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Increased oxidative stress tolerance by cambialistic superoxide dismutase overexpression in *Mesorhizobium japonicum* MAFF303099

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ABSTRACT

Oxidative stress is a common denominator underlying many environmental insults. As a global response, the increment of intracellular reactive oxygen species leads to the activation of the antioxidant system to maintain cellular homeostasis. The *Mesorhizobium japonicum* MAFF303099 mlr7636 SOD gene was constitutively overexpressed to test if the enhanced superoxide dismutase (SOD) activity contributes to oxidative stress tolerance in free-living cells. Salt stress, decreases in the osmotic potential, and superoxide generation by killing assays were assayed in cultures of *M. japonicum* with a constitutive SOD overexpression under the *nptII* promoter of pFAJ1708 plasmid to evaluate bacterial survival. The results showed that the strain carrying an additional *mlr7636* copy had five-fold increased SOD activity in the periplasmic space using Fe as a cofactor, and that its higher tolerance to oxidative stress was related to high SOD activity *per se*, which contributes to fast superoxide dismutation associated with hydrogen peroxide reduction by increases in Catalase activity.

To our knowledge, this is the first report of homologous superoxide dismutase overexpression in *M. japonicum*, which contributes to the description of its role in the tolerance to oxidative stress under free-living conditions.

Keywords: oxidative stress, superoxide dismutase, overexpression, *Mesorhizobium japonicum Mesorhizobium loti*.

RESUMEN

El estrés oxidativo es un común denominador que subyace a muchas agresiones ambientales. Como respuesta global, el aumento de especies reactivas de oxígeno intracelular desembocan en la activación del sistema antioxidante para mantener la homeostasis celular. El gen SOD mlr7636 de Mesorhizobium japonicum MAFF303099 fue sobreexpresado constitutivamente para evaluar el efecto en la tolerancia del rizobio al estrés hídrico y oxidativo en vida libre. Se llevaron a cabo ensayos de estrés salino, osmótico y oxidativo por generación de ion superóxido en cultivos de M. japonicum con sobreexpresión de SOD bajo el promotor nptII del plásmido pFAJ1708 para evaluar la supervivencia bacteriana. Se observó un incremento cinco veces mayor en la actividad de la mutante que llevaba una copia adicional del gen mlr7636, localizada principalmente en el espacio periplásmico, mediante el uso de Fe como cofactor. El incremento en la tolerancia de la cepa M. japonicum SOD al estrés oxidativo se relacionó con el incremento a la actividad SOD per se, al contribuir a una rápida dismutación del superóxido a peróxido de hidrógeno, asociada a incrementos de la actividad catalasa.

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Hasta donde sabemos, este es el primer reporte del efecto de la sobreexpresión homóloga de la superóxido dismutasa en M. japonicum, el cual contribuye a la descripción de su rol en la tolerancia al estrés oxidativo en condiciones de vida libre.

Palabras clave: *estrés oxidativo, fisiología microbiana, superóxido, dismutasa sobreexpresión, Mesorhizobium japonicum, Mesorhizobium loti.*

INTRODUCTION

Reactive oxygen species (ROS) are unavoidable by-products of aerobic life and their signaling role during plant-microbe interactions has been extensively demonstrated. ROS are constantly produced during normal metabolic processes, but their levels increase under abiotic stress conditions, which can damage DNA, lipids, membranes, and proteins.

In rhizobia, the antioxidant system allows bacteria to modulate ROS levels produced during both the free-living stage and the symbiotic interaction. One of the key enzymes involved in the redox modulation of these processes is Superoxide dismutase (SOD, EC 1.15.1.1). SODs are metalloenzymes that have been found in nearly all organisms examined to date. They act as the primary line of defense against the first free radical produced in the ROS cascade, the superoxide radical (O_2^-) (Santos *et al*., 2000).

In bacteria, there are three general classes of SODs, which differ in their metal co-factors: manganese-containing MnSOD (SOD A), Fe-containing FeSOD (SOD B) and cambialistic Fe/Mn SODs (CamSOD); the three SODs share a high protein sequence and structure similarity (Santos *et al*., 1999; Asencio *et al*., 2011).

Rhizobiales have shown variability in their superoxide dismutase enzymes: in *Sinorhizobium meliloti* Rm5000, *sodA* is the only gene encoding a cambialistic SOD activity with either manganese or iron as a co-factor (Santos *et al*., 1999). In *Bradyrhizobium diazoefficiens* USDA 110, four genes encoding superoxide dismutases have been reported (http://genome.annotation.jp/rhizobase/). Two of these genes, *bll7559*, a Fe-Mn SOD (ChrC), and *bll7774* SOD, *sodF*, have been reported as inducible under drought stress (Cytryn *et al*., 2007).

In *Mesorhizobium japonicum* MAFF303099, formerly known as *Mesorhizobium loti* (Martínez-Hidalgo *et al*., 2016), the SOD is encoded by the *mlr7636* gene and it has been described as a Mn/Fe cofactor binding protein (Kaneko *et al*., 2000). In *M. japonicum*, SOD activity, like other cellular processes, produces hydrogen peroxide, whose scavenging occurs through the activity of two different catalases, a monofunctional catalase and a bifunctional catalase-peroxidase encoded by *mlr2101* and *mlr6940*, respectively (http://genome.annotation.jp/rhizobase/Mesorhizobium).

It has been reported that the disruption of the SOD gene in *S. meliloti* affects its symbiotic properties with alfalfa, with the mutant bacteria being affected during the stage of nodulation, infection and bacteroid development (Santos *et al*., 2000). However, there are no reports about the effect of rhizobial SOD overexpression on tolerance to oxidative and salt stress conditions.

In this work, a SOD overexpressing *M. japonicum* was obtained, and its free-living phenotype was investigated. Our results suggest that overexpression of SOD in *Mesorhizobium japonicum* improves the survival of this bacterium during oxidative stress induced by drought and salinity.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Mesorhizobium japonicum MAFF303099 (WT), 1708 and sod1708 strains were grown in YEM medium (Vincent, 1970) aerobically at 28°C. For saline and osmotic stress conditions, the YEM medium was supplemented with 150 mM NaCl and 15% (W/V) polyethylene glycol 8000 (PEG) to obtain a water potential equal to -0.84 MPa. Values of water potential were measured using isopiestic thermocouple psychrometry (Wescar® Point Microvoltmeter HR-337 Dew). *Escherichia coli* DH5α and S17-1 strains were grown in LB medium. Strains harboring plasmids pFAJ1708 and pFAJsod were grown on medium supplemented with Tetracycline (Tc) 10 µg/mL. The bacterial strains and plasmids used are listed in table 1.

Bacterial Strains	Relevant characteristics	Source
***M. japonicum* strains**		
WT	Wild type MAFF303099 sequenced strain	(Cytryn *et al*., 2007)
1708	MAFF303099 with pFAJsod, Tc^R	This work
sod1708	MAFF303099 with pFAJ1708, Tc^R SOD	This work
***E. coli* strains**		
S17-1	RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	(Kaneko *et al*., 2000)
DH5α	F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17(rk-, mk+)	(Hanahan, 1985)
Plasmids		
pFAJ1708	Broad-host-range plasmid, Tc^R, containing *ntpII* promoter	(Dombrecht *et al*., 2001)
pFAJsod	pFAJ1708 containing *mlr7636* cloned downstream *nptII* promoter for gene expression	This work

Table 1. Bacterial strains and plasmids used in this study.

Growth kinetics

Cell growth was evaluated in triplicate using a pre-inoculum of 0.8 (OD_{600}) with an initial OD_{600}~ 0.01 in 50 mL fresh media at 28°C in an orbital shaker (160 rpm). Growth was monitored at OD_{600} in a DU-640 Beckman spectrophotometer (Beckman Coulter, USA) for 64 hours.

Determination of tolerance to oxidative stress

Tolerance to O_2^- was determined by conventional bacterial killing assays, in which bacteria were exposed to exogenous O_2^- generated by the oxidation reaction of xanthine by Xanthine oxidase enzyme (Melillo *et al*., 2009). Cells were grown up to 0.5 OD_{600} in YEM; then they were centrifuged and resuspended at 10^9 CFU/mL in sterile phosphate-buffered saline (PBS) and treated with 250 µM hypoxanthine and 0.1 U/mL of Xanthine oxidase. In order to discriminate the effect of O_2^- and the H_2O_2, produced by SOD activity, bovine catalase (1 U/mL) was added to hypoxanthine/xanthine oxidase reaction to reduce the H_2O_2 presence and subsequent Fenton chemistry, and to ensure that killing of bacteria was only in response to O_2^- radicals. The number of viable bacteria was determined at 0, 30, 60, and 120 min of incubation by plating serial dilutions on YEM agar plates. A similar protocol was used for examining the susceptibility to H_2O_2. The cultures were treated with 1 mM H_2O_2 and number of viable bacteria was determined at the same time intervals. Bacterial colonies were counted after 48 h and expressed as $\log_{10}$ CFU/mL.

Superoxide dismutase cloning and plasmid construction

After genomic DNA extraction by CTAB method (Wilson *et al*., 2001), the *mlr7636* gene was amplified by polymerase chain reaction (PCR), using the specific forward primer SOD-XbaI-F (5'-ATATTCTAGACCACGAGGGAGTACTACCCATGG-3', the *XbaI* restriction site is underlined and the bold letters represent the start codon of *mlr7636* gene) and SOD-BamHI-R (5'-ATATGGATCCTCACTTTGCCTTTTCGTAGAGC 3', the *BamHI* restriction site is underlined), for directional cloning in pFAJ1708 downstream of the constitutive *nptII* promoter. PCR reaction was performed as follows: initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 20 sec, annealing at 65°C for 20 sec and extension at 68°C for 1 min. The 639-bp PCR product and the pFAJ1708 plasmid were digested by *XbaI* and *BamHI* enzymes, and the DNA fragments were purified from agarose using the Qiagen Gel Extraction QiaexII kit. The *mlr7636* gene was cloned into pFAJ1708 with T4 DNA ligase (Promega) at 4°C during 16 h. The resulting recombinant plasmid pFAJsod was transformed into *E. coli* DH5α using the heat shock method. The clones were sequenced to confirm the insertion of *mlr7636* in frame with the *nptII* promoter and used to transform electrocompetent *E. coli* S17-1 by electroporation with a Gene Pulser Xcell, Bio Rad, 2.5 KV /5 ms.

Transformation of *M. japonicum*

Biparental conjugation was performed in YEM medium using *M. japonicum* MAFF303099 (WT) as the recipient and *E. coli* S17-1 carrying pFAJsod or pFAJ1708 as donors (Simon *et al*., 1983). The donor/recipient ratio was 1:1. Transconjugant *M. japonicum* cells were selected from the halo of *E. coli* inhibition around the disc containing 30 µg of nalidixic acid on YEM medium supplemented with Tc 20 µg/mL.

Protein extraction

For the analysis of the whole cell fraction, cultures of *M. japonicum* were grown up to 0.1 OD_{600} in 50 mL of liquid YEM medium. The cells were harvested by centrifugation at 15000 *g* at 4°C for 15 min, washed with 5 mL of 0.9% (W/V) NaCl and resuspended in 200 µL of 50 mM KH_2PO_4 pH 7.8 buffer. The cells were sonicated (Ultrasonic Vibracell VCX600) at 33% amplitude by pulses of 3 seconds with intervals of 3 seconds for 2 min. The samples were maintained in an ice-water bath during sonication. Then, the extracts were centrifuged at 14000 g for 20 min and the supernatant was recovered.

The periplasmic proteins were obtained by hypo-osmotic shock treatment (Krehenbrink *et al*., 2011). The cells were harvested by centrifugation at 15000 *g* and at 4°C for 15 min, washed with 5 mL of 0.9% (W/V) NaCl and briefly resuspended in 0.2 mL of hyperosmotic buffer containing 20% (W/V) sucrose in 30 mM Tris/HCl pH 8.0 supplemented with 5 µL lysozyme (100 mg/mL in 30 mM Tris/HCl, pH 8). After 1 hour of incubation in ice, the cells were pelleted by centrifugation for 5 min (10000 g at 4°C) and resuspended in 0.2 mL of ice-cold distilled water. After additional 15 min on ice, the cells were pelleted by centrifugation at 10000 *g* at 4°C for 30 min. The supernatant contained the periplasmic proteins. The protein concentrations were determined by the Lowry method using bovine serum albumin (BSA) as a standard (Lowry *et al*., 1951).

Polyacrylamide gel electrophoresis and western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Non-denaturing polyacrylamide gel electrophoresis (ND-PAGE), for zymograms, was performed using the same buffer system with the omission of SDS from all buffers. The western blot analysis was conducted in a submerged system (Bio-Rad); the proteins separated by PAGE were blotted onto a nitrocellulose membrane (Amersham Biosciences). Polyclonal antibodies rabbit anti-FeSOD (Agrisera AS 06 125) and anti-MnSOD (N-20, Santa Cruz, SC18503) were used as primary antibodies. Goat anti-rabbit and rabbit anti-goat Alkaline phosphatase conjugates were used as secondary antibodies.

Enzyme activities

SOD activity was determined spectrophotometrically at 560 nm (Beauchamp and Fridovich, 1971). One Unit of SOD was defined as the amount that inhibits the photoreduction of nitrotetrazolium blue chloride (NBT) by 50%. The reaction mixture was composed of 50 mM potassium phosphate pH 7.8 containing 777 µM methionine, 448 µM NBT, 0.54 µM EDTA and 3.32 µM riboflavin. In the absence of the enzyme, the mixture was calibrated to reach an OD_{560} = 0.25 after 10 min incubation in UV light (360 nm) at 25°C. Specific activity was expressed in SOD units (µg of total protein)$^{-1}$.

The SOD zymogram was obtained after electrophoresis in 12% ND-PAGE. SOD activity was developed by incubating gels with 2.5 mM NBT during 25 min in the dark, followed by incubation for 20 min in 50 mM potassium phosphate pH 7.8 containing

28 µM riboflavin and 28 mM tetramethyl-ethylene diamine (TEMED). The reaction was continued for 10 to 15 min under white light and then stopped by a brief wash in water (Rao *et al*., 1995).

To determine the metallic cofactor of SOD in the zymogram, the ND-PAGE was pre-incubated with potassium cyanide (KCN 5 mM) or hydrogen peroxide (H_2O_2 10 mM) at RT for 30 min to inhibit CuZnSOD and FeSOD, respectively. After incubation, enzyme activity was assayed as described above.

Catalase (CAT) activity was determined with a reaction mixture containing 50 mM potassium phosphate pH 7.4 with the sample, and the reaction was started by adding 5 mM H_2O_2. The activity was determined spectrophotometrically by measuring the decreasing rate of H_2O_2 at 240 nm at 37°C for 1 min. Specific activity was expressed in CAT units (µg of total protein)$^{-1}$ (Jamet *et al*., 2003).

Both SOD and CAT activities were evaluated in rhizobia grown in NaCl 150 mM and PEG 15%.

Glucose-6-Phosphate Dehydrogenase (G6PDH) activity: The absence of cytoplasmic content in the periplasmic fraction was evaluated by measuring G6PDH activity. The reaction mixture contained 100 mM Tris-ClH buffer pH 8, 10 mM $MgCl_2$, 0.18 mM $NADP^+$ and 1 mM D-Glucose-6-Phoshate. The reaction was initiated by adding the sample and followed by the formation of reduced NADPH at 340 nm in spectrophotometer at 25°C. One Unit of G6PDH was defined as the amount of enzyme required to catalyze the reduction of 1 µmole of $NADP^+$ per min at 25°C. Specific activity was expressed in G6PDH units (mg of total protein)$^{-1}$ Banerjee and Fraenkel, 1972).

Phylogenetic analysis

A phylogenetic tree was constructed with the Mlr7636 amino acid sequence based on the alignment of the 11 SOD sequences from the Rhizobase database sharing more than 50% of identity. The analysis was performed by the neighbor joining method (1000x bootstrap replications) using Mega 5.2 software (Tamura *et al*., 2011).

Bioinformatic analysis for signal peptide prediction

A protein sequence corresponding to the open reading frame of the locus *mlr7636* of *M. japonicum* MAFF303099 was obtained from http://genome.annotation.jp/rhizobase and was entered as the query sequence in the SignalP 4.1 for Gram negative and TatP 1.0 algorithms (Petersen *et al*., 2011; Bendtsen *et al*., 2005), using the default criteria.

Statistical analysis

The values of enzymatic activity were expressed as mean ± standard error (SE). In all experiments, the replicates were analyzed statistically via ANOVA using the DGC Test at $p \leq 0.05$ in InfoStat software (Di Rienzo *et al*., 2016).

RESULTS

Effect of saline and osmotic stress on *M. japonicum* growth

The effect of saline and osmotic stress conditions on *M. japonicum* overexpressing *sod* gen (sod1708), the wild-type strain (WT) and the transformed strain with the empty vector (1708) were evaluated in YEM media (control), YEM containing 150 mM NaCl and 15% PEG. Under control conditions, the three strains had equivalent growth rates, whereas in saline treatment, all strains showed a slightly higher growth rate than those in control conditions. PEG treatment showed a 50% decrease in bacterial growth rate, indicating that the strains were sensitive to osmotic stress (fig. 1).

Effect of saline and osmotic stress on SOD and CAT activities

To evaluate the effect of salt and osmotic stress on *M. japonicum* SOD and CAT enzymes, total activities were determined in extracts from whole cells grown 72h under 150 mM NaCl and 15% PEG. Under salt stress, the WT strain showed higher levels of SOD and CAT activities than in control conditions, with approximately 4.8 and 2.8-fold increments, respectively (table 2). However, under osmotic stress conditions, only CAT activity showed an increase, whereas SOD decreased. The overexpression of *mlr7636* gene in sod1708 strain led to a 3.4-fold increase in SOD activity with respect to WT strain and 3.6-fold with respect to 1708 strain under control conditions. Under salt stress conditions, no differences in SOD activity were found between the overexpressor sod1708, WT and 1708 strains (table 2). Similar responses were found under PEG-induced osmotic stress treatment, with a 4.1 and 4.5-fold increase in SOD activity being detected in sod1708 strain compared with WT and 1708 strains, respectively.

CAT activity increased significantly in sod1708 strain in control conditions and saline stress treatment, but it showed no differences after osmotic treatment.

Increase of periplasmic SOD activity in *M. japonicum* sod strain

Specific SOD activity determined at an early growth stage (DO_{600} ~ 0.1) was lower than at the late exponential phase; however, no differences in whole cell lysates were found between strains (data not shown). The analysis of specific SOD activity in the periplasmic fraction revealed a 5-fold increase of enzyme specific activity in sod1708 strain compared to non-overexpressing strains (fig. 2 A). In order to assess the cytosolic contamination of the periplasmic extract, the specific G6PDH activities of both the whole cell extract and the periplasmic fraction were determined (fig. 2 B). The results showed low specific G6PDH activity levels for the periplasmic fractions, with less than 12, 6 and 2% of the specific activity of the whole cell lysate for WT, 1708 and sod1708, respectively.

Isoforms and localization of SOD in *M. japonicum* sod

The expression of SOD isoforms revealed in the zymograms where whole cell and periplasmic protein fractions were used showed changes in the activity patterns among the WT, 1708 and sod1708 strains. A unique activity band, which we named basal sod (BSOD) in this report, was observed in whole cell extracts of all the strains, whereas the sod1708 strain showed three extra bands (fig. 3 A). In periplasmic fractions, BSOD was absent in WT, whereas the sod1708 and the 1708 strains shared the same activity profile, with a similar relative mobility (Rf) to those found in whole cell extracts, including the three extra bands. Moreover, the three extra activity bands were inhibited by 10 mM H_2O_2, indicating that these isoforms used iron as cofactor (fig. 3 B).

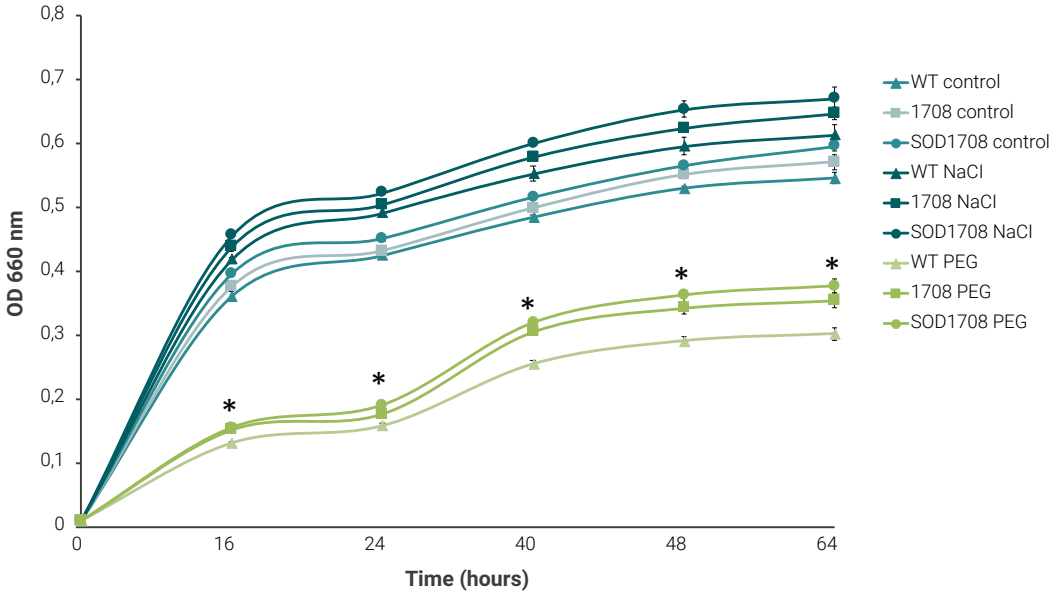


Figure 1. Growth curves of the WT, 1708 and sod1708 strains under osmotic and saline stress conditions. YEM medium was supplemented with 15% PEG or 150 mM NaCl, both of which lowered water potential to -0.84 Mpa. Data are the mean of three independent replicates with the error bars indicating the standard error. * $p \leq 0.05$ DGC test.

Strains	SOD activity			CAT activity		
	Control	NaCl	PEG	Control	NaCl	PEG
WT	0.20±0.04a	0.97±0.11a	0.10±0.01a	0.13±0.05a	0.37±0.09a	0.58±0.08a
1708	0.19±0.04a	1.01±0.09a	0.09±0.03a	0.16±0.04a	0.36±0.02a	0.54±0.06a
sod1708	0.69±0.06b	1.16±0.13a	0.41±0.05b	0.45±0.11b	0.68±0.10b	0.74±0.05a

Results of enzyme activities are presented in U (µg protein)$^{-1}$

Each value is expressed as mean ± SE (n=3)

Different letters in each column indicate significant differences ($p \leq 0.05$ DGC test)

Table 2. Specific activities of SOD and CAT of WT, 1708 and sod1708 under NaCl and PEG conditions 72 h.

WESTERN-blot analysis

The western blot analysis with FeSOD antiserum detected bands of 20.1 kDa in periplasmic extracts from all strains (fig. 3 C). On the other hand, this band was not detected in whole cell extracts. However, the molecular weight of the protein in periplasmic extracts, 20.1 kDa, does not coincide with the theoretical molecular weight of 22.7 kDa, predicted for the protein encoded by *mlr7636* gene. Therefore, the difference in 2.6 kDa mass obtained in the periplasmic extracts could be due to scission of a signal peptide. Nevertheless, the presence of a signal peptide for Sec or TAT pathway could not be predicted in the first 70 amino acids of the N-terminal end of the amino acid sequence (data not shown).

Phylogenetic analysis of rhizobia *Sod* genes

The consensus phylogenetic tree, constructed using 11 SOD sequences from RhizoBase database which had ≥50% identity with Mlr7636 sequence, showed two clusters (fig. 3 D). Cluster I included the product of the *mlr7636* gene between putative *sodB* of *Rhizobium leguminosarum* bv. viciae 3841 and *sodB* of *Rhizobium etli* CFN42 (RHE CH01203). Moreover, this subgroup was related to *SodB* FeSOD of *Sinorhizobium meliloti* 1021 (SMc00043) and *SodB* Mn-SOD of *Rhizobium sp*. NGR234 (NGR c07300). Cluster I also included three SODs capable of using Fe or Mn as cofactor: SODB from *Bradyrhizobium sp*. BTAi1 (BBta 1335), SOD of *Bradyrhizobium sp*. ORS278 (BRADO6273) and SOD of *B. diazoefficiens* USDA110 (bll7774). Cluster II included sequences that showed lower identity levels with the Mlr7636 sequence; this cluster was composed of FeSOD of *K. pneumoniae* Kp342 (GKPORF B1093), SOD of *C. taiwanensis* LMG19424 (RALTA A0566) and Fe/Zn/MnSOD of *Azospirillum sp*. B510 (AZL014560).

Bacterial tolerance to superoxide and H_2O_2 in killing assay

To assess the effect of the SOD overexpression on *M. japonicum* survival under excess of ROS production, we performed

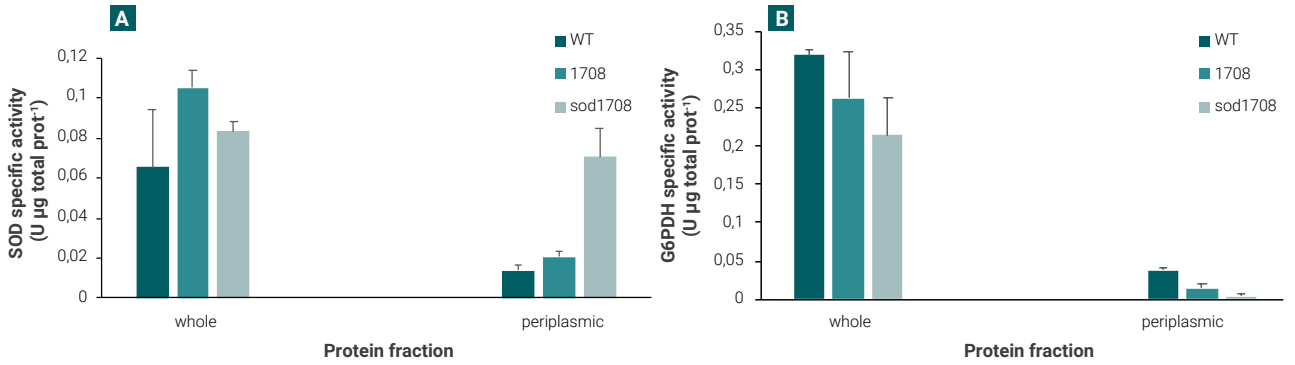
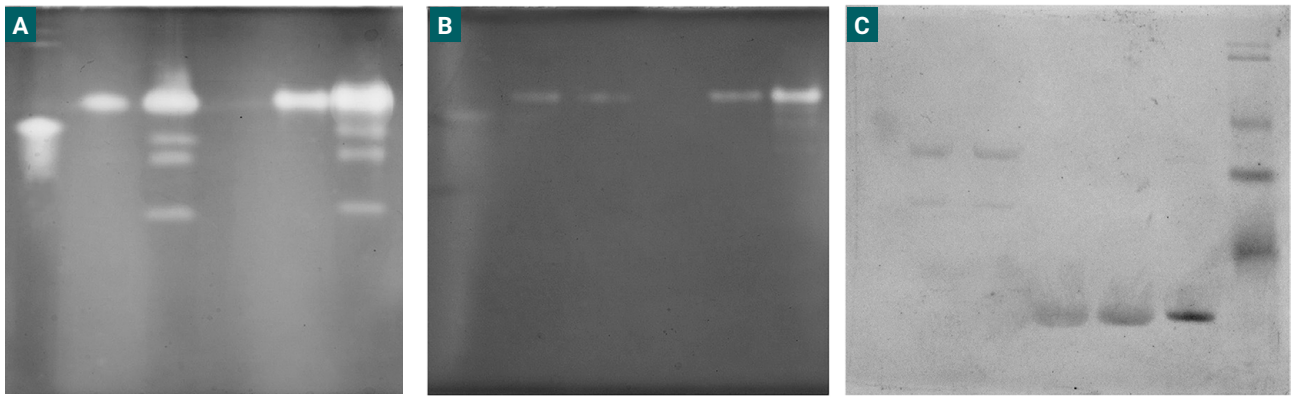


Figure 2. A. Specific SOD activity of the WT, 1708 and sod1708 strains in whole and periplasmic extracts. Data are representative of at least three independent replicates. Different letters indicate significant differences in the mean ($p \leq 0.05$ DGC test). B. G6PDH activity as cytoplasmic marker and control of contamination in periplasmic preparation of the *M. japonicum* strains. Data are representative of three independent replicates. Error bars represent the standard error.



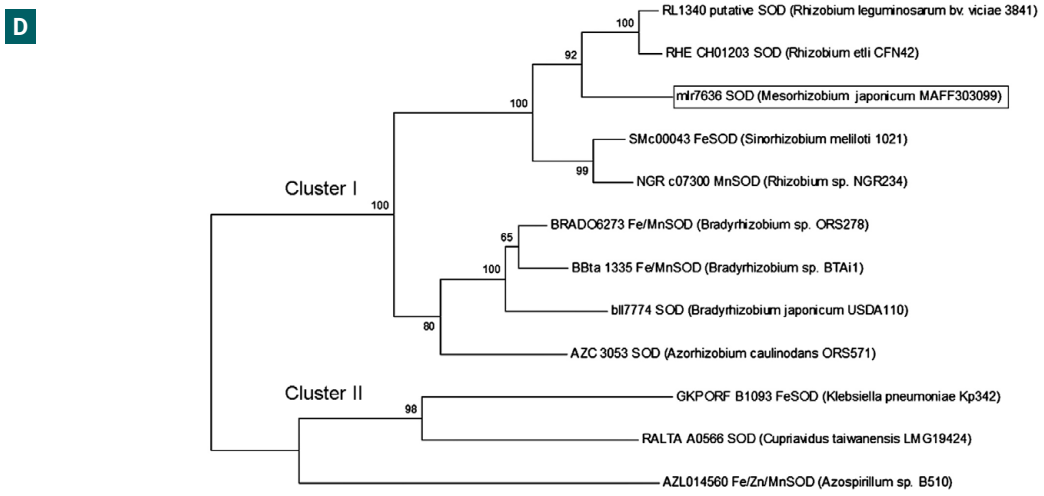


Figure 3. A. Zymogram of total SOD activity of the WT, 1708 and sod1708 strains. Lanes 1-3 correspond to whole cell (WC) and lanes 4-6 correspond to periplasmic (PP) extracts. B. Zymogram of SOD inhibition by 10 mM H2O2. C-Western-blot analysis using anti-FeSOD antibody of the WT, 1708 and sod1708 strains. Lanes 1-3 correspond to whole cell (WC), lanes 4-6 correspond to periplasmic (PP) extracts. Lane M corresponds to prestained SDS-PAGE broad range standard (Bio-Rad). D. Neighbor-joining phylogenetic tree based on aligned sequences of 11 sod amino acid sequences from the Rhizobase database, sharing more than 50% identity with the mlr7636 product.

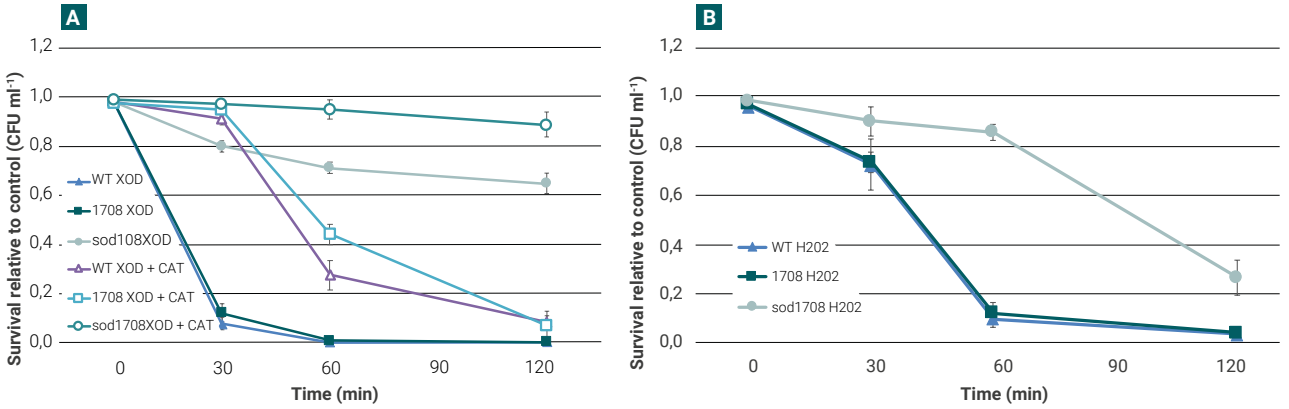


Figure 4. A. Bacterial killing assay of extracellular superoxide anion generated by the hypoxanthine/xanthine oxidase reaction (XOD) against WT, 1708 and sod1708 strains alone, and with addition of 1U Catalase. B- Survival of the WT, 1708 and sod1708 strains to 1 mM H_2O_2. Data are the mean of three independent replicates. Error bars represent the standard error.

a bacterial killing assay using cultures of sod1708, WT and 1708 strains, subjected to exogenously generated O_2^- by the addition of hypoxanthine and xanthine oxidase. The results showed a 20% reduction of CFU/mL in sod1708 strain after 30 min of treatment. This survival reduction was not statistically significant with respect to the untreated control and no further decrease was recorded up to 120 min (fig. 4 A). In contrast, both WT and 1708 strains showed a reduction of more than 90% in viability compared to the untreated control 30 min after O_2^- treatment, with no viable cells being detected, even after 60 min. Catalase supplementation (1 U/mL) to the hypoxanthine/xanthine oxidase system restored the growth of the overexpressing sod1708 strain to similar levels to those of the untreated control (fig. 4 A). On the other hand, exogenously added catalase restored the CFU/mL values to those of untreated cells up to 30 min, whereas a decrease of approximately 55% was observed starting at 60 min.

To test whether increased superoxide tolerance of the sod1708 strain may be due to enhanced H_2O_2 tolerance, a survival percentage was determined by adding 1 mM H_2O_2 to the growth media. Compared with the untreated control, the survival of *M. japonicum* sod strain was not significantly affected up to 60 min, whereas at 120 min, only 26% of bacteria survived. In contrast, WT and 1708 strains showed a reduction of 27% and 90% of CFU/mL after 30 and 60 min, respectively, of hydrogen peroxide treatment (fig. 4 B).

DISCUSSION

Rhizobia are found in the soil, where they are subjected to abiotic stress conditions until legume plants roots are available to infect. Both drought and salinity are severe environmental stress conditions affecting those non-spore forming bacteria. Stress promotes a cascade of reactive oxygen species (ROS), and their accumulation can lead to death by ROS toxicity. The genome of *M. japonicum* MAFF303099 includes the *mlr7636*, *mlr2101* and *mlr6940* genes, which encode a superoxide dismutase, a catalase, and a catalase/peroxidase, respectively (http://genome.annotation.jp/rhizobase/). The complex ROS detoxification mechanism in *M. japonicum* MAFF303099 involves the catabolism of superoxide and H_2O_2, therefore, superoxide dismutase and catalase are two of the main enzymes involved in superoxide and hydrogen peroxide catabolism.

Our results showed that the exposure of *M. japonicum* to saline stress increased both SOD and CAT activities. Moreover, under osmotic stress, SOD activity was reduced, and CAT presented the highest induction in response to both saline and osmotic stress. The induction of SOD and CAT activities under stress conditions coincides with the results reported by Cytryn *et al*. (2007), who described the induction of genes of *B. japonicum* exposed to reduced water activity.

The effects of rhizobial *kat* deletion have been studied in *S. meliloti*. Jamet *et al*. (2003); it demonstrated that *kat* genes are crucial for protection against peroxide and needed for symbiosis establishment with *M. sativa*, whereas the homologous *kat* genes expression, *mlr2101* and *mlr6940*, in *M. japonicum* MAFF303099, showed that these catalases play crucial roles during symbiotic interaction with *Lotus japonicus* (Hanyu *et al*., 2009). On the other hand, Santos *et al*. (1999), reported that the heterologous expression of *S. meliloti* Rm5000 SOD complemented a deficient *E. coli* mutant, restoring the aerobic growth. However, the homologous expression of SOD in rhizobia and its effects on growth and protection against ROS has not been reported yet. In this study, we overexpressed the homologous *mlr7636* sod gene in *M. japonicum* MAFF303099. The zymogram, the western blot and the enzymatic assays demonstrated that the *sod* gene was successfully expressed in sod1708. Therefore, the sod1708 strain showed a 3.4 and 4.1-fold increase in SOD activity in comparison with the WT and 1708 strains under control and osmotic conditions respectively, whereas no differences were found between strains exposed to salt stress. SOD was detected in the periplasmic fractions from the three strains, but sod1708 strain showed higher SOD activity than the non-overexpressing strains, indicating that it was exported to the periplasmic space. Furthermore, the zymograms revealed the presence of three additional SODs in the overexpressing *M. japonicum* sod strain. These bands were inhibited by H_2O_2, indi-

cating that they were FeSOD (Beyer and Fridovich, 1987). In contrast, the lack of inhibition with H_2O_2 demonstrated that the common BSOD corresponded to a MnSOD. It is difficult to explain why the BSOD migration in *M. japonicum* 1708 and *M. japonicum* sod was somewhat different from the BSOD migration observed in the wild-type strain; one possible reason could be metabolic changes occurring in transformed cells (Wang *et al*., 2006). The homologous gene overexpression and the multiple activity bands, differentially inhibited by H_2O_2 in the zymograms, support the assumption that SOD from *M. japonicum* MAFF303099 could have a cambialistic behavior able to form a complex with either Fe or Mn^2; further investigation is necessary to elucidate this aspect. On the other hand, the western blot analysis revealed the presence of single bands of approximately 20.1 kDa in the periplasmic extracts of all strains. In addition, the calculated molecular weight of the detected protein was 2.6 kDa lower than what was theoretically predicted for the *mlr7636* product. Although the signal peptide prediction showed no detectable Sec or TAT signal peptide (Petersen *et al*., 2011; Bendtsen *et al*., 2005), the difference could be due to scission of a signal peptide during the translocation to periplasmic space using an alternative mechanism, as it was described in *R. leguminosarum bv viciae* (Krehenbrink *et al*., 2011). The consensus phylogenetic tree of Mlr7636 revealed that the sod sequence from *M. japonicum* was highly related to *R. leguminosarun bv viciae* 3841, and clustered with FeSOD of *Sinorhizobium meliloti* 1021 (SMc00043) as well as with MnSOD of *Rhizobium* sp. NGR234 (NGR c07300) sequences. The other subgroup in cluster I included sodB from *Bradyrhizobium sp*. BTAi1 and BRADO6273 from *Bradyrhizobium sp*. ORS 278; both of them can use Fe and Mn as co-factor.

SOD overexpression protects against oxidative stress by inducing catalase activity

The protective effect of superoxide dismutase overexpression against oxidative stress in free-living *M. japonicum* has been demonstrated in killing assays. In the presence of exogenously generated superoxide, the sod1708 strain showed a substantially higher tolerance than the controls WT and 1708 strains. The CFU reduction caused by superoxide in the *M. japonicum* sod strain was 20% under oxidative stress conditions, whereas the control strains were non-viable.

The dismutation of superoxide anion by SOD produces H_2O_2 and water; therefore, the addition of catalase to the hypoxanthine/xanthine oxidase reaction in the bacterial killing assay restored the initial CFU values to those reached by the untreated strain. A similar effect was observed in control strains when catalase was added, but in these cases the restored CFU values reached only 45%. These results clearly show that the resulting H_2O_2 from superoxide dismutation induced by SOD activity was more lethal to bacteria than superoxide anion *per se*. Furthermore, our data suggest that homologous superoxide dismutase overexpression increase tolerance to oxidative stress in *M. japonicum* because the high SOD activity *per se* contributes to fast superoxide dismutation to H_2O_2 and O_2; additionally, the induced catalase activity reduces the hydrogen peroxide levels. These findings may conclude that the SOD overexpression in *M. japonicum* MAFF303099 can increase the bacterial tolerance to exogenously generated oxidative stress conditions in free-living stage.

Finally, interaction studies will be required to determine the effect of sod overexpression in sod1708 strain on symbiosis with *L. japonicus*.

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COMPLIANCE WITH ETHICAL STANDARDS

No animals were used in the study.

CONFLICT OF INTEREST

The data acquisition for this work has not been in legal conflict with the authorities where the work was carried out. The authors have no conflicts of interest to declare.

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