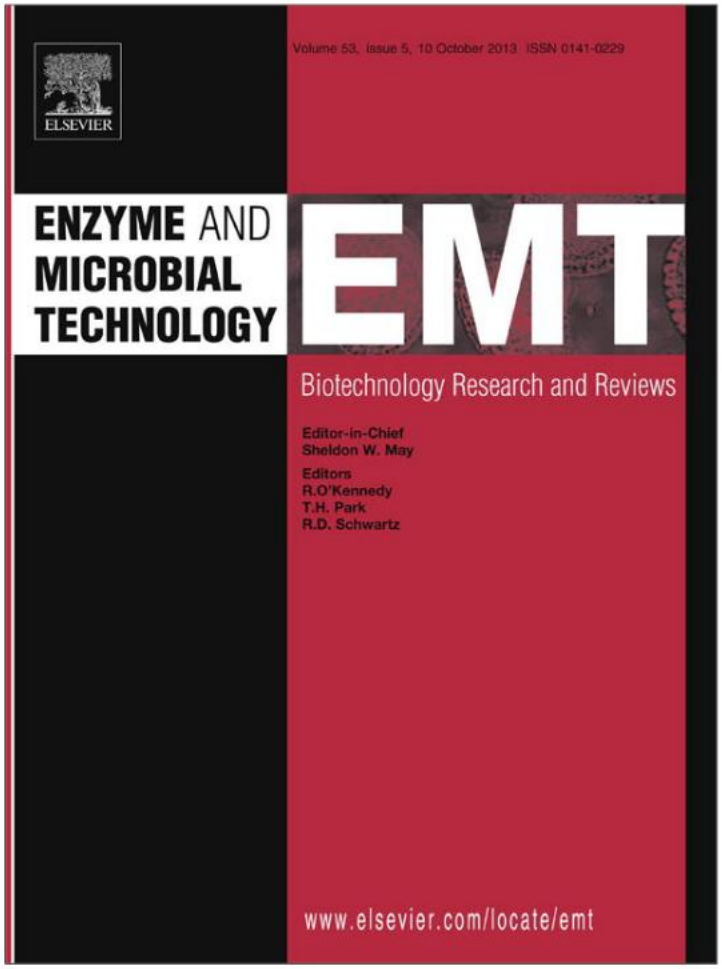

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Antioxidant defense system responses and role of nitrate reductase in the redox balance maintenance in *Bradyrhizobium japonicum* strains exposed to cadmium

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ABSTRACT

In this work, we evaluated the effects of cadmium (Cd) on the antioxidant defense system responses and the role of nitrate reductase (NR) in the redox balance maintenance in *Bradyrhizobium japonicum* strains. For that, *B. japonicum* USDA110 and its NR defective mutant strain (GRPA1) were used. Results showed that the addition of 10 μM Cd did not modify the aerobic growth of the wild type strain while the mutant strain was strongly affected. Anaerobic growth revealed that only the parental strain was able to grow under this condition. Cd reduced drastically the NR activity *in B. japonicum* USDA110 and increased lipid peroxide content in both strains. Cd decreased reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in *B. japonicum* USDA110 although, a significant increased was observed in the mutant GRPA1. GSH-related enzymes were induced by Cd, being more evident the increase in the mutant strain. This different behavior observed between strains suggests that NR enzyme plays an important role in the redox balance maintenance in *B. japonicum* USDA 110 exposed to Cd.

1. Introduction

Nitrogen is a basic element for life being a constituent of main macromolecules, proteins and nucleic acids. Despite de fact that nitrogen is essential for living organisms and consist in 78% of the atmosphere, this form cannot be used by animals or by plants due to the stability conferred by a molecular triple bound. Thus, for use in metabolic processes it must be oxidized or reduced to combined and assimilable forms, such as nitrate (NO_3^-) or ammonium (NH_4^+) [1]. The *N*-cycle includes both reductive and oxidative processes, in which prokaryotes play a predominant role. Nitrogen fixation and nitrate assimilation are assimilatory pathways that generate ammonium that is further incorporated into cell, mainly by the glutamine synthetase–glutamate synthase (GS–GOGAT) route. The first pathway is carried out by free living or symbiotic diazotrophic prokaryotes, while the second pathway is carried out by bacteria, fungi, algae and higher plants, and almost all living organisms able to incorporate ammonium into carbon skeletons. In contrast, some prokaryotes can obtain metabolic energy by redox processes involving nitrogen compounds, such as nitrification and denitrification [2]. The denitrification constitutes one of the three main branches of the biogeochemical nitrogen cycle, in which prokaryotes take place since they have all the enzymes able to carry out this process [3,4]. The nitrate reductase (NR) enzyme catalyze the first step of the denitrification pathway where nitrate (NO_3^-) is reduced to nitrite (NO_2^-). Denitrifying bacteria not only can reduce NO_3^- to NO_2^- but also to nitric oxide (NO) and nitrous oxide (N_2O) to dinitrogen (N_2).

Two types of dissimilatory nitrate reductases have been found in denitrifying bacteria. One is known as the respiratory membrane-bound nitrate reductase (Nar), and the other is the periplasmic nitrate reductase (Nap) [5–7]. It is known that Nap system has different physiological functions such as redox balancing to dissipate excess reducing power in *Paracoccus* and *Rhodobacter* species [8–10]. It catalyses the first step of aerobic denitrification in *Paracoccus denitrificans* [11], and could also participate in adaptation to anaerobic metabolism in *Ralstonia eutropha* [12,13]. Considering many functions of Nap activity, *nap* gene expression can also be different depending on the organism [5]. In *Escherichia coli*, *nap* gene is maximally expressed at low nitrate concentrations under anaerobic conditions [14–17]. On the other hand, the Nap system from *R. eutropha* is maximally expressed under aerobic conditions at the stationary phase of growth and is not induced by nitrate [13]. In

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Paracoccus pantotrophus, maximal *nap* expression is found in cells growing aerobically with butyrate, a highly reduced carbon source, even in the absence of nitrate [8].

Bradyrhizobium japonicum belongs to a group of bacteria collectively referred as rhizobia. It is a Gram negative soil bacterium with high agronomic importance since it is able to establish a symbiotic nitrogen-fixing association with soybean plant, contributing to soil fertility. Once introduced into soils, bradyrhizobia can survive for years and the population level will depend on soil characteristics and soybean cropping history [18]. Denitrification pathway is rare in rhizobia strains since most species do not contain the entire set of denitrification genes. *B. japonicum* is the only true denitrifier rhizobia. It has been shown that it reduces NO_3^- simultaneously to NH_4^+ and N_2 when cultured microaerobically with nitrate not only as a source of nitrogen but also as a terminal electron acceptor. Moreover, *B. japonicum* USDA110, is the only rhizobium in which the denitrification genes (*nap*EDABC, *nir*K, *nor* CBQD and *nos*RZDFYLX) have been isolated and characterized [19].

Cadmium (Cd) is a chemical element that does not have any biological function known and is highly toxic even at low concentration. It can be introduced into agricultural soils through phosphate fertilizers, sewage sludge, and atmospheric fallout from industrial and urban activities [20,21]. In this way, Cd can not only affect soil microorganisms and plants that have a high economic value but also it can accumulate in organisms, transfer from one trophic level to the next and multiply its concentration along the food chain [22]. This metal is known to induce oxidative status alterations in microorganisms, increasing the levels of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals ($^-$OH) [23]. Studies performed on soil bacterial communities exposed to Cd are scarce even more on rhizobia. In a previous work, we have demonstrated that Cd caused oxidative stress, modification of intracellular inclusions, and alterations of the antioxidant defense system in *Bradyrhizobium* sp. (peanut microsymbiont) [24,25].

Enzymatic and non enzymatic defense systems allow microorganisms to maintain their cellular redox status reducing deleterious effects of Cd [26]. GSH is the most abundant intracelular thiol that may complex heavy metals and is involved in ROS sequestration by its oxidation to GSSG [27]. Moreover, enzymatic defense involved in GSH metabolism such as GSH peroxidase (GPx), GSH reductase (GR) and GSH-S-transferase (GST) contribute to mitigate Cd effects [25].

Studies on soil microorganisms growing in heavy metal contaminated environment are scarce. Moreover, it is known that NR enzyme participates in the redox balance dissipating excess reducing power under certain metabolic conditions, however there is no report regarding NR enzyme in redox balance maintenance under heavy metal stress. Therefore, we decided to evaluate the role of NR activity as well as the participation of the antioxidant defense system in *B. japonicum* exposed to Cd.

2. Materials and methods

2.1. Bacterial strains and growth media

B. japonicum USDA110, able to infect soybean (*Glycine max* L.) (USDA, Belstville), and the nitrate reductase mutant (GRPA1), obtained by disruption of the *nap*A gene [28], were used in this study.

Cultures were grown in yeast extract-mannitol (YEM) medium [29] supplemented with different concentrations (0–20 μM) of Cd (as $CdCl_2\,2.5H_2O$) and incubated on an orbital shaker (150 rpm) at 28° C during 72 h.

Antibiotics were added to *B. japonicum* GRPA1 culture at the following concentration: 200 μg/ml of spectinomycin and 200 μg/ml of streptomycin. The number of viable cells was determined at different time points of bacterial growth as colony forming units (CFU)/ml by the drop-plate method [30] on YEM solid medium (15% agar w/v).

Anaerobic cultures were kept at 28° C in YEM medium supplemented with 10 mM KNO_3 in completely filled, rubber-stoppered serum bottles. Antibiotics were added to *B. japonicum* USDA110 GRPA1 culture.

2.2. Determination of nitrate reductase activity

Cells of *B. japonicum* grown aerobically in YEM medium were harvested by centrifugation (8000 × g for 10 min at 4 °C), washed twice with YEM, resuspended in 150 ml of the same medium supplemented with 10 mM KNO_3, and finally incubated under anaerobic conditions for 96 h. After incubation, the cells were washed with 50 mM Tris/HCl buffer (pH 7.5) and then resuspended in 1 ml of the same buffer. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 10 mM KNO_3, 200 mM methyl viologen ($MV^{\bullet}$) and 50 ml cell suspension (0.3–0.5 mg protein). The reaction was started by the addition of 50 ml of freshly prepared sodium dithionite solution (8 mg ml^{-1}) in Tris/HCl buffer. After incubation for 10 min at 30° C, the reaction was stopped by vigorous shaking until the samples had lost their blue color. Nitrite was estimated after diazotation by adding the sulfanilamide/naphthylethylene diamine dyhydrochloride reagent [31].

2.3. Assessment of oxidative damage

The quantification of lipid peroxide was estimated by determining MDA (malondialdehyde) reacting to TBA (thiobarbituric acid)-reactive substance following the method described by [32] with modifications. Bacterial pellets were obtained from cultures in late exponential phase of growth by centrifugation for 10 min at 10,000 × g at 4°C and washed twice with 0.85% sterile NaCl, as previously described [24]. Samples were resuspended in 50 mM phosphate buffer at pH 7 and 28 μl of trichloroacetic acid (TCA), vortexed, sonicated (width: 80; time: 2 min, pulse, every 6 s) and then centrifugated at 2000 × g at 4°C for 15 min. Finally, 100 μl of supernatant were added to an assay mixture of 100 μl of 0.1 M EDTA and 600 μl of a solution of 1% (w/v) TBA and 50 mM NaOH. Samples were kept in boiling water for 15 min and, after cooling, the absorbance at 532 nm was measured.

2.4. Determination of glutathione content

Cell pellets were obtained from bacterial cultures at late exponential phase of growth by centrifugation for 10 min at 10,000 × g at 4°C. Cells were homogenized in 5% 5-sulfosalicilic acid using a homogenizer with plunger and centrifuged for 10 min at 10,000 × g at 4° C to remove cell debris and then washed twice with 0.85% sterile NaCl. The supernatants were used to measure total GSH content [33]. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated as the difference between the two forms. The supernatant (15 μl) was added to 0.174 mg ml^{-1} NADPH, 0.6 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and distilled water to complete 1 ml. This mixture was incubated for 15 min at 30°C. Then, 5 μl of glutathione reductase (266 U ml^{-1}) were added. The reaction was followed at 412 nm and the total GSH content was calculated from a standard curve using GSH (0–30 μmol). In order to measure GSSG the supernatant was incubated with 2 μl of 2-vinylpyridine followed by 5 μl of triethanolamine (TEA), the latter being placed on the side of the tube above the level of the liquid. 2-Vinylpyridine and TEA were added to ensure that GSSG is the only form of glutathione that can react with the DTNB reagent. The solution was vortex-mixed for 30 s and left at room temperature for 60 min. Afterwards, 100 μl of the resultant solution was assayed as described above. Calibration curves were carried out using GSSG samples (0–10 μmol) treated exactly as above. The intracellular GSH content was expressed as μmol GSH/mg protein. Protein was estimated by the Coomassie blue staining method with bovine serum albumin as standard [34].

2.5. Enzymatic assays

Bacterial pellets were obtained as described in assessment of oxidative damage. Pellets were resuspended in 2 ml of extraction buffer (50 mM phosphate potassium buffer, EDTA pH 7.5) and sonicated (as described before). The supernatants were used to determine enzymatic activities.

GPx activity was determined as described by Flohe and Gunzler [35]. The assay mixture in 1 ml contained 100 μl of protein extract, 0.1 M phosphate buffer at pH 7.4, 0.24 U GR and 10 mM GSH. Then 1.5 mM NADPH and 1.5 mM H_2O_2 were added. The reaction was measured following NADPH oxidation at 340 nm. One unit of GPx is defined as the quantity of enzyme needed to produce 1 μmol $NADP^+$/min. GR activity was determined as described by Sheadle and Bassham [36]. The assay mixture in 1 ml contained 300 μl protein extract, 0.5 mM GSSG and reaction buffer (50 mM Tris–HCl, 0.15 mM NADPH, 3 mM $MgCl_2$ at pH 7.5). A reaction without GSSG was performed in order to determine the non-specific consumption of NADPH. The reduction of GSSG by GR was followed by the NADPH oxidation at 340 nm. One unit of GR is defined as the quantity of enzyme needed to produce 1 μmol $NADP^+$/min. GST activity was assayed by Habig et al. [37] technique, measuring GSH conjugates with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay mixture in 1 ml contained 0.1 M phosphate buffer at pH 7, 0.25 mM CDNB, 4 mM GSH and 200 μg protein extract. The enzymatic activity was followed continuously at 340 nm. One unit of GST is defined as the quantity of enzyme needed to produce 1 μmol conjugated CDNB-GSH/min.

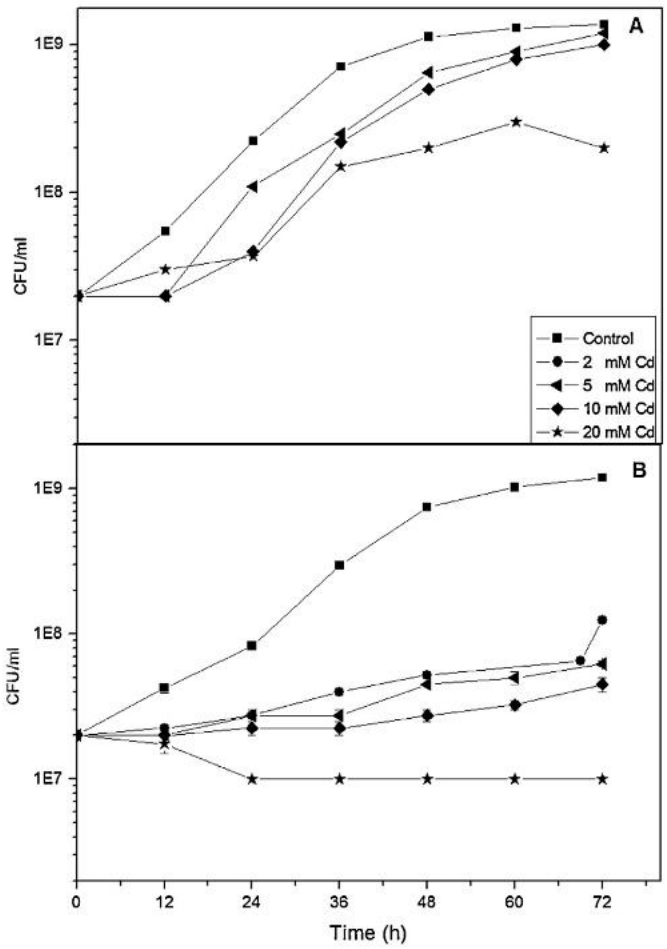

Fig. 1. Aerobic growth of wild type *B. japonicum* USDA110 (A) and *napA* mutant derivative GRPA1 (B) at different cadmium concentrations.

2.6. Statistical analysis

Differences among treatments were analyzed by two-way ANOVA at $P < 0.05$ as significant according to Duncan's test.

3. Results

3.1. Effects of cadmium on cell viability

Bradyrhizobium sp. strains were grown on YEM complex medium to analyze whether Cd affects their growth by measuring the viable cell number each 24 h. The wild-type strain (*B. japonicum* USDA110) grew up to 20 μM Cd (Fig. 1A) meanwhile the NR defective mutant strain (GRPA1) was unable to grow at this concentration (Fig. 1B). At the Cd concentration evaluated, both strains showed an extended lag growth phase, being clearly more evident in the *napA* mutant. Anaerobic growth revealed that only the parental strain was able to grow under this condition (Fig. 2).

3.2. Nitrate reductase activity in response to Cd

NR activity of the wild type was 777.42 ± 0.67 and 307.28 ± 0.99 nmol nitrite/min/mg protein in control and Cd-treated conditions, respectively. This results showed a 60% of significant reduction of NR activity when strain was exposed to Cd. In order to corroborate the mutation of *napA* gene, nitrate reductase activity was measured on the mutant strain. In control conditions, the activity found in *B. japonicum* GRPA1 (95.94 nmol nitrite/min/mg protein) represent only 12% of total activity observed in the wild type strain at the same condition.

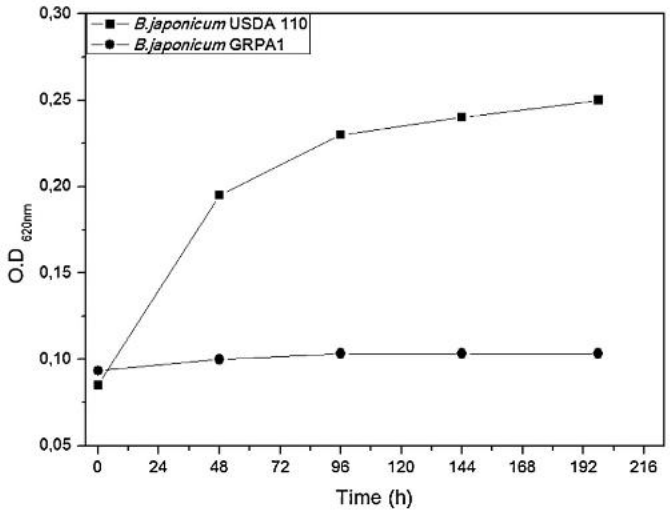

Fig. 2. Nitrate-dependent anaerobic growth of wild-type *B. japonicum* USDA110 and *napA* mutant derivative GRPA1.

3.3. Lipid peroxidation

Quantification of MDA content in *B. japonicum* USDA110 showed a significant increased when exposed to 10 μM of Cd compared to control conditions. This behavior was also observed in the mutant strain GRPA1 (Fig. 3).

3.4. Biothiols induced by cadmium in bacterial cells

GSH content of the wild type and the mutant strains showed a significant increased when exposed to Cd. However, GSSG remained unchanged in both strains.

Comparison between strains exposed to metal showed that *B. japonicum* GRPA1 was able to induce more GSH than the wild type strain. In control condition, GSSG content was higher in the mutant strain than the wild type one and this behavior was also observed after Cd treatment (Table 1).

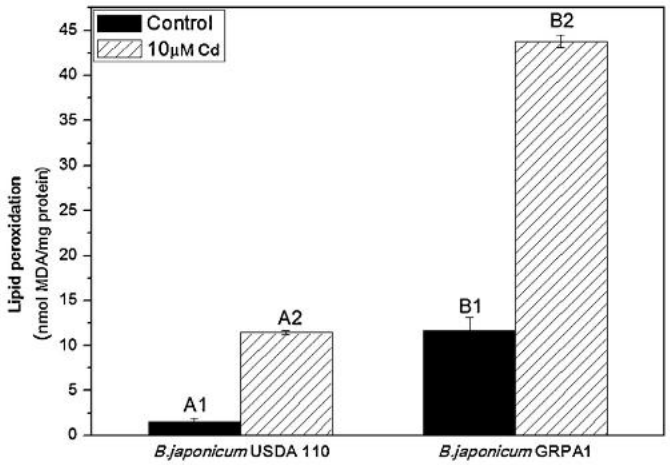

Fig. 3. MDA content in *Bradyrhizobium japonicum* exposed to 10 μM cadmium. Data represent the mean ± SE ($n = 4$). Different letters in each column indicate significant differences between strains for a same treatment. Different numbers in each column indicate significant differences between treatments for each strain ($P < 0.05$) according to the Duncan's test.

Table 1
Glutathione content in *Bradyrhizobium japonicum* strains exposed to cadmium.

Strains	GSH (nmol/mg protein)		GSSG (nmol/mg protein)		GSH/GSSG ratio	
	Cadmium addition (μM)					
	0	10	0	10	0	10
USDA 110	1.77 ± 0.12 A1	3.35 ± 0.36 A2	0.01 ± 0.01 A1	0.03 ± 0.01 A1	177	111
GRPA1	1.60 ± 0.01 A1	14.11 ± 1.96 B2	0.19 ± 0.01 B1	0.19 ± 0.01 B1	8.42	74.2

Data represent the mean ± SE ($n = 4$). Different numbers in each row indicate significant differences between treatments for each strain. Different letters in each column indicate significant differences between strains for a same treatment ($P < 0.05$) according to Duncan's test.

3.5. Glutathione related enzymes

The determination of GSH-related enzymes (GPx, GR and GST) of *B. japonicum* strains showed different responses to Cd exposure (Fig. 4A–C). GPx activity was strongly induced by Cd in both strains however, the enzyme basal activity in the mutant strain was higher than the wild type one. Even when this behavior was also observed for GR, increased enzyme activity induced by Cd was greater in the mutant strain.

Cd caused an increase of GST activity in both strains. However, this increase was higher in the wild type strain (5 fold). In contrast, no differences were observed when comparing the strains between equivalent treatments.

4. Discussion

It is documented that one of the main effects of Cd on microorganisms is the alteration of the cell oxidative status, increasing ROS production [23] and inducing lipid, protein and DNA alterations [38,39]. Until now, there is no experimental evidence that involve NR with Cd oxidative stress. It is known that this enzyme is active both aerobically and anaerobically and is involved in the maintenance of the redox status of the cells in *Rhodococcus* [40]. Growth rate differences observed in *B. japonicum* strains could be due to the oxidative cell status modified by the presence of the metal that induced oxidative stress. Nevertheless, the wild type strain grew up to 20 μM Cd, suggesting that the presence of NR allows cells to maintain its redox status and therefore confers a higher Cd tolerance.

Denitrification is an important process in the biological cycle of nitrogen, where bacteria play an important role as they have all the enzymatic machinery to carry out this process [3,4]. Liu et al. [41] studied Cd and Pb effects on urease, phosphatase, catalase and invertase enzymatic activities of soil bacteria and fungi. The results obtained showed that the most negative effects were observed after Cd treatment. Our results demonstrated that Cd decreased NR activity of *B. japonicum* USDA110. Considering that Cd induce oxidative stress by depletion of the enzymatic defense system [42], it could be suggest that in the wild type strain, the reduction of NR activity is related to an increase in ROS production affecting its functionality.

Literature reviewed indicates that Cd is able to induce lipid peroxide in eukaryotic cells [43,44] however, studies on prokaryotic cells are scarce. Cd does not experiment redox changes, so it is not involved in ROS production in a direct way. ROS production could be due to a displacement of bounding Fe^{2+} protein that leaves this ion available to participate on Fenton-type reactions, causing oxidative stress. Pacheco et al. [45] reveled that 30 μg/ml Cd induced lipid peroxides on *E. coli* K-12 BW25113. Similar results were found in the sensitive peanut microsymbiont *Bradyrhizobium* sp. SEMIA6144 strain, exposed to 10 μM Cd [25]. Results obtained in this study are in agreement with these authors since Cd induced an increase in the MDA content in *B. japonicum* strains. Cd damage on membrane lipids of the wild type strain was similar to the

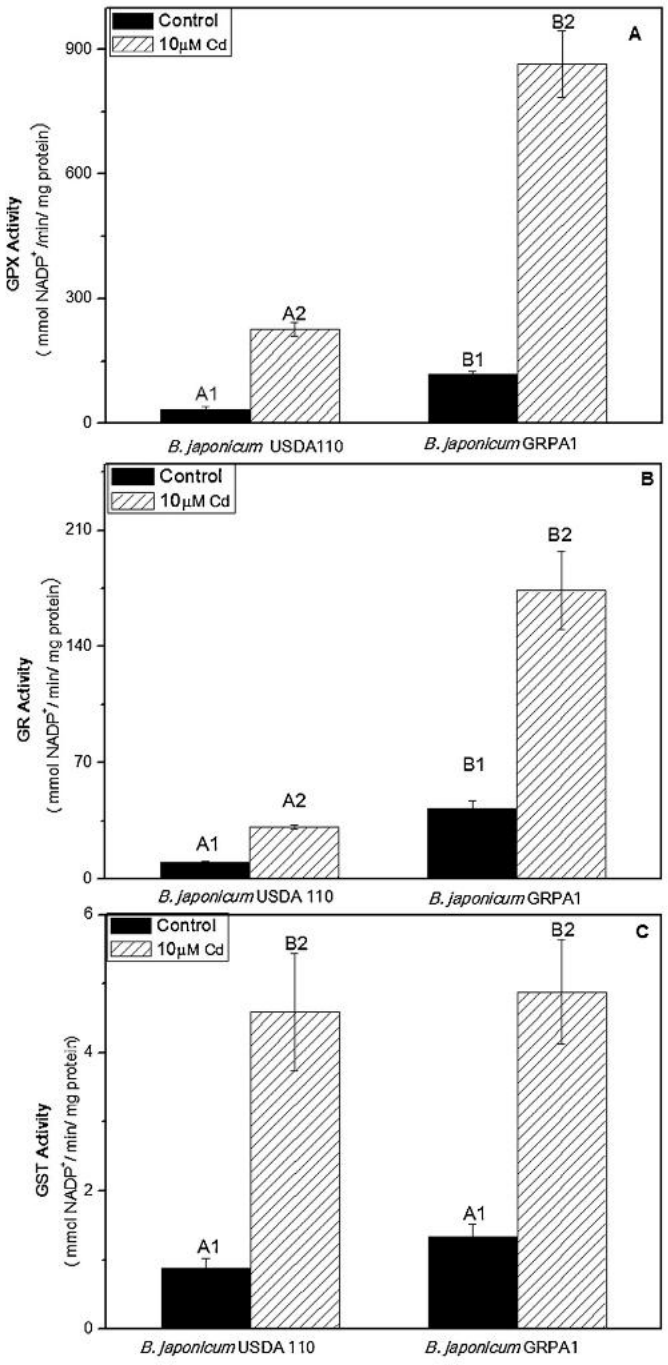

Fig. 4. Activities of GSH related enzymes in *Bradyrhizobium japonicum* USDA 110 and GRPA1 exposed to 10 μM cadmium. (A) GPx. (B) GR. (C) GST. Data represent the mean ± SE ($n = 4$). Different letters in each column indicate significant differences between strains for a same treatment. Different numbers in each column indicate significant differences between treatments for each strain ($P < 0.05$) according to the Duncan's test.

mutant strain, but is important to highlight that in control condition *B. japonicum* GRPA1 had higher lipid peroxide content than the wild type strain. Cd reduced *B. japonicum* growth and induced lipid peroxide, being more evident in the mutant strain. Therefore, it could indicate that NR is involved in cellular redox balance contributing to a better adaptation against metal. Probably, in the wild type strain the NR enzyme and the antioxidant defense system could be playing an important role that allows *B. japonicum* USDA110 to have a high viability, even when lipid peroxide content is enhanced, under Cd stress.

GSH is the most abundant intracellular thiol that may complex heavy metals and is involved in ROS sequestration by its oxidation to GSSG [27]. This molecule keeps a strong reducing environment in the cell and participates in different stressing conditions such as acidity, salinity, H_2O_2 and methylglyoxal reducing cellular damage [46,47]. Corticeiro et al. [48] demonstrated that Cd addition produced a totally oxidation of GSH in *Rhizobium leguminosarum* biovar *viciae*. Moreover, studies performed on *Bradyrhizobium* sp. revealed that GSH contribute to the tolerance of the peanut microsymbiont strains against Cd toxicity [24]. In both strains, Cd induced GSH synthesis although the GSH/GSSG ratio was different, since in *B. japonicum* USDA110 this ratio was decreased while the opposite result was observed in the mutant strain. This differential response of the studied strains is due to a significant increment on GSH content while the level of GSSG kept unaltered in the mutant strain compared with the wild type one. Furthermore, GSSG content was significantly higher in *Bradyrhizobium* GRPA1 strain than in the wild type one, under control condition. Taken together, the increase in biothiols content and the elevated amount of lipid peroxide found on the mutant strain suggest that NR enzyme contributes to cellular redox balance in bacteria. The elevated GSH/GSSG ratio observed in the mutant strain could result in higher concentrations of free GSH, allowing to cell to maintain a reduced environment and mitigate ROS effects.

In order to evaluate whether GPx, GR and GST enzymes are involved against Cd toxicity, their specific activities were analyzed on *B. japonicum* strains exposed to metal. GPx and GR enzymes have an important role in GSH/GSSG maintenance being crucial to keep cellular redox status. Our results showed that GPx and GR activity were significantly induced by Cd, being the increment of GR activity higher in the mutant strain than in the wild type one. These results are in agreement with those obtained by Bianucci et al. [25] and Corticeiro et al. [48] where a strongly induction of the GSH-related enzymes was observed in rhizobia strains exposed to Cd. Our results revealed that the GR enzyme is being strongly induced to transform GSSG to GSH. Thus, free GSH is available to be complexed to Cd ions and mitigate ROS induced damage.

Glutathione-S-transferases (GSTs) are detoxifying enzymes present in all aerobic organisms [49]. However, less information is available on the biological functions of bacterial GSTs, being mainly related to growth and degradation of aromatic compounds [49,50]. It was already demonstrated in *S. cerevisiae* that GSTs appear to be crucial against ROS as well as in overall cellular detoxification of toxic oxidants such as H_2O_2 and arsenic species [51]. Studies of GST activity performed on prokaryotic cell exposed to heavy metals are scarce. Results obtained by Bianucci et al. [25] revealed that 10 μM Cd decreased GST activity in *Bradyrhizobium* sp. strains. In this study, GST activity increased in both *B. japonicum* strains, although this increment was higher in the wild type strain. GST enzymatic induction by Cd suggest that this enzyme could be forming conjugates with GSH and oxidative stress products (oxidized lipids, proteins and DNA) avoiding metal cellular damage. Particularly, GSH content and GR activity in the mutant strain were higher than in the wild type strain. Possibly, a still undescribed mechanism allows cells to increase free GSH availability instead of being used by GST enzyme.

The differential behavior observed in the *B. japonicum* strains suggest that NR enzyme would have an important role, allowing the microorganism to maintain a correct redox balance and limiting metal caused damage, particularly on ROS generation.

5. Conclusion

In *B. japonicum* USDA110, NR activity would have an important role in the cellular redox balance maintenance against Cd toxicity. Thus, NR activity, together with the antioxidant system, could allow to bradyrhizobial cells to have a better growing, avoiding oxidative damage to macromolecules by Cd exposure.

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References

[1] Taiz L, Zeiger E. Plant physiol. The Benjamin-Cumming Publishing Company. Inc.; 1991. p. 559.
[2] Cabello P, Roldan MD, Moreno-Vivian C. Nitrate reduction and the nitrogen cycle in archaea. Microbiology 2004;150(11):3527–46.
[3] Zumft WG. Cell biology and molecular basis of denitrification. MMBR 1997;61(4):33–616.
[4] Richardson DJ, Watmough NJ. Inorganic nitrogen metabolism in bacteria. Curr Opin Chem Biol 1999;3(2):207–19.
[5] Moreno-Vivián C, Cabello P, Martínez-Luque M, Blasco R, Castillo F. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J Bacteriol 1999;181(21):6573–84.
[6] Richardson DJ, Berks BC, Russell DA, Spiro S, Taylor CJ. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell Mol Life Sci 2001;58(2):165–78.
[7] Potter L, Angove H, Richardson D, Cole J. Nitrate reduction in the periplasm of Gram-negative bacteria. Adv Microb Physiol 2001;45:51–112.
[8] Sears HJ, Sawers G, Berks BC, Ferguson SJ, Richardson DJ. Control of periplasmic nitrate reductase gene expression (*nap*EDABC) from *Paracoccus pantotrophus* in response to oxygen and carbon substrates. Microbiology 2000;146(11):2977–85.
[9] Ellington MJK, Bhakoo KK, Sawers G, Richardson DJ, Ferguson SJ. Hierarchy of carbon source selection in *Paracoccus pantotrophus*: strict correlation between reduction state of the carbon substrate and aerobic expression of the *nap* operon. J Bacteriol 2002;184(17):4767–74.
[10] Gavira M, Roldan MD, Castillo F, Moreno-Vivián C. Regulation of nap gene expression and periplasmic nitrate reductasa activity in the phototrophic bacterium *Rhodobacter sphaeroides* DSM158. J Bacteriol 2002;184(6):1693–702.
[11] Richardson DJ, Wehrfritz JM, Keech A, Crossman LC, Roldan MD, Sears HJ, Butler CS, Reilly A, Moir JW, Berks BC, Ferguson SJ, Thomson AJ, Spiro S. The diversity of redox proteins involved in bacterial heterotrophic nitrification and aerobic denitrification. Biochem Soc Trans 1998;26(3):401–8.
[12] Warnecke-Eberz U, Friedrich B. Three nitrate reductase activities in *Alcaligenes eutrophus*. Arch Microbiol 1993;159(5):405–9.
[13] Siddiqui RA, Warnecke-Eberz U, Hengsberger A, Schneider B, Kostka S, Friedrich B. Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. J Bacteriol 1993;175(18):5867–76.
[14] Darwin AJ, Ziegelgoffer EC, Kiley PJ, Stewart V. FNR, NarP and NarL regulation of *Escherichia coli* K-12 *nap*F (periplasmic nitrate reductase) operon transcription in vitro. J Bacteriol 1998;180(16):4192–8.
[15] Grove J, Tanapongpipat S, Thomas G, Griffiths L, Crooke H, Cole J. *Escherichia coli* K-12 genes essential for the synthesis of c-type cytochromes and a third nitrate reductase located in the periplasm. Mol Microbiol 1996;19(3):476–81.
[16] Potter LC, Cole JA. Essential roles for the products of the *nap*ABCD genes, but not *nap*FGH, in periplasmic nitrate reduction by *Escherichia coli* K-12. Biochem J 1999;344:69–76.
[17] Wang H, Tseng CP, Gunsalus RP. The *nap*F and *nar*G nitrate reductase operons in *Escherichia coli* are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. J Bacteriol 1999;181(17):5303–8.
[18] Catroux G, Revellin C, Hartman A. Practical Aspects of legume inoculation: inoculant quality and inoculation efficacy Consecuences for the soil microflora. In: Laich, Gonzales, Etcheverría, editors. Actas del Seminario microorganismos útiles para la agricultura y la forestación. La Pampa. Balcarce: INTA/CERBAS; 1996. p. 108–25.

[19] Bedmar EJ, Robles EF, Delgado MJ. The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. Biochem Soc Trans 2005;33:141–4.
[20] McGrath SP, Chaudri AM, Giller KE. Long-term effects of metals in sewage sludge on soils, microorganisms and plants. J Ind Microbiol 1995;14(2):94–104.
[21] Giller KE, Witter E, McGrath SP. Toxicity of heavy metals to microorganisms and microbial process in agricultural soils: a review. Soil Biol Biochem 1998;30(10–11):1389–414.
[22] De Acevedo F. Toxicologia do Mercurio. Sao Paulo: RiMa; 2003. p. 292.
[23] Sandalio LM, Dalurzo HC, Gomez M, Romero-Puertas MC, del Río LA. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot 2001;52(364):2115–26.
[24] Bianucci E, Fabra A, Castro S. Cadmium accumulation and tolerance in *Bradyrhizobium* spp. (peanut microsymbionts). Curr Microbiol 2011;62(1):96–100.
[25] Bianucci E, Fabra A, Castro S. Involvement of glutathione and enzymatic defenses system against cadmium toxicity in *Bradyrhizobium sp.* strains (peanut symbionts). Biometals 2012;25(1):23–32.
[26] Silver S, Phung LT. A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. J Ind Microbiol Biotechnol 2005;32(11–12):587–605.
[27] Sies H. Glutathione and its role in cellular functions. Free Radic Biol Med 1990;27(9–19):916–21.
[28] Delgado MJ, Bonnard N, Tresierra-Ayala A, Berdman EJ, Muller P. The *Bradyrhizobium japonicum napEDABC* genes encoding the perisplamic nitrate reductasa are essential for nitrate respiration. Microbiology 2003;149(12):3395–403.
[29] Vincent J. A manual for the practical study of root-nodule bacteria. In: International biological programme. Handbook No. 15. Oxford: Blackwell Scientific Publication; 1970.
[30] Somasegaran P, Hoben H. Quantifying the growth of rhizobia. In: Handbook for rhizobia. New York: Springer; 1994. p. 47–57.
[31] Nicholas DJD, Nason A. Determination of nitrate and nitrite. Methods Enzymol J 1957;3:981–4.
[32] Steels EL, Learmonth RP, Watson K. Stress tolerance and membrane lipid unsaturation in *Saccharomyces cerevisiae* grown aerobically or anaerobically. Microbiology 1994;140(3):569–76.
[33] Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 1985;113:548–55.
[34] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[35] Flohe L, Gunzler W. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114–21.
[36] Sheadle M, Bassham J. Chloroplast glutathione reductase. Plant Physiol 1977;59(5):1011–2.
[37] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249(22):7130–9.
[38] Romero-Puertas MC, Palma JM, Gómez M, del Río LA, Sandalio LM. Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ 2002;25(5):677–86.
[39] Waisberg M, Joseph P, Hale B, Beyersmann D. Molecular and cellular mechanism of cadmium carcinogenesis. Toxicology 2003;192(2-3):95–117.
[40] Richardson DJ, King GF, Kelly DJ, McEwan AG, Ferguson FJ, Jackson JB. The role of auxiliary oxidants in maintaining redox balance during phototrophic growth of *Rhodobacter capsulatus* on propionate or butyrate. Arch Microbiol 1988;150(2):131–7.
[41] Liu S, Yang Z, Wang X, Zhang X, Gao R, Liu X. Effects of Cd and Pb pollution on soil enzymatic activities and soil microbiota. Front Agric China 2007;1(1):85–9.
[42] Pinto E, Sigaud-Kutner TCS, Leitão M, Okamoto OK, Morse D, Colepicolo P. Heavy metal-induced oxidative stress in algae. J Phycol 2003;39(6):1008–18.
[43] Howlett NG, Avery SV. Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and influence of plasma membrane fatty acid unsaturation. Appl Environ Microbiol 1997;63(8):2971–6.
[44] Sarkar S, Yadav P, Bhatnagar D. Lipid peroxidative damage on cadmium exposure and alterations in antioxidant system in rat erythrocytes: a study with relation to time. Biometals 1998;11(2):153–7.
[45] Pacheco CC, Passos JF, Castro AR, Moradas-Ferreira De Marco P. Role of respiration and glutathione in cadmium-induced oxidative stress in *Escherichia coli* K-12. Arch Microbiol 2008;189(3):271–8.
[46] Sobrevals L, Muller P, Fabra A, Castro S. Role of glutathione in growth of *Bradyrhizobium* sp. (peanut microsymbiont) under different environmental stresses and in symbiosis with the host plant. Can J Microbiol 2006;52(7):609–16.
[47] Bianucci E, Fabra A, Castro S. Growth of *Bradyrhizobium* sp. SEMIA 6144 in response to methylglyoxal: role of glutathione. Curr Microbiol 2008;56(4):371–5.
[48] Corticeiro SC, Lima AI, Figueira EM. The importance of glutathione in oxidative status of *Rhizobium leguminosarum* biovar *viciae* under Cd exposure. Enzyme Microb Technol 2006;40(1):132–7.
[49] Vuilleumier S, Pagni M. Bacterial glutathione S-transferases: new lessons from bacterial genomes. Appl Microbiol Biotechnol 2002;58:138–46.
[50] Allocati N, Federici L, Masulli M, Di Ilio C. Glutathione transferases in bacteria. FEBS J 2009;276(1):58–75.
[51] Todorova D, Nedeva D, Abrashev R, Tsekova K. Cd (II) stress response during the growth of *Aspergillus niger* B 77. J Appl Microbiol 2007;104(1):178–84.