Two distinct manganese-containing superoxide dismutase genes in *Bacillus cereus*: their physiological characterizations and roles in surviving in wheat rhizosphere

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Bacillus cereus; superoxide dismutase; colonization.

Abstract

Rhizosphere inhabitants interact intricately with plant host. *Bacillus cereus* 905 isolated from wheat rhizosphere colonized wheat rhizosphere with large population size. In this work, the role of superoxide dismutases (SODs) of *B. cereus* 905 in surviving in wheat rhizosphere was analyzed. Two genes, *sod*A-1 and *sod*A-2 encoding two distinct manganese SODs (MnSODs), were isolated from the bacterium. The amino acid sequence similarity between the two peptides is 58.43%. Through homologous recombination, three mutant strains have been created, each lacking either *sod*A-1, *sod*A-2 or both. Analysis of these mutant strains revealed differences in transcription and enzymatic activity of SOD. MnSOD2, encoded by *sod*A-2, plays a more important role in antioxidative stress. MnSOD1, the product of *sod*A-1 gene, is expressed at lower level. The function of the two MnSODs appears to be essential in colonization of wheat rhizosphere.

Introduction

Plant rhizosphere-colonizing bacteria potentially promote growth of host plants and have been used to develop biopesticides and/or biofertilizer (Whipps, 2001). *Bacillus cereus* is a ubiquitous facultative anaerobic bacterium. It prefers oxygen as the terminal electron acceptor during respiration to effectively produce ATP (Bruce & Amábile-Cuevas, 1991). This aerobiosis often generates toxic reactive oxygen species (ROS) as by-products that include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^\bullet$OH). Superoxide anion, formed when oxygen receives an electron, may cause the release of Fe^{2+} from enzymes that contain 4Fe-4S clusters (Halliwell & Gutteridge, 1986). Subsequently, the released Fe^{2+} may lead to the generation of $^\bullet$OH through Fenton reaction (Halliwell & Gutteridge, 1986; Imlay & Linn, 1988; Keyer & Imlay, 1996). One of the 4Fe-4S-containing enzymes, 6-phosphogluconate dehydratase, is essential in the Entner–Deudenoff pathway (Gardner & Fridovich, 1991a). Other 4Fe-4S-containing enzymes include aconitase and fumarase of the TCA cycle, dihydroxyacid dehydratase of branched amino acid biosynthesis pathway (Woods *et al.*, 1988; Gardner & Fridovich, 1991b; Flint *et al.*, 1993). Inactivation of these enzymes results in growth inhibition. Hydroxyl radical may cause lethal damage in DNA and cell membrane. Mechanism scavenging O_2^- to eliminate the toxicity is required by aerobic organisms.

Superoxide dismutases (SODs, EC 1.15.1.1) specifically catalyze the conversion of O_2^- to H_2O_2 and O_2. Since the first discovery of these metalloenzymes in 1969 (Fridovich, 1975), SODs have been considered as key enzymes defending against oxidative stress. According to their metal cofactors, metalloenzymes are classified into manganese SOD (MnSOD), iron SOD (FeSOD) and copper zinc SOD (Cu/ZnSOD) (Fridovich, 1995). All three SODs exist in *Escherichia coli* and function at different locations. Both FeSOD and MnSOD are present in the cytoplasm while Cu/ZnSOD is located in the periplasm (Benov *et al.*, 1995; Fridovich, 1995). The *sod*B-encoded FeSOD is thought to play a house-keeping role because of its constitutive expression. The expression of *sod*A-encoded MnSOD fluctuates in different growth phase responsive to internal O_2^- levels. The *sod*C-encoded Cu/ZnSOD is expressed in a minimal level to protect the periplasmic and membrane constituents from exogenous superoxide (Benov et al., 1995). A single copy for each gene has been found in *E. coli* genome. Double mutant of *sod*A and *sod*B in *E. coli* is highly sensitive to internal

O_2^- generation, exhibiting a growth arrest on the medium containing 50 μM paraquat (Carlioz & Touati, 1986). Similarly, *Pseudomons putida* double mutant of *sod*A and *sod*B is impaired in growth on root surfaces, being more sensitive to oxidative stress than wild type (Young *et al.*, 2000).

*sod*A is a single-copy gene in majority of bacteria (Carlioz & Touati, 1986; Sanders *et al.*, 1995; Takashi *et al.*, 1998; Young *et al.*, 2000; Ryan & Scandalios, 2002). Although the authors have previously identified two different MnSODs in *B. cereus* 905 crude extract by chemical inhibition methods (Droillard *et al.*, 1989), the role of the two *sod*A in *B. cereus* 905 is unclear. In this paper, two *sod*A genes were identified and their roles in defense against oxidative stress in *B. cereus* was elucidated. Since *B. cereus* 905 is able to colonize the oxic or microoxic habitat 'wheat rhizosphere' in large quantity where also the plant may actively contribute to the production of active oxygen species, the regulation and roles of these two *sod*A genes in survival in the wheat rhizosphere were investigated.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and vectors used in this study are listed in Table 1. *Bacillus cereus* 905 was isolated from wheat rhizosphere and stored in $-80\,^{\circ}C$.

Bacillus cereus and *E. coli* were grown aerobically in Luria–Bertani (LB) broth (Sambrook *et al.*, 1989) at 32 and at 37 °C, respectively. Minimal medium [50 mM K_2HPO_4, 50 mM KH_2PO_4, 2.5 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$] supplemented with different carbon source was used as a growth medium in some of the experiments. Wheat root wash medium was prepared using modified methods of previous publication (Albert *et al.*, 1986). Briefly, 100 g of 20-day-old intact wheat roots were washed in sterile water for 15 min. The wash was centrifuged at 10 000 ***g*** for 10 min to remove the insoluble material. The root wash was lyophilized and 0.5 g (dry weight) of the lyophilized root wash was dissolved in 5 mL of sterile minimal medium solution. The wash was stored frozen at $-20\,^{\circ}C$ and used as the growth medium after sterilization by membrane filtration through 0.22 μm (pore-size) filters.

Viable counts were determined by serial dilution of cultures with phosphate-buffered saline (PBS) and plating on LB agar (1% w/v). Results are representative of at least three independent experiments of $< 10\%$ variation.

Preparation of cell extracts, polyacrylamide gel electrophoresis (PAGE) and SOD activity determination

Cultures of *B. cereus* were harvested by centrifugation at 5000 ***g***, and the pellets were resuspended in 50 mM phosphate-sodium buffer (pH 7.8) and disrupted by sonication. Cell debris was removed by centrifugation at 12 000 ***g*** for 15 min. The cell extract was analyzed following nondenaturing PAGE. The SOD activity was visualized by tetrazolium red negative staining. Inhibition experiments with SOD isoenzymes were carried out with a supplement of H_2O_2 and KCN as described previously (Droillard *et al.*, 1989).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source/reference
Strains		
Bacillus cereus		
905	Wild-type strain	This lab
KO1	905 *sod*A-1	This study
KO2	905 *sod*A-2	This study
KOS	905 *sod*A-1 *sod*A-2	This study
Escherichia coli		
DH5α	F^- Δ(*lac-arg*F)*U169 rec*A-1 *end*A-1 *hsd*R (r_K^- m_K^+) *sup*E-44 *gyr*A-1 *rel*A-1 *deo*R *thi-1* (Φ80d*lac*-Z ΔM15)	Strategene
GC4468	F^- Δ*lacU169 rps*L	Carlioz & Touati (1986)
QC779	As GC4468. (*sod*A :: MudPR13)25 Φ(*sod*B-kan)1-Δ_2 Cm^R Sm^R Kan^R *Lac*$^-$	Carlioz & Touati (1986)
Plasmids		
pBluescript KS(−)	Cloning vector, colE1 origin, Ap^R	Promega
pHP14	Bifunctional replicon, 4.2 kb, Em^R, carrying the MCS of m13 mpll.	Smith *et al*. (1987)
pLARF-5	Cosmid vector Tc^R	Keen *et al*. (1988)
pEBS	pBluescript KS(−) inserted a 1.1-kb E1/E2 amplified fragment containing the erythromycin-antibiotic cassette from pHP14 at ClaI site, Em^R	This study

Ap, ampicillin; Em, erythromycin; Tc, tetracycline.

Molecular cloning techniques

Molecular cloning was performed essentially as described by Sambrook *et al.* (1989). Primers used in this paper were listed on online supplementary documents. Plasmid DNA was introduced in *E. coli* and *B. cereus* by electroporation (Belliveau & Trevors, 1989; Sambrook *et al.*, 1989). A cosmid library of *B. cereus* 905 was prepared to clone the *sod* genes following the protocol recommended by Packagene® Lambda DNA Packaging System (Promega). DNA and its deduced peptide sequences were analyzed with the BLAST program of the National Center for Biotechnology Information (NCBI).

SOD assays

Cell extracts were prepared from cultures in 250 mL bottles with 50 mL of medium. SOD activity was determined with the standard SOD assay, which involves steady-state generation of superoxide by means of bovine xanthine oxidase plus xanthine (Flohé *et al.*, 1988; Jenney *et al.*, 1999). One unit of activity is calculated as the amount of protein necessary to inhibit the rate of superoxide-dependent reduction of cytochrome *c* by 50%. The mass of protein was estimated by gel filtration-HPLC on a Bio-Sil SEC 250 column (Bio-Rad), using the Bio-Rad BioLogic HR system.

Construction of the *sod* mutants

The two-step allelic exchange strategy was used to knock out the *sod* genes (Shi *et al.*, 2004). To fit with the antibiotic characteristics of *B. cereus*, a 1.1 kb erythromycin-antibiotic cassette from pHP14 was amplified with primers E1/E2 and was inserted into the ClaI site of pBluescript KS (+), yielding pEBS (Smith *et al.*, 1987).

To knock out the *sod*A-1 genes in *B. cereus*, the plasmid pEBS-KO1 was constructed in two steps. First, a 1.3-kb fragment was obtained by PCR from the upstream region of *sod*A-1 with the primers S1/S2 (Fig. 2a). It was digested with PstI and XhoI and inserted into pEBS. Then, a 1.2-kb fragment was obtained by PCR from the *sod*A-1 with primers S3/S4 (Fig. 2a). The amplified fragment was digested by PstI and BamHI and inserted between the PstI and BamHI sites of the previous construction, yielding the plasmid pEBS-KO1. With the same procedure, the PstI- and XhoI-digested S5/S6 amplified fragment and the PstI- and BamHI-digested S7/S8 amplified fragment were inserted in the pEBS, yielding the plasmid pEBS-KO2 (Fig. 2b).

The pEBS-derivative plasmids for making the knockouts of *sod*A-1 and *sod*A-2 were transformed into *B. cereus* by electroporation. Transformants of single crossovers were selected on erythromycin plates. These transformants were then grown in LB medium for 4–6 days with regular subculturing to allow for the excision and loss of the plasmid. Cultures were properly diluted and plated onto LB plates, and the knockout mutants (double-crossover recombinants) were obtained as erythromycin-sensitive colonies. Chromosomal DNA was isolated from selected mutants, and PCR analysis and DNA sequencing were performed to confirm the replacements of the wild-type genes by double-homologous recombination events in the *sod*A-1 and *sod*A-2 loci. To obtain *sod*A-1 and *sod*A-2 double mutant strain of *B. cereus*, one of the single mutants was transformed with another plasmid construct.

Root colonization

Wheat seeds (*Triticum aestivum* L.) were surface sterilized with 10% sodium hypochlorite for 3 min and washed extensively with sterile distilled water, then germinated at sterilized condition overnight. Inocula were grown to an $OD_{600\,nm}$ of 3 in LB broth containing 5 μg mL^{-1} erythromycin, centrifuged and resuspended in sterile 50 mM potassium phosphate buffer (pH 7.5) to an $OD_{600\,nm}$ of 0.5 (about 10^8 CFU mL^{-1}). The sterilized seeds were soaked for 1 h in the bacterial suspensions. Each seed was then planted into the growth matrix constituted with 10 parts of peat moss, seven parts of vermiculite, three parts of sand and seven parts of Perlite (v/v) (Young *et al.*, 2000). The growth matrix (pH 7.0) was autoclaved at 121 °C twice for 90 min on 2 successive days. At defined times, five seedlings were gently removed. Excised roots were transferred into 20 mL of sterile 50 mM potassium phosphate buffer, and vortexed vigorously for 2 min. After serial dilution, the dilutions were plated on LB plates containing 5 μg mL^{-1} erythromycin. CFU g^{-1} of root were scored after incubation at 32 °C. Identities of wild-type strains and SOD mutants were checked by staining gels for SOD activity of extracts from these cells.

Bacterial populations were assessed at each time point using ANOVA of a two-way factorial in a completely randomized design. Means of the CFU g^{-1} of root was compared using of Tukey mean comparisons with no significant difference at $P > 0.05$.

Results

Cloning of *sod* genes of *B. cereus* 905

To identify genes of *B. cereus* 905 that are involved in resistance to oxidant, a cosmid library of the bacterium was constructed and the plasmids from the cosmid library were further transferred into *E. coli* QC779 (*sod*A *sod*B), a strain that cannot survive in medium containing 50 μM paraquat (Sigma). Eight colonies of *E. coli* QC779 carrying putative complementary plasmid restored SOD^+ phenotype of the bacterium. The eight cosmids were subcloned to pBluescript KS (+). The resulting eight plasmids were sequenced and designated as pBS1–8.

The SOD activity encoded by the plasmids in *E. coli* QC779 was visualized following nondenaturing PAGE. Two

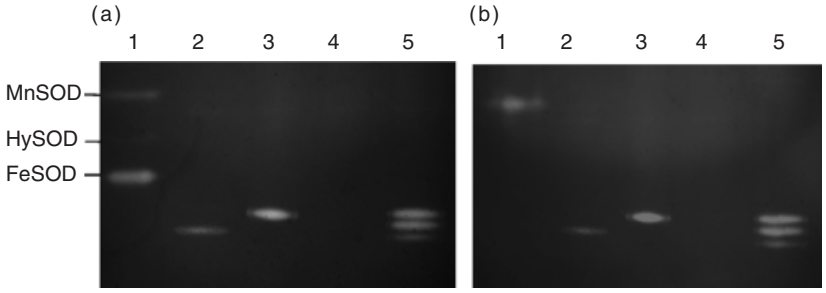

Fig. 1. Detection of SOD activity in *Escherichia coli* and *Bacillus cereus* on nondenaturing polyacrylamide gels (a) No inhibitors, (b) with 5 mM H_2O_2. Lanes: 1, *E. coli* GC4468; 2, *E. coli sodA sodB* strain QC779/pBSsodA1with *B. cereus* sodA-1 gene; 3, QC779/pBS-sodA2 with *B. cereus* sodA-2 gene; 4, QC779; 5, *B. cereus* 905. Lanes contain crude extracts of stationary cultures (30 g of total protein). The *E. coli* MnSOD, hybrid SOD (HySOD), and FeSOD are indicated.

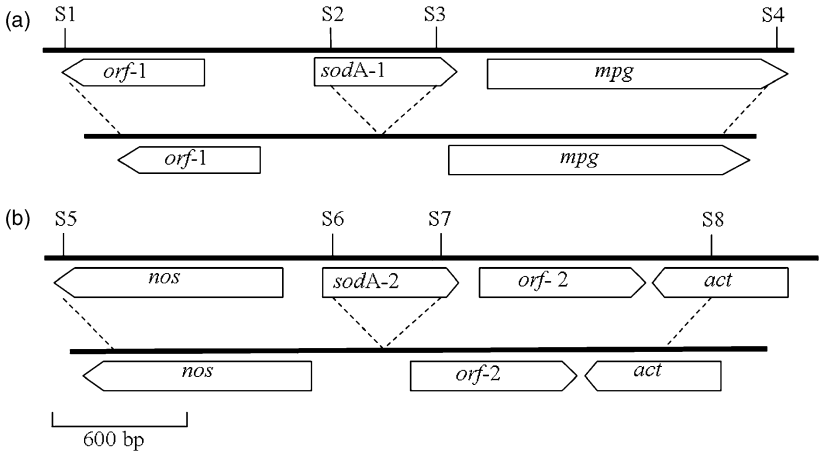

Fig. 2. Chromosomal organization surrounding (a) *sodA*-1 (b) *sodA*-2 in *Bacillus cereus* and strategy for homologous exchange mutagenesis to obtain SOD-deficient mutants. Upper line represents the genetic map of *sodA*-1 or *sodA*-2 region. Lower line represents the genetic map of corresponding mutant. Genes: mpg, membrane protein gene; nos, nitric oxide synthase; act, acetamide transpoter; orf-1 and orf-2, conserved hypothetical proteins with unknown functions. Primers used in this study were marked as P1 through P4 and S1 through S8.

distinct band sites of the SOD activity were identified among the eight clones (pBS1–8). Clones pBS1 and pBS3 were chosen for further study. The SOD activity of crude exact of *E. coli* QC779 harboring pBS1 and pBS3 was not inhibited by H_2O_2 (Fig. 1), indicated pBS1 and pBS3 containing MnSOD gene, redesignated as pBS-*sod*A1 and pBS-*sod*A2, respectively.

Nucleotide sequences of the two *sod*A genes from *B. cereus* 905

The inserts of pBS-*sod*A1 and pBS-*sod*A2, of 3.3 and 3.6 kb in size, respectively, were completely sequenced for both strands (GenBank accession nos. EF075931 and EF075932). A complete ORF of 654 bp (Fig. 2a) encoding a polypeptide of 238 amino acids was identified in pBS-*sod*A1. Similarly, a 624 bp ORF encoding a polypeptide of 208 amino acids was identified in pBS-*sod*A2 (Fig. 2b). The deduced amino acid sequences of the two peptides were highly similar to the SOD family of proteins. Thus, the two loci are designated as *sod*A-1 and *sod*A-2, respectively. The amino acid sequence encoded by *sod*A-1 is 58.4% identical to that encoded by *sod*A-2. The critical residues commonly used to predict manganese specificity of SODs (Parker & Blake, 1988) are present in both the peptides (Fig. 3). The signature amino acid residues of *sod*A gene were identified in both *sod*A-1 (Gly^{92}, Gly^{93}, Phe^{100}, Gln^{165}, Asp^{166}) and *sod*A-2 (Gly^{77}, Gly^{78}, Phe^{85}, Gln^{150}, Asp^{151}). Therefore, the polypeptides encoded by *sod*A-1 and *sod*A-2 were termed MnSOD1 and MnSOD2, respectively.

Construction of the *B. cereus* SOD-deficient mutants

To investigate the physiological roles of the *sod*A-1 and *sod*A-2 genes in *B. cereus*, three mutant strains KO1, KO2 and KOS were constructed. Strains KO1 and KO2 are knock-out mutant for *sod*A-1 and *sod*A-2, respectively. KOS is a double mutant for both *sod*A-1 and *sod*A-2. The mutants were verified by PCR and DNA sequencing by the outside flanking region from the according mutants (data not shown). Crude extracts from these mutants and wild-type strain were assessed for SOD activity in nondenaturing polyacrylamide gels. Both KO1 and KO2 showed a single SOD band of SOD activity (Fig. 4a). However, the electrophoretic mobility of SOD in KO2 is faster than that in KO1. They respectively correspond to the fast band and the slow

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MnSOD1      MSLKWQYINWEEYNNGKTRIYQIYPYAYDALEPHFDKETM      40
MnSOD2      ..............MSSFQLPKLS.YDYDELEPYIDSNTL      25

MnSOD1      NIHHTKHHNTYITNLNAALEGHAELADKSVEELVANLNEV      80
MnSOD2      SIHHGKHHATYVNNLNAALENYSELHNKSLEELLCNLEAL      65
                     **          *
MnSOD1      PEAIRTAVRNNGGGHANHTFFWTILSPNGGGQPVGELATA     120
MnSOD2      PKEIVTAVRNNGGGHYCHSLFWEVMSPRGGGEPNGDVAKV     105

MnSOD1      IEAKFGSFDAFKEEFAKAGATRFGSGWAWLVVNNGELEVT     160
MnSOD2      IDYYFNTFDNLKDQLSKAAISRFGSGYGWLVLDGEELTVM     145
              **
MnSOD1      STPNQDSPLTEGKTPVIGLDVWEHAYYLNYQNRRPDYIGA     200
MnSOD2      STPNQDTPLQEGKIPLLVIDVWEHAYYLKYQNRRPEFVTN     185

MnSOD1      FWNVVDWNAAEKRYQEAK....                       218
MnSOD2      WWHTVNWDRVNEKYLQAIQSQK                       207
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Fig. 3. Alignment of deduced amino acid sequences of *Bacillus cereus* 905 *sodA*-1 and *sodA*-2. Sequence alignment was obtained using the DNAMAN (Version 6) program. The conserved residues in the two sequences are boxed. *Signature residues that determines specificity for metal ligand (Fe or Mn) bound to the SOD apoprotein.

band of SOD activity in wild-type type *B. cereus* strain. The middle band of SOD activity in wild type was not observed in either KO1 or KO2. The double mutant of *B. cereus* KOS (*sod*A-1 *sod*A-2) was completely devoid of SOD activity (Fig. 4b). These data suggest that the two MnSODs make up the majority of SOD activity in *B. cereus*.

SOD activity in *B. cereus*

The SOD activity in KO1 in LB (18.6 U mg^{-1}) is 2.5-fold of that in KO2 (7.4 U mg^{-1}), suggesting that the activity of MnSOD2 in *B. cereus* is higher than MnSOD1 (Fig. 4b). The wild type has the highest SOD activity (23.4 U mg^{-1}). Addition of superoxide anion generator paraquat in culture medium induced SOD activity in wild type, KO1 and KO2 strains. However, either with or without paraquat challenge, none of KO1 and KO2 could have a SOD activity comparable with wild type. Therefore in KO1 strain, the lost SOD activity of *sod*A-1 in KO1 was not fully compensated by *sod*A-2. Similarly, it was in KO2 strain. The KOS strain could not survive the paraquat challenge.

Sensitivity of the SOD-deficient mutants to oxidative stress

The growth of the three mutants in LB without oxidative stress was similar to that of the wild-type strain under the same condition. To test the roles of the two MnSODs in the defense against O_2^- stress, the SOD-deficient mutants and the wild type were treated with paraquat to generate O_2^- within the cell. When treated with 50 μM paraquat, both the wild type and KO1 continued to grow on LB plate, whereas KO2 and KOS could not grow on LB plates (data not shown). When cultured in LB containing 20 μM parquat at aerobic condition, strain KO1 grew at a rate similar to wild type, while KO2 displayed significant growth arrest. The data were shown in the online supplementary documents.

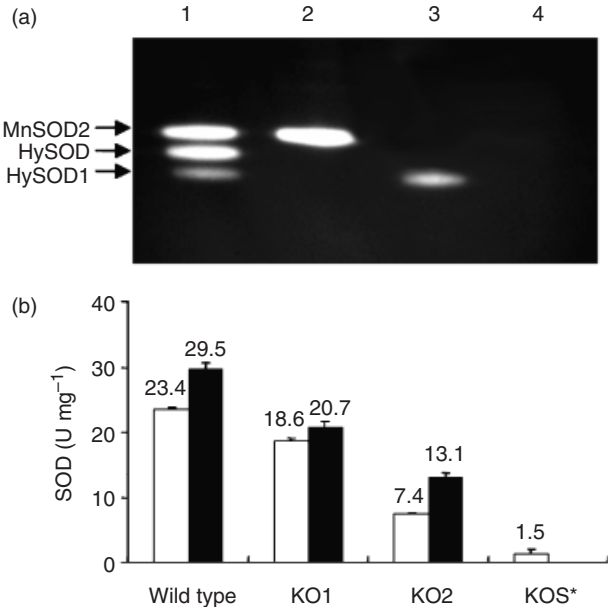

Fig. 4. SOD activity in *Bacillus cereus* 905 and its *sodA* mutants. Bacterial cells were harvested at exponential growth phase, 4 h after inoculation at 1 : 100 (v/v). (a) Detection of SOD activity on nondenaturing polyacrylamide gels with negative staining. Lanes: 1, wild type (905); 2, *B. cereus* KO1; 3, *B. cereus* KO2; 4, *B. cereus* KOS. Lanes contain crude extracts of stationary cultures (35 g of total protein). MnSOD1, hybrid SOD (HySOD), and MnSOD2 are indicated. (b) SOD activity in the crude extracts in presence (black bars) or absence (white bars) of 10 M paraquat. **Bacillus cereus* KOS was unable to grow well in presence of paraquat, therefore its SOD activity was not assayed. Values are the means and SEs of five experiments.

The roles of the two MnSODs in *B. cereus* in surviving in the wheat rhizosphere

Nutrition utilization is the key factor of bacterial colonization in rhizosphere. To study the roles of the two MnSODs in survival in the wheat rhizosphere, the survival of mutants in minimal medium supplemented with 0.2% (w/v) glucose, succinate or myoinositol, media extremely

poor in carbon source was analyzed. Regardless of supplementation, wild type and KO1 sustained the scarce-nutrient condition up to 6 h, whereas KO2 lost a half of viable cells. The double mutants KOS scarcely survived 6 h (Fig. 5a–c). Similar survival profiles were obtained for the four strains when tested in wheat root wash medium (10% w/v) (Fig. 5d).

The colonization data shown in Fig. 6 are the means of five different studies. On the wheat roots, populations of the wild type and KO1 fluctuate slightly (from the maximum cell densities, *c.* 10^8 CFU g^{-1} of root 5 days after planting to *c.* 10^6 CFU g^{-1} of root 20 days after planting). At the same periods, the density of KO2 on the wheat roots was lower, by about 10-fold, than that of the wild type and the KO1. Among the four strains, population of KOS was statistically lowest. It decreased to *c.* 10^3 CFU g^{-1} of root 20 days after planting. To visualize colonization, the bacteria with a stable plasmid expressing green flourescent protein (GFP) was tagged constitutively, pHP14-GFP (Dunn & Handelsman, 1999). The online version of this paper contains a supplementary figure showing the difference of the fluorescence intensities at the root colonized with the four strains. The fluorescence intensities at the root colonized with the wild type (458 ± 32) was similar to that colonized with the KO1 (412 ± 45), fluorescence with the KO2 (254 ± 42) was weaker, and it is weakest of KOS (103 ± 23), being comparable with controls lacking any inoculum (39 ± 9).

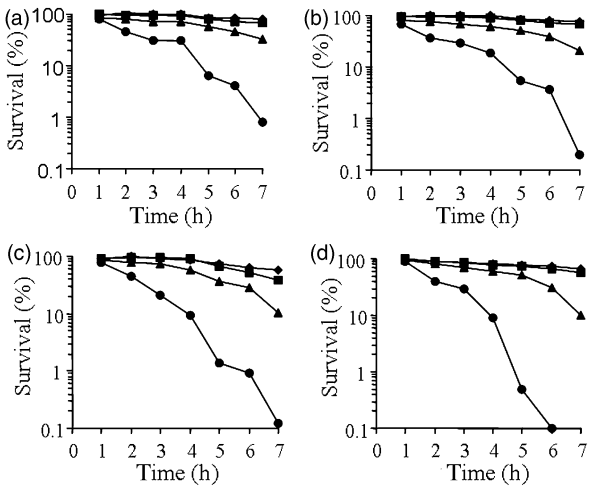

Fig. 5. Survival of *Bacillus cereus* and its *sodA* mutants in minimal media containing 0.2% different carbon sources and wheat root wash medium. (a) Glucose. (b) Succinate. (c) Myoinositol. (d) Wheat root wash (see 'Materials and methods'). Two milliliters 108 CFU mL^{-1} of wild type (♦), KO1 (■), KO2 (▲) and KOS (●) washed by sterile water two times were inoculated in 10 mL the culture medium, and viable cells were determined by plating serial dilution on LB agar plates. Each data point in the survival curves represents the average of five independent experiments.

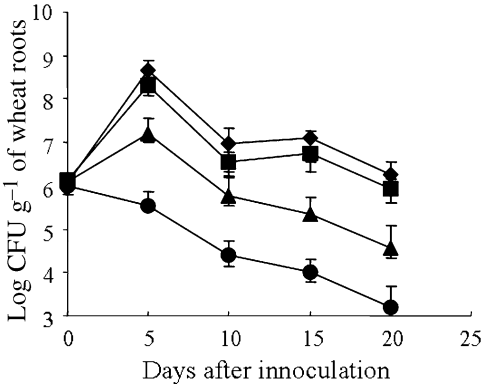

Fig. 6. Colonization of *Bacillus cereus* and its *sodA* mutants on wheat roots. At defined times, cell numbers of wild-type strain (♦), KO1 (■), KO2 (▲) and KOS (●) were determined by plating serial dilutions of root washes on LB agar plates containing 5 μg mL^{-1} erythromycin and the means of five separate experiments are presented.

Discussion

In this paper, the identification of two distinct *sod*A genes in *B. cereus* 905 and their roles in antioxidant function in the cell were described. Insensitivity of their coding products to H_2O_2 suggested that both the two *sod*A genes belong to Mn-containing superoxide dismutase genes. And the five conserved amino acids are presented at the due positions, which ensure specific binding to manganese ion (Parker & Blake, 1988). The low similarity (58.84%) between the two *sod*A genes unarguably indicates their distinction. Analysis of *B. cereus* genome with PCR revealed that both a Cu/ZnSOD gene and a Fe/ZnSOD gene exist in the chromosome of *B. cereus* 905. However, their antioxidant roles could not be significant as their activity was not detectable with methods used in this study (data not shown). Therefore, MnSODs coded for by the *sod*A-1 and *sod*A-2 genes play a major role in protection against oxidative stress in *B. cereus*. An intriguing question is whether the two *sod*A genes entirely overlap in their spectra of function. As knock-out mutations in either *sod*A-1 or *sod*A-2 cause partial loss of SOD activity in comparison with the wild-type bacterium, it is highly likely that each one has developed certain unique function that is not replaceable by the other. The survival of the null mutation strains further supports this notion. Similar to these functional divergence *sod*A genes in *B. cereus*, the *sod* genes in *B. anthracis* do not work cooperatively to protect superoxide stress (Passalacqua *et al.*, 2006).

Many prokaryotic microorganisms have single MnSOD and FeSOD related genes in their genome to protect themselves against oxygen toxicity. In *E. coli*, MnSOD expression can be rapidly and pronouncedly induced by superoxide anion (Hassan & Fridovich, 1977). Data showed that the SODs types and enzymatic activities in *B. cereus* are

different in comparison with *E. coli*. First, two distinct MnSODs, not one MnSOD and one FeSOD, fulfilled the antioxidant function in *B. cereus*. The middle SOD activity band was thought as a heterodimer constituted by both of the two MnSOD monodimers as it did not present in *B. cereus* KO1 and *B. cereus* KO2. And then, each enzymatic activity of MnSOD1 and MnSOD2 increases slightly when challenged by superoxide anion. At the upstream of the two *sod*A genes, Fur box, an element exist at 5′ flanking region of *E. coli sod*A gene (Niederhoffer *et al.*, 1990), and other known regulation boxes (Compan & Touati, 1993) could not be found. These could explain the difference of enzymatic activity regulation in response to oxidative stress.

Essential to rhizosphere colonization of bacteria is the ability to grow on nutrients from rhizosphere released by roots. Plant roots produce exudates containing sugars, organic acids, amino acid and phenolic compounds (Weller, 1988; Gamliel & Katan, 1992). Catabolism of these components in rhizosphere resident bacterium is vital to its predominant colonization. It is known that excessive O_2^- cause inactivity of some key enzymes of cell metabolism. Colonization and the growth of plant roots is accompanied by changes in the rhizosphere. Bloomfield *et al.* (1998) and Dodd *et al.* (1997) pointed out that transfer of bacteria from one environment to another causes an imbalance in metabolism, instantaneously producing O_2^- and free radicals. Apparently, the *sod*A-1 and *sod*A-2 activities play unique and important roles in colonizing the rhizosphere of *B. cereus*.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Sequences of the primers used.

Fig. S1. Sensitivity of the SOD-deficient mutants to oxidative stress.

Fig. S2. Confocal images of GFP-tagged strains of *Bacillus cereus* on wheat root 5 days after planting.

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