SOD isoforms have been identified, varying in their metallic cofactors, including Cu/ZnSOD, MnSOD, FeSOD, NiSOD, and Fe/ZnSOD. Most cyanobacteria have the FeSOD and MnSOD isoforms varying in their metallic cofactors (Li et al. 2002), but the marine cyanobacterium *Synechococcus* sp. has both FeSOD and Cu/ZnSOD (Chadd et al. 1996), whereas *Synechocystis* sp. contains only the FeSOD (sodB; Tichy and Vermaas 1999). The cyanobacterium *Synechococcus* sp. strain PCC7942 has two SODs. The MnSOD is thylakoid-associated, whereas FeSOD is cytosolic (Herbert et al. 1992). Although FeSOD and MnSOD share many attributes (e.g., primary sequence and structural homology) which suggest a common evolutionary trend, they are considered as distinct isoforms because of many differential analysis outcomes. For instance, they showed different antigenic properties; in addition, H_2O_2 was found to inhibit only the FeSOD isoform rather than MnSOD (Grace 1990). They were also hypothesized to have different protective functions within the cyanobacterial cell, i.e., the FeSOD protects against O_2^- formed within the cytosol, whereas the MnSOD protects against O_2^- formed in the thylakoid lumen (Thomas et al. 1998).

The cyanobacterium *Spirulina* (*Arthrospira*) *platensis* is widely distributed and is considered an ideal model system for the investigation of the environmental regulation of oxidative stress responses (Desai and Sivakami 2007) because of its fast growth and ease of laboratory culture and manipulation. Moreover, *Spirulina* spp. are a valuable resource for natural antioxidants, such as phycocyanin pigments, carotenoids, and phenolic compounds in addition to the antioxidant enzymes, superoxide dismutase, catalase, and peroxidase. Consequently, investigations were carried out with the aims of assessment, production, and utilization of these antioxidants under abiotic stresses (Dhiab et al. 2007; Ürek and Tarhan 2012).

Herbert et al. (1992) were the first to propose the protective role of cyanobacterial SOD in photo-oxidative damage. Although SOD activity has been reported under different stresses including light (Kim and Suh 2005), nutrient limitation (Ürek and Tarhan 2012), heavy metals (Choudhary et al. 2007), and salinity (Singh and Kshatriya 2002), the regulation and expression of superoxide dismutase under abiotic stresses still need further examination especially in terms of SOD isoenzymes.

On the other hand, the taxonomy of cyanobacteria has historically been based on their morphology and physiological characteristics. However, the life cycle stage and environmental conditions may influence morphology and make identification difficult especially with those of highly similar species (Teneva et al. 2012). While phylogenetic analysis using 16S rRNA sequences is a standard method to address the relationship between taxa, it may have some shortcomings (Le Roux et al. 2004), and phylogenetic analysis using other genes (e.g., FeSOD) would be alternative methods.

The goals of this study were to (1) investigate FeSOD induction under stress conditions in *S. platensis*, (2) examine the phylogenetic relationship between the type strains based on the FeSOD sequences and confirm the utility of the gene as a molecular marker, and (3) overexpress the enzyme in a bacterial host for preliminary characterization.

Materials and methods

Spirulina platensis (Gomont) Geitler MIYE 101 (currently regarded as a taxonomic synonym of *Arthrospira platensis* Gomont) was obtained from the Phycology Lab, Faculty of Science, Zagazig University, Egypt. Voucher specimens have been deposited in the Faculty of Science of Zagazig University. Culturing of *S. platensis* MIYE 101 was performed according to Zarrouk (1966). The stress concentrations were chosen following a preliminary screening and included the addition to the growth medium of 0.035 mM (control) up to 1 mM Fe, ($FeSO_4$-chelated, in a molar ratio 1:2 of Fe: Na_2-EDTA; Estevez et al. 2001) and 17 mM (control) to 340 mM of NaCl. Following inoculation with a mid log phase culture (ca. 0.7 mg dry weight), the culture flasks were incubated at 31±0.5 °C while illuminated with continuous fluorescent light (Duro-Test 20 W Vita-Lite) at 60 μmol photons m^{-2} s^{-1} for 14 days. Cells were harvested by centrifugation (at 10,000 rpm for 10 min at 4 °C, Sorvall Legend X1R, Thermo Scientific) and washed with 10 mM Na_2-EDTA and twice with distilled water. The biomass yield (DW) was determined by placing the harvested pellets in clean crucibles and drying at 105 °C until a constant weight was achieved (APHA 1985). After harvest, algal pellets were homogenized with an equal volume of glass beads (0.45–0.50 mm in diameter) in 50 mM phosphate buffer (pH 7.0), 1 % polyvinylpyrrolidone, 0.5 % Triton X-100, and 1 mM Na_2-EDTA at 4 °C. After removal of cell debris by centrifugation, the supernatant was assayed for SOD activity (Beauchamp and Fridovich 1971). One unit of SOD activity is defined as the amount that causes 50 % inhibition in the rate of NBT reduction measured at 560 nm.

Cloning and expression of FeSOD

The total RNA from *S. platensis* MIYE 101 was extracted using Trizol® (Invitrogen, USA), treated with DNase I (Invitrogen) and checked by 1 % agarose gel electrophoresis. The RNA was reverse-transcribed to cDNA using the RevertAidTM H Minus First Strand cDNA synthesis kit (Fermentas, USA). A cDNA fragment encoding a portion of FeSOD was isolated by PCR amplification of the cDNA

library (Guo et al. 2004; acc. no. AY282414). The DNA sequence was confirmed using BigDye v. 3.1 as previously described)Doering and Piercey-Normore 2009), and the fragment was then used to isolate the full-length FeSOD using the 5′ RACE System (Version 2.0 for Rapid Amplification of cDNA Ends kit, Invitrogen) and 3′ RACE (First Choice RLM-RACE kit, Ambion, USA). The coding region of the FeSOD was amplified in 20-μL reaction mixtures containing primers SPI_SOD_F and SPI_SOD_R (0.4 μM of each primer, designed to have the *Bam*HI and *Hind*III sites, respectively; Table 1), 1X supplied PCR buffer, 200 μM of each dNTPs, 2 mM $MgCl_2$, 350 ng cDNA template, 1 U *Taq* DNA polymerase (Invitrogen), and the following PCR thermal cycle: 94 °C for 4 min followed by 35 cycles of 94 °C for 50 s, 58 °C for 60 s, and 72 °C for 60 s, followed by 1 cycle of 72 °C for 10 min. The PCR product (633 bp, Fig. S1) was gel-purified and then cloned into the expression vector pET28c (Novagen, USA). The sequences were verified for another time before transformation into *E. coli* BL21 (DE3) pLysS cells (Novagen, USA). Finally, FeSOD protein was expressed in the *E. coli* cells induced at OD_{600}=0.6 with the addition of 25 μM isopropyl-β-*D*-thiogalactopyranoside (IPTG), followed by incubation for 15 h at 28 °C. The expressed protein was purified by affinity chromatography on a HiTrap HP column (GE Healthcare) and its expression and purity confirmed on 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). All the procedures were done according to the manufacturers' instructions. The DNA manipulations were carried out by using standard procedures (Sambrook et al. 1989).

Phylogenetic analysis

The phylogenetic tree of selected FeSOD sequences was constructed by maximum parsimony methods implemented in PAUP 4.0 (Swofford 2003) after alignment using ClustalX (Jeanmougin et al. 1998). Maximum parsimony was performed using the tree bisection and reconnection branch swapping option, 1,000 random heuristic searches, and 500 bootstrap replicates (Felsenstein 1985). Bootstrap was done using the MP option in PAUP and values greater than 70 are reported in the phylogenies.

Quantification of FeSOD gene expression by RT-PCR

The FeSOD mRNA expression by the *S. platensis* MIYE 101 under the different iron and salt stress conditions was measured by quantitative real-time RT-PCR. As described earlier, total RNA was extracted, treated with DNase I, and reverse-transcribed into cDNA (RevertAid^{TM} H Minus First Strand cDNA synthesis kit, Fermentas, USA). RT-PCR assays were performed in a 20-μL mixture which contained 1X SYBR Green Master Mix (iQTM SYBR® Green Supermix, Bio-Rad, USA), 75 nM of each primer (SgF2 and SPD_FESOD-R, product size 338 bp; Table 1), and the corresponding cDNA (transcribed from 100 ng total RNA). PCR amplification was carried out in an MJ Mini™ thermal cycler equipped with an MJ Mini-Opticon RT-PCR detector (Bio-Rad). The thermal cycling profile was 95 °C for 4 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Single-product PCR was verified by performing a melting curve analysis as well as gel electrophoresis and ethidium bromide/UV visualization of the final RT-PCR product. DEPC water was used as negative control by replacement of the template. The intensity of SYBR Green dye fluorescence was measured, and the relative ratios were normalized with the value of 16S rRNA (primers RTrrn16sF and RTrrn16sR, product size 222 bp; Table 1) and represented as means of data from 12 replicates (four biological and three technical) for each sample . The relative levels of gene expression were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Table 1 List of oligonucleotide primers used in this study

Reaction	Primer	Sequence (5′ to 3′)	Reference
cDNA synthesis & 3′ RACE	SPD_FESOD-F	GAGGATTTGACACGATGGCATTTG	Guo et al. 2004
cDNA synthesis, 5′ RACE & RT-PCR	SPD_FESOD-R	*CCAGA*TAGTAGGCGT*G*TTCCCAAAC	Guo et al. 2004, changed nucleotides italicized
3′ RACE	SgF1	TGGCTGATATGTCCCTGGAAG	This study
3′ RACE	SgR1	GCTCCTGTAGGTTGACCACCA	This study
3′ RACE & RT-PCR assay	SgF2	CAAAGCCACTTACAATGATCCT	This study
5′ RACE	SgR2	CAAAGCCACTTACAATGATCCT	This study
ORF cloning/overexpression	SPI_SOD_F	AGAGGAGAAAACA*GGATCC*CTTTTGAACTTCC	This study, *Bam*HIsite italicized
ORF cloning/overexpression	SPI_SOD_R	AGCAGACGCGA*AAGCTT*AAGCAGACGCGAG	This study, *Hind*III site italicized
Reference gene (RT-PCR)	RTrrn16sF	CAGCTCGTGTCGTGAGATGT	Ohmori et al. 2009
Reference gene (RT-PCR)	RTrrn16sR	TTACGGGATTGGCTCAGACT	Ohmori et al. 2009

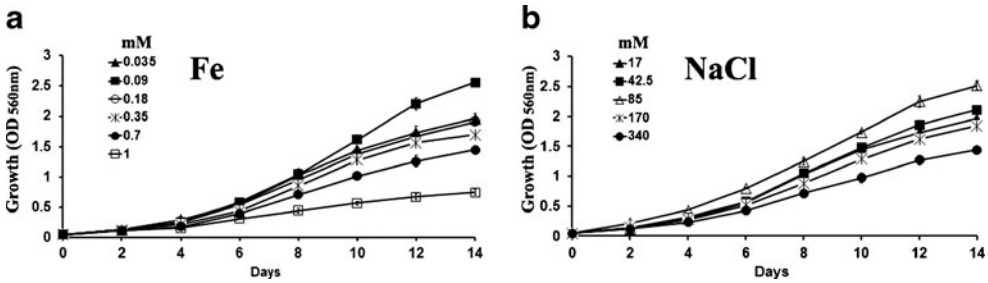

Fig. 1 Growth of *S. platensis* under different iron (**a**) and salinity (**b**) stresses. The control medium contains the standard ion concentrations (0.035 mM Fe and 17 mM NaCl). Data are the mean ± SE; n=3

Characterization of the *S. platensis* SOD

In order to (1) analyze the FeSOD activity (either native or recombinant), (2) check for any additional SOD isoform presumable expression, and (3) perform SOD isoform differentiation analysis, non-denaturing (native) PAGE was used. Accordingly, crude extracts and purified FeSOD protein were fractionated by non-denaturing PAGE prior to staining with riboflavin–nitro blue tetrazolium (NBT) (Beauchamp and Fridovich 1971). To differentiate among the SOD isoforms, the samples were incubated with 5 mM H_2O_2 for 30 m to inactivate both Cu/ZnSOD and FeSOD or with 3 mM KCN to inactivate Cu/ZnSOD. MnSOD is unaffected by either treatment (Butow et al. 1997).

Biochemical properties of the recombinant FeSOD

The optimum pH for the FeSOD activity was determined by assaying the purified enzyme (2 U) directly in 50 mM citrate buffer (pH 3.0, 4.0, 5.0, and 6.0), 50 mM phosphate buffer (pH 7.0), 50 mM Tris HCI buffer (pH 8.0 and 9.0), and 50 mM glycine–NaOH buffer (pH 10.0 and 11.0) (Beauchamp and Fridovich 1971). The pH stability of the FeSOD was determined by incubating the FeSOD (2 U) at different pH values for 1 h prior to assay. The thermal stability of the FeSOD was determined by incubating the enzyme (2 U) for varying times up to 1 h at 50, 70, 90, and 100 °C in 50 mM phosphate buffer, pH 7.8, prior to assay. The enzyme activity was calculated as a percentage of that of FeSOD activity under the standard assay conditions.

Statistical analysis

The data are presented as mean ± standard error (SE) of at least three independent experiments. All of the statistical analyses were carried out using SPSS 10.0 software (SPSS, USA) as described by Dytham (1999). One-way analysis of variance (ANOVA) with Duncan's multiple-range tests was used for comparison of the significance level between values at P <0.05. Different uppercase letters above the bars, within each assay, are significantly different.

Results

Effect of iron and salinity stress on the growth of *S. platensis* and expression of FeSOD

The growth of *S. platensis* MIYE 101 challenged with different iron and salinity conditions was optimal at 0.09 mM Fe and 85 mM NaCl (Figs. 1 and 2) and slowest at 1.0 mM Fe and 340 mM NaCl. The fact that growth is enhanced by the addition of up to 0.09 mM Fe and 85 mM of NaCl is reflection of the need for certain minimal levels of Fe and Na ions as either enzyme cofactors or to establish optimal charge gradients across the membrane. The levels of SOD (shown to be

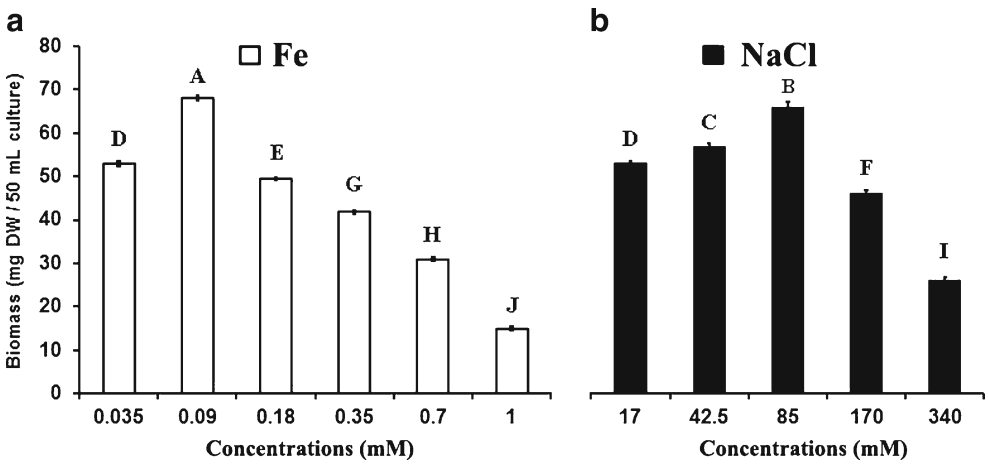

Fig. 2 **a**, **b** Biomass productivity of *S. platensis* under different iron and salinity stresses after the 14th day. *Bars* are mean ± SE; n=3. *Different letters* represent significant differences at P<0.05 (Duncan's)

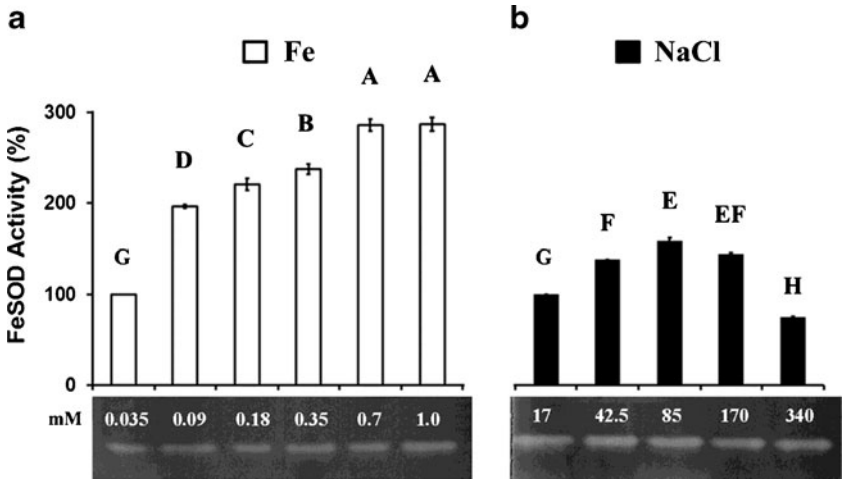

Fig. 3 FeSOD activity of *S. platensis* under different iron (**a**) and salinity (**b**) stresses. *Bars* are mean ± SE, $n=3$, which were normalized to the control value (activity at normal ion concentrations in the medium, 0.035 mM Fe and 17 mM NaCl, set at 100). *Different letters* represent significant differences at $P<0.05$ (Duncan's). FeSOD activity stained gel (12 %); each *lane* was loaded with 30 μg protein extract of *S. platensis*

FeSOD later) were increased threefold with increasing Fe and 2.5-fold with increasing NaCl (Fig. 3). Transcription of the FeSOD gene also increased with increasing Fe (fourfold increase) and NaCl (fivefold increase) (Fig. 4). Both maximum activity and maximum transcript expression occurred at 1.0 mM Fe and 85 mM NaCl.

Cloning, characterization, and phylogenetic analysis of the FeSOD from *S. platensis*

A cDNA fragment (633 bp) containing the *S. platensis* MIYE 101 FeSOD gene was cloned into pET28c vector and sequenced, revealing a 603-bp open reading frame encoding a 201 amino acid protein (accession number KC999472) with a mass of about 23 kDa. When this study was initiated, there were no other full-length FeSOD genes available, and the partial fragments lacked a large part of the N- and C-terminals. A more recent GenBank search revealed full-length sequences from *Spirulina* species (accession numbers EKD08081, EKN80783, and EDZ93736), all 201 amino acids in length. A sequence alignment was done to these sequences (data not shown), and it was found that these sequences showed complete similarity (100 %) to the FeSOD gene of *S. platensis* MIYE 101 isolated by this study. The sequence of the *S. platensis* MIYE 101 FeSOD gene was compared to other FeSOD genes to generate a phylogenetic tree (Fig. 5) in which the cyanobacterial FeSOD gene formed a clade with 91 % bootstrap support. The phylogenetic position of *Nostoc* and *Anabaena* supports their relatedness. Furthermore, FeSOD gene forms two clades for the investigated Cyanobacteria and Chlorophyta, which reflects the large taxonomic divergence and also the essential conservation of the gene.

The deduced amino acid sequence of *S. platensis* FeSOD was further aligned with other 18 highly similar FeSODs (Supplemental Fig. S3 and Table S1). The *S. platensis* MIYE 101 FeSOD showed high similarity at 79 and 78 % to that of *Lyngbya* sp. PCC 8106 (ZP_01619231) and *Oscillatoria* sp. PCC 6506 (ZP_07113911), respectively. We found several highly conserved amino acid residues among the FeSODs, which may be involved in ligand binding and/or the catalytic mechanism. In *S. platensis* FeSOD, these are Pro-

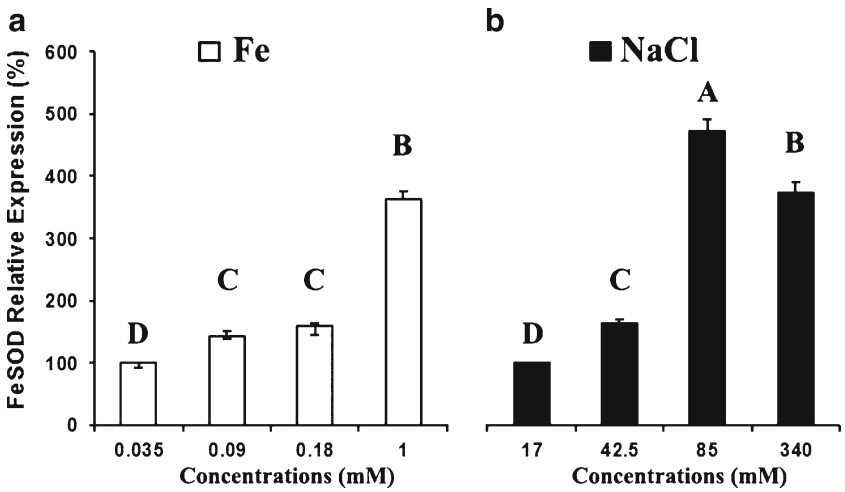

Fig. 4 Relative expression of FeSOD transcript of *S. platensis* under different iron (**a**) and salinity (**b**) stresses. *Bars* are mean ± SE, $n=12$, which were normalized to the control value (activity at normal ion concentrations in the medium, 0.035 mM Fe and 17 mM NaCl, set at 100). *Different letters* within each parameter represent significant differences at $P<0.05$ (Duncan's)

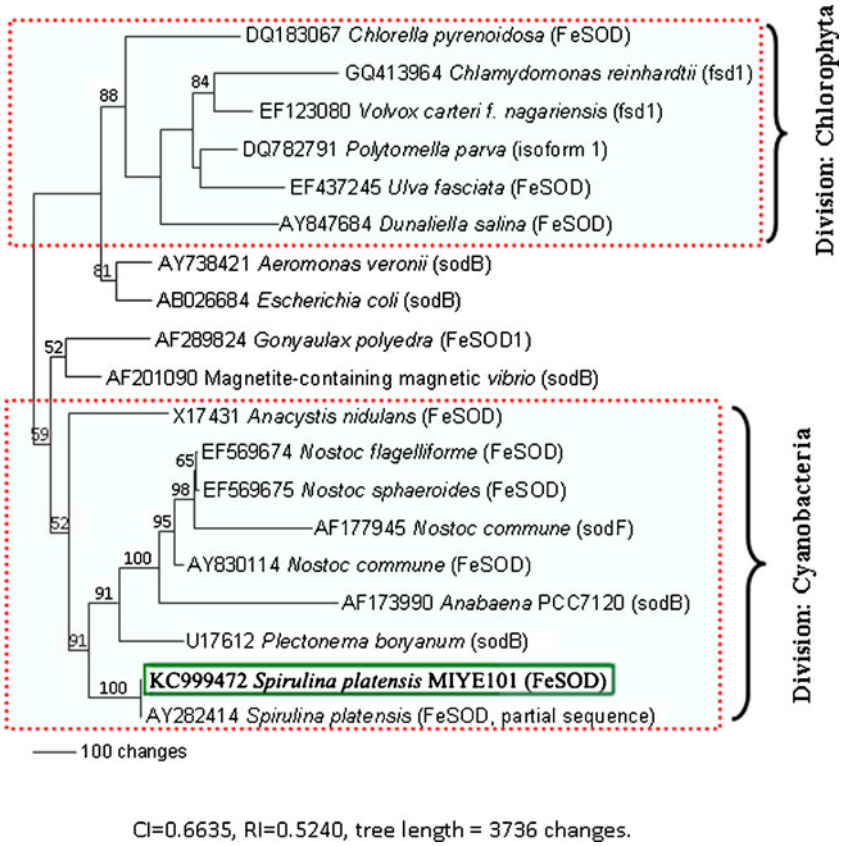

Fig. 5 Phylogenetic tree for FeSOD nucleotide sequences. The tree is midpoint-rooted, and bootstrap values >50 % are shown *above* the branches' points. NCBI GenBank accession numbers precede the name of each species

9, Leu-15, His-28, Lys-31, His-32, His-33, Tyr-36, Val-37, Leu-45, Thr-47, Glu-55, Glu-56, Asn-73, Phe-83, Gly-93, Gly-94, Phe-110, and Val-193 (Supplemental Fig. S3).

Expression, purification, and characterization of recombinant FeSOD

The *S. platensis* MIYE 101 FeSOD clone was expressed in *E. coli* BL21 (DE3), and an excellent yield of FeSOD protein was realized in response to 25 μM IPTG at 28 °C for 15 h. The protein was purified to homogeneity by Ni-affinity chromatography, revealing a protein with the expected mass of ~23 kDa (Fig. 6a). The *S. platensis* FeSOD was insensitive to the KCN at 3 mM but inhibited by 5 mM H_2O_2 (Fig. 6b). It retained more than 60 % of maximal activity over a relatively broad pH range from 6 to 9 even after prolonged incubation at those pH values (Fig. 7). The FeSOD was rapidly inactivated at 100 °C but retained 82 % activity after 1 h at 50 °C (Fig. 8). Surprisingly, it retained 45 % activity after incubation at 90 °C for 30 min.

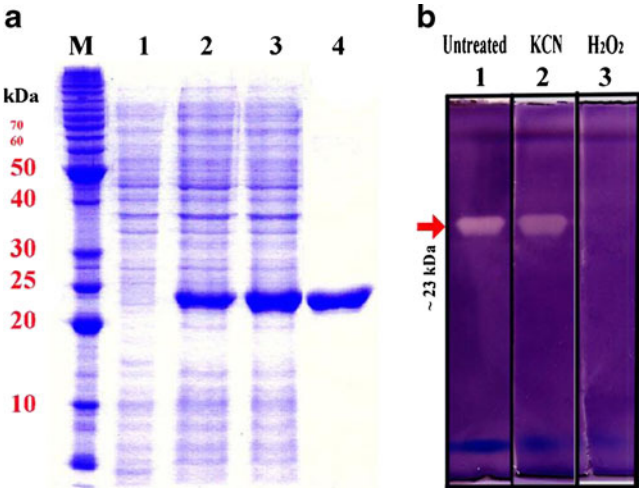

Fig. 6 **a** Expression of recombinant *S. platensis* FeSOD in *E. coli* BL21 (DE3) pLysS. Proteins (10 μg/lane) were analyzed by SDS-PAGE (12 %) and stained with Coomassie® Brilliant Blue R-250. *M* molecular weight size markers (Invitrogen, kDa); *lane 1*, uninduced *E. coli* cells lysate; *lane 2*, 25 μM IPTG-induced cells lysate; *lane 3*, 1 mM IPTG induced cells lysate; after incubation at 28ºC for 15 h, *lane 4* represents the purified FeSOD recombinant protein (5 μg/lane) by HiTrap™ ion exchange column filtration. **b** Inhibitor profile by native-PAGE stained gel (12 %) of the purified recombinant FeSOD of *S. platensis* enzyme activity (2 μg/lane). The enzyme activity was significantly inhibited by 5 mM H_2O_2 (lane 3) but not inhibited by 3 mM KCN (lane 2). Control contained FeSOD without any inhibitor

Discussion

The wide distribution of Cyanobacteria in different environments may suggest that they are susceptible to stress factors

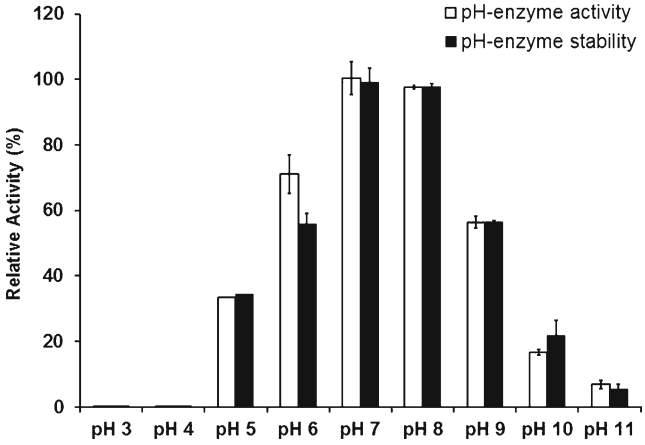

Fig. 7 Effect of pH on activity and stability of recombinant *S. platensis* FeSOD. The relative activity (%) was calculated referring to the activity of the standard activity conditions (mean ± SE, n=3)

such as extreme conditions of light, temperature, heavy metals, and salinity. Although iron is needed for the growth of all phytoplanktons as it serves essential metabolic functions in the photosynthetic electron transport, respiratory electron transport, nitrate and nitrite reduction, sulphate reduction, dinitrogen (N_2) fixation, and detoxification of reactive oxygen species (e.g., superoxide radicals and hydrogen peroxide) (Sunda and Huntsman 1997), higher concentrations may be harmful. The growth of *Microcystis aeruginosa*, for example, was limited by iron below 12.3 μM and inhibited when the iron concentration was 24.6 μM (Wang et al. 2010). Salinity stress causes an imbalance of the cellular ions, resulting in ion toxicity and osmotic stress, leading to retardation of growth either directly by salt or indirectly by oxidative stress induced by ROS (Shalaby et al. 2010). The decrease in growth with increasing salinity has been recorded (Deniz et al. 2011). Growth of *Spirulina* sp. and chlorophyll *a* content exhibited decreases at most exposure levels of sodium chloride, i.e., from 200 mM (Deniz et al. 2011) up to 1.0 M (Vonshak et al. 1988). By contrast, Dhiab et al. (2007) reported the

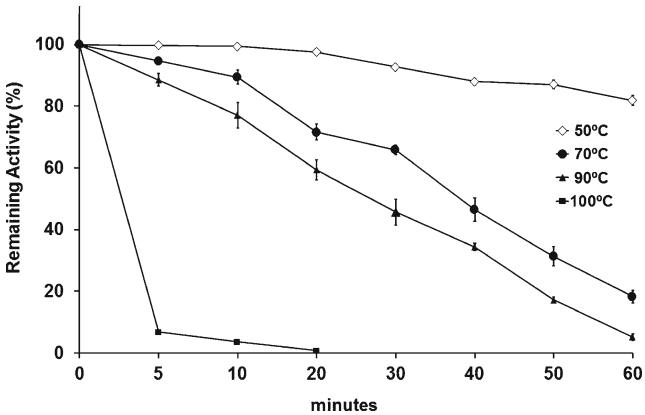

Fig. 8 Thermostability of recombinant FeSOD of *S. platensis*. Data are mean ± SE, n=3

enhancement of chlorophyll *a* content of *S. platensis* by salt concentrations (17–500 mM NaCl). They explained their contradictory results by genetic and environmental factors of different strains of *S. platensis*, which may use different strategies to respond to salt stress.

The protective mechanisms of *S. platensis* against heavy metals and salinity stresses have been reported previously. These include the effects on the growth, pigment content, proline, malondialdehyde, photosynthetic activity, and antioxidant enzyme activity (Choudhary et al. 2007; Dhiab et al. 2007); however, these studies disregard the expression levels of the SOD isoforms under these conditions. In this study, the analysis of FeSOD activities under the described stresses was performed using two methods, the non-denaturing PAGE analysis and the direct NBT method assay (Fig. 3). The purpose of the non-denaturing PAGE is to examine any presumable expression of other SOD isoform activity under the described stresses and to examine their individual activity (by susceptibility tests to KCN and H_2O_2; data not shown). The possibility that there is one or more of SOD isoform expressions under stress conditions cannot be predicted. For instance, *E. coli* constitutively produces FeSOD but expresses an additional MnSOD only under aerobic conditions (Gregory and Fridovich 1973). Furthermore, gel analysis suggested that additional isoenzymes of MnSODs of *Haematococcus pluvialis* (green alga) were induced or up-regulated under stress conditions (high light, high levels of iron and sodium acetate; Wang et al. 2011).

In this study, *S. platensis* MIYE 101 regulated the FeSOD isoform only, and there was no induction of any other SOD isoform under the tested stresses. Moreover, the stress conditions induced elevated levels of FeSOD gene transcription and enzymatic activity (Figs. 3 and 4) especially with Fe concentrations (0.09–1 mM), which may be due to the necessity of iron for optimal FeSOD activity (Campbell and Laudenbach 1995). This finding may vary according to the species tested; for example, Ürek and Tarhan (2012) reported that SOD activities of *S. maxima* reached their maximum at 0.036 mM Fe, whereas the maximum SOD activity of *Anabaena variabilis* was recorded at 0.02 mM Fe (Padmapriya and Anand 2010).

The decreased FeSOD activity found at high salinity (340 mM NaCl) may be due to the collapse of intracellular defense against the increased toxic oxygen radicals. This failure of the intracellular defense mechanism imposed reduction effects on all cellular enzyme activities including SOD (Singh and Kshatriya 2002). Thus, FeSOD gene expression in *S. platensis* is part of a generalized stress response that can mitigate the effects and protect against a broad range of stresses. Because multiple stresses are often experienced simultaneously, this imparts not only a level of safety and efficiency but also a level of inefficiency if, for example, elevated SOD is not required.

The ORF sequence of the obtained FeSOD gene in the NCBI Genbank database showed that the deduced amino acid sequences in this study have complete similarity (100 %) with SOD sequences of other strains namely *A. platensis* C1 (acc. no. EKD08081), *A. platensis* str. Paraca (EKN80783), and *A. maxima* CS-328 (EDZ93736), but these sequences were submitted as SOD and do not confirm that the isoform is FeSOD. Our results showed that the SOD sequences of these *Spirulina* (*Arthrospira*) species belong to the FeSOD isoform. It also showed a high degree of sequence similarity with other cyanobacterial SOD genes which group closely in a phylogenetic analysis (Fig. 5; Supplemental Fig. S3 and Table S1). The comparison of homologous sequences allows the functional relationships among proteins to be determined in addition to their predicted structure. Indeed a majority of freshwater Cyanobacteria express just one FeSOD, while most marine Cyanobacteria also encode a single SOD with 30–40 % identity and 50–60 % similarity to NiSOD of a *Streptomyces* species (Youn et al. 1996). Because many organisms encode multiple SODs, the presence of FeSOD alone in *S. platensis* suggests that it may be expressed very efficiently or, alternatively, that superoxide has not been a serious challenge during *S. platensis* evolution. The homology of FeSOD gene sequences of the species in this study confirms the conservation of the gene and supports previous conclusions (based on other genes) that Cyanobacteria are monophyletic (Zhaxybayeva et al. 2006). This homology may be useful for construction of degenerate primers (for the purpose of isolation of other SODs) or to provide an advantage for the FeSOD gene to be used as a molecular marker in physiological, phylogenetic, and taxonomic studies (together with 16S rRNA sequences). For instance, *Anabaena* PCC7120 formed a 100 % sub-clade with the other investigated *Nostoc* species (Fig. 5). This may reveal the phylogenetic distance between these species such as the separation of *Nostoc* and *Anabaena* into two distinct genera which has been controversial. The 16S rRNA studies have emphasized their close relatedness (Teneva et al. 2012). Therefore, additional analyses are required to clarify the phylogenetic relationships among Cyanobacteria. The separation of *S. platensis* from other Cyanobacteria may demonstrate that its different taxonomic position or the divergence of the Cyanobacteria and the Chlorophyta based on FeSOD gene may indicate the gene divergence rather than the taxonomic divergence. It may suggest that these groups of FeSOD genes are of bacterial origin which originated from two independent horizontal gene transfer events (Dufernez et al. 2008).

The successful expression of the *S. platensis* MIYE 101 FeSOD in an *E. coli* host has made possible an initial partial characterization of the recombinant enzyme. In particular, its pH and thermal stabilities are similar to those previously reported for native FeSOD from *S. platensis* and for MnSOD from *Rhodopseudomonas spheroids* (Lumsden et al. 1976; Desai and Sivakami 2007). In addition, these properties are consistent with the general observation that FeSOD activities are optimum at slightly alkaline pH (Aydemir and Tarhan 2001). Grace (1990) showed that the optimum catalytic rates of FeSOD and MnSOD fall within a pH range of 6.0–8.0. However, Cu/ZnSOD has a broader pH range of 5.2–9.5. This may be related to their metal cofactor coordination in the active site of the enzyme. Reduction in total SOD activity may occur when the cyanobacterium *Synechocystis* PCC 6803 was grown for 24 h at 43 °C (El-Sheekh and Rady 1995), while the purified FeSOD of the thermophilic cyanobacterium *Thermosynechococcus elongatus* showed increased activity at 50 °C. The increased activity is expected in thermophilic species (Kerfeld et al. 2003), yet to the best of our knowledge, there is little information available on the thermostability of the cyanobacterial FeSOD. Nevertheless, the thermostability of SOD has been investigated in many other species including the aquatic plant *Hydrilla verticillata* at 45 °C (Panda and Khan 2004). In higher temperatures, activity falls as reported for many terrestrial plants, potentially due to the heat denaturation of the enzyme (Panda and Khan 2004). The endurance of SOD under several stress conditions (i.e., pH and heat) gives it an advantage for further application as a powerful antioxidant.

Our results (PAGE analysis) showed that the recombinant FeSOD has a size of about 23 kDa (Fig. 6a) with a high specific activity (530 U mg^{-1}, in vitro assay). Our findings also provide additional support to previously published literature; for example, Priya et al. (2007) reported that the amino acid range in cyanobacterial FeSOD is 199–229 residues with a molecular weight of 21–25 KDa; Li et al. (2002) reported that the purified native FeSOD of the Cyanobacterium *Anabaena* sp. strain PCC 7120 has a molecular mass of 22.3 kDa; Bhattacharya et al. (2004) reported that the recombinant SOD from the cyanobacterium *Synechocystis* sp. PCC 6803 has a size of about 22 kDa; and Xia et al. (2003) have purified the native FeSOD from *S. platensis* which has a size of 21 kDa. However, contradictory observations have also been recorded. Lumsden et al. (1976) reported that the *S. platensis* FeSOD has a molecular weight of about 37 kDa, which is composed of two non-covalently joined subunits of equal size. Desai and Sivakami (2007) reported that the purified SOD from *S. platensis* appeared to be a homodimer with a molecular weight of 30 kDa consisting of two 15-kDa protein subunits, with a specific activity of 290 U mg^{-1} protein. If this particular enzyme were to be exploited for commercial applications, a more extensive characterization would be necessary.

In conclusion, this study has shown that FeSOD expression in *S. platensis* MIYE 101 is controlled as part of a generalized stress response that includes iron and salinity induction as well as predicted oxidative stress induction. The overexpression and partial characterization of the *S. platensis* MIYE 101 FeSOD is also reported. The current study suggests (based

on the FeSOD gene) that cyanobacterial taxa are monophyletic. FeSOD could be used as a molecular marker for many scientific investigations; in addition, its high activity and stability give it an advantage (as a powerful antioxidant) for many applications.

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