Effect of Copper on Expression of Functional Genes Associated with *Bradyrhizobium diazoefficiens* Denitrification

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Abstract

Link to the GitHub repository: https://github.com/pachecopedrojose025/proyecto_final

Nitrous oxide (N_2O) is a powerful greenhouse gas that contributes to climate change. Denitrification is one of the largest sources of N_2O in soils. The soybean endosymbiont *Bradyrhizobium diazoefficiens* is a model for rhizobial denitrification studies since, in addition to carry out the biological nitrogen fixation process, it has the ability to grow anaerobically under free-living conditions by reducing nitrate from the medium through the complete denitrification pathway. This bacterium contains a periplasmic nitrate reductase (Nap), a copper (Cu)-containing nitrite reductase (NirK), a *c*-type nitric oxide reductase (cNor), and a Cu-dependent nitrous oxide reductase (Nos) encoded by the *napEDABC*, *nirK*, *norCBQD* and *nosRZDFYLX* genes, respectively. In this work, the involvement of Cu in growth and denitrification genes expression has been investigated in *B. diazoefficiens*. A notable reduction in growth as well as in *nirK*, *nor* and *nos* gene expression was observed under oxygen-depleted and Cu-limiting conditions. However, *nap* expression was not affected under these conditions. Our results demonstrate, for the first time, the role of Cu in transcriptional control of *B. diazoefficiens* denitrification. Thus, this study will contribute by proposing useful strategies for reducing N_2O emissions from agricultural soils.

Keywords

Cu-containing nitrite reductase; enzymatic activity; gene expression; nitric oxide reductase; nitrous oxide reductase; periplasmic nitrate reductase

1 Introduction and State of Art

With a 300-fold greater global warming potential than carbon dioxide (CO_2), nitrous oxide (N_2O) is one of the main biogenic greenhouse gases (GHG), and has also been described as the biggest single cause of ozone depletion[1]. N_2O emissions from human activities, fundamentally Agriculture, Forestry and Other Land Use (AFOLU), have notably increased since the Green Revolution in the early 60s. During the period 2007–2016, these activities represented 81% of the anthropogenic emissions of N_2O, according to the last special report by the Intergovernmental Panel on Climate Change[2]. In particular, agriculture has become the major source of N_2O emissions, accounting for approximately 78% of the anthropogenic N_2O sources[2] because of a global agricultural intensification and a great increase in the non-synchronized use of synthetic nitrogen fertilisers[3][4][5]. Several biological pathways occurring in agricultural soils are involved in N_2O emissions. Among all of them, nitrification and denitrification are the main microbial N_2O sources directly affected by soil nitrogen fertilisation, but only denitrification is known to be the largest source of N_2O[6].

Apart from other organisms, such as archaea and fungi, some facultative bacteria possess the ability to adapt their metabolism to an oxygen-depleted environment in the presence of nitrate as a respiratory substrate through the activation of denitrification. This pathway consists of the dissimilatory reduction of nitrate or nitrite to dinitrogen (N_2) via the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O). In this process, specific metalloenzymes are sequentially involved: periplasmic (Nap) or membrane-bound

(Nar) nitrate reductases, copper (Cu)-containing (NirK) or cytochrome cd_1-containing (NirS) nitrite reductases, nitric oxide reductases (cNor, qNor or Cu_ANor), and nitrous oxide reductase (Nos). The majority of denitrifiers are found in the phylum Proteobacteria, within the domain Bacteria. The alpha-proteobacterium *Paracoccus denitrificans* and the gamma-proteobacteria *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* are the first model organisms where denitrification were widely studied. Reviews covering the physiology, biochemistry and molecular genetics of denitrification have been published elsewhere[7][8][9][10][11][12].Over recent years, several reports about denitrification in plant endosymbiotic bacteria emerged[13][14][15]. Thanks to their capacity to establish a N_2-fixing symbiotic relationship with plants, these bacteria can contribute to natural N soil enrichment, while reducing the need for chemical fertilisation. Therefore, symbiotic N_2 fixation is considered a process with economic, ecological and agricultural importance. In this process, a mutualist association between soil bacteria, commonly known as rhizobia, and plants of the Fabaceae family is established. Rhizobia may induce the formation of nodules in the legume roots and on the stems of some aquatic legumes; nodules are specialized structures where N_2 fixation takes place[16].

Bradyrhizobium diazoefficiens, which establishes N_2-fixing symbiosis with soybean (*Glycine max*), is considered a model organism in the study of denitrification in rhizobia because it is the only known rhizobia species able to grow under oxygen-limiting conditions with nitrate as sole electron acceptor and, also, to perform the complete denitrification pathway under both free-living and symbiotic conditions[13]. Denitrification in *B. diazoefficiens* is carried out by a periplasmic nitrate reductase (Nap), encoded by the *napEDABC* operon[17], a Cu-containing nitrite reductase (NirK), encoded by the *nirK* gene[18], a cytochrome *c*-type nitric oxide reductase (cNor), encoded by the *norCBQD* operon[19], and a Cu-dependent nitrous oxide reductase (Nos), encoded by the *nosRZDFYLX* genes[20]. Nap is a functional heterodimer comprising the catalytic subunit NapA of about 90 kDa that contains a bis molybdopterin guanine dinucleotide ($Mo[MGD]_2$) cofactor and a [4Fe-4S] centre, and NapB (15 kDa) that contains 2 heme *c* groups and receives electrons from the membrane-bound NapC (25 kDa) which binds 4 heme *c* groups. NirK is a homotrimer with a predicted molecular mass of about 35 kDa per monomer that contains type 1 and type 2 Cu centres. The catalytic subunit of cNor, NorB, contains heme *b* and a binuclear active centre (heme b_3 and Fe_B). NorC is a membrane-anchored protein (16 kDa) that contains heme *c*. Finally, the catalytic subunit of Nos, NosZ (120–160 kDa), is a homodimer Cu-containing enzyme with two distinct Cu centres (Cu_A and Cu_Z).

Similarly to many other denitrifiers, expression of denitrification genes in *B. diazoefficiens* requires both oxygen limitation and the presence of nitrate or a derived nitrogen oxide (NOx), this control being mediated by the FixLJ-$FixK_2$-NnrR regulatory cascade[21][22][23]. In fact, the expression of *napEDABC*, *nirK* and *nosRZDFYLX* genes requires microoxic conditions and directly depends on the transcriptional regulator $FixK_2$[23][24], while expression of *norCBQD* genes relies on NO, being NnrR the transcriptional regulator which directly interacts with the *norCBQD* promoter[23][25]. In this context, the molecular discriminatory determinants for selective $FixK_2$ recognition and target activation were recently unveiled[26].

Besides being a source of N_2O, the ecological and environmental importance of denitrification lies in the fact that Nos is the only known enzyme able to remove N_2O from ecosystems[4], the expression and activity of this enzyme becoming a natural target to effectively reduce N_2O emissions from agricultural soils. Increasing knowledge of the regulation and biochemistry of N_2O metabolism in rhizobia will raise opportunities for the design of effective mitigation strategies to reduce N_2O emissions from legume crops[27].

Nowadays, new environmental factors are emerging as candidates for controlling denitrification, such as pH[28][29] or Cu[30]. In the case of Cu, it is an essential cofactor in critical enzymes, such as multicopper oxidases, as well as the Nos and NirK denitrification enzymes. The role of this metal in denitrification has been studied in a wide range of non-symbiotic microorganisms, such as *Pseudomonas perfectomarinus*[31], *P. stutzeri*[30], *P. denitrificans*[32][33] and *Achromobacter xylosoxidans*[32]. Regarding rhizobia, Serventi et al. (2012)[34] investigated the role of Cu in cytochrome oxidase biogenesis in *B. diazoefficiens*. Nevertheless, studies covering Cu influence on the denitrification pathway in rhizobia are scarce. In this study, the effect of different Cu regimes on growth and gene expression is analysed in free-living cultures.

2 Materials and Methods

2.1 Bacterial strains and growth conditions

Bacterial strains used in this study are compiled in Table 1.

Table 1: *B. diazoefficiens* strains used in this study.

Strains	Relevant description	Reference
110spc4	Wild-type, Cm^r, Spc^r	[35]
BG0602	*napE-lacZ* fusion-containing strain derived from 110spc4, Cm^r, Spc^r, Tc^r	[36]
RJ2498	*nirK-lacZ* fusion-containing strain derived from 110spc4, Cm^r, Spc^r, Tc^r	[21]
RJ2499	*norC-lacZ* fusion-containing strain derived from 110spc4, Cm^r, Spc^r, Tc^r	[21]
BG0301	*nosR-lacZ* fusion-containing strain derived from 110spc4, Cm^r, Spc^r, Tc^r	[24]

B. diazoefficiens cells were cultivated routinely under oxic conditions at 30℃ in peptone–salts–yeast extract (PSY) medium supplemented with 0.1% L-arabinose, essentially as described by Mesa et al. (2008)[22]. Buffered Vincent's minimal medium, here defined as vitamin-free modified Vincent's minimal medium (BVM,[37][38]) was used in this study, containing the following ingredients (per litre): KH_2PO_4, 2 g; K_2HPO_4, 2 g; NH_4Cl, 840 mg; $MgSO_4$, 246.48 mg; $CaCl_2$, 67.63 mg; $FeCl_3$, 10 mg; MOPS, 2.09 g. This medium was supplemented with 3 g of 1 M arabinose and 1 mL from a mineral solution[39] consisting of: H_3BO_3, 145 mg; $ZnSO_4$, 108 mg; Na_2MoO_4, 125 mg; $MnCl_2$, 4 mg; $FeSO_4$, 125 mg; $CoSO_4$, 70 mg; nitrile triacetate, 7 g; $CuSO_4$, 5 mg. When needed, the medium was supplemented with 10 mM KNO_3 (referred here as BVMN). Final pH was adjusted around 6.8 with 2 M NH_3.

Final Cu concentration in BVM or BVMN as indicated in the original recipe[37] was 0.02 μM, referred to in this manuscript as Cu-standard medium (Cu-S). In this study, 13 μM Cu was used as high Cu conditions (Cu-H); this concentration was also used as Cu-H in previous studies[32][33]. In the case of the Cu-limiting medium (Cu-L), $CuSO_4$ was omitted from the mineral solution, and 10 μM bathocuproine disulfonic acid (BCS) (Cu(I) chelator) and 1 mM L-ascorbate (reducer from Cu(II) to Cu(I)) were added to the medium in order to lower Cu availability[32][34]. Only for the Cu-L medium, glassware was treated overnight with 0.1 M HCl and rinsed afterwards with double-distilled water[34].

After growing under oxic conditions in the PSY medium, *B. diazoefficiens* cells were collected by centrifugation (8000x *g*, 8 min, 4℃). Next, cells were washed twice with BVM or BVMN and inoculated at an optical density at 600 nm (OD_{600}) of 0.05 (or 0.2 when needed). For oxic conditions, 3 mL of medium were added to 17-mL tubes. For anoxic conditions, 17-mL tubes were completely filled with medium. For microoxic conditions, 3, 50 and 100 mL of medium were added to 17-mL, 250 and 500-mL rubber stoppered tubes or Erlenmeyer flasks, respectively. The headspace was then filled with a gas mixture consisting of 2% (v/v) oxygen and 98% (v/v) N_2 and both, tubes and flasks, were incubated at 30℃ with agitation at 170 rpm.

When needed, antibiotics were added to *B. diazoefficiens* cultures at the following concentrations (μg/mL): spectinomycin (Spc), 200 (solid cultures), 100 (liquid cultures); tetracycline (Tc), 100 (solid cultures), 25 (liquid cultures); chloramphenicol (Cm), 20.

2.2 Determination of β-galactosidase activity

β-galactosidase activity was analysed using permeabilised cells from at least three independent cultures (3 mL), assayed in triplicate for each strain and condition, as previously described[40]. Specific activity was calculated in Miller Units (MU)[41] applying the formula shown in (1):

$$MU = [1000 * (OD_{420} - OD_{550})]/[t(min) * V(mL) * OD_{595}] \tag{1}$$

where:

OD_{420} and OD_{550} are the optical density values from the reaction,
OD_{595} is the optical density from the culture used in the reaction,
t is the time of incubation for all the reactions, always expressed in minutes,
and V is the volume of the reaction, always expressed in mL.

2.3 Analysis of gene expression by qRT-PCR

Expression of *napE*, *nirK*, *norC* and *nosR* was analysed by qRT-PCR using a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific,Waltham, MA, USA). *B. diazoefficiens* 110spc4 was grown microaerobically in the BVMN medium under Cu-L or Cu-S conditions for 48 h. Cell harvest, isolation of total RNA and cDNA synthesis were performed as described previously[21]. Primers for the PCR reactions (Table 2) were specifically designed with the Clone Manager Suite software to have melting temperatures between 57 and 62°Cin order to generate PCR products of 75–100 base pairs. Each PCR reaction contained 9.5 μL of iQTM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 2 mM (final concentration) of individual primers and appropriate dilutions of different cDNA samples in a total volume of 19 μL. Reactions were run in triplicate. Melting curves were generated to verify the specificity of the amplification. Relative changes in gene expression were calculated as described by Pfaffl (2001)[42]. Expression of the 16S *rrn* gene was used as reference for normalization (Table 2).

Table 2: Primers used in this study for qRT-PCR experiments.

Primers	DNA sequence 5'-3'
bsr7036(*napE*)-for4	GCCTTCCTGTTCCTGAC
bsr7036(*napE*)-rev4	CCGGCAAACATCTGGTAGA
nirK-6for	AGCCTTCACCGACACCGAAGAG
nirK-6rev	GAGCGCATTCTTGCCGGTAAGC
norC-3for	GCAGATGCCGCAGTTCAAC
norC-3rev	TGATCGTGCTCACCCATTG
nosR-for	ATGATCCAGGTGCGGCTGAAG
nosR-rev	CCGGCTGTGATGATTGTGTTCG
16S-for	GCAGGCTTAACACATGCAAGTC
16S-rev	AGGTACGTTCCCACGCGTTACTC

2.4 Statistical analysis

The total number of replicates is given in each figure. Data were checked for normal distribution according to Kolmogorov–Smirnov and Shapiro–Wilk tests. We then performed inferential statistics to test null hypotheses applying a parametric ANOVA for unpaired treatments. Next, a *post-hoc* Tukey HSD test at $p < 0.05$ with SPSS software was performed.

3 Results

3.1 Cu effect on *B. diazoefficiens* 110spc4 growth under different oxygen conditions

B. diazoefficiens 110spc4 was grown under oxic, anoxic and microoxic (2% initial O_2 concentration) conditions in Buffered Vincent's medium[34] supplemented with nitrate (BVMN) and different Cu concentrations: Cu limitation, *i.e.*, chelated (Cu-L), Cu standard (Cu-S, 0.02 μM) or high Cu (Cu-H, 13 μM) (Figure 1). Under oxic conditions, cultures reached an optical density at 600 nm (OD_{600}) of around 1.5 after 7 days of incubation, regardless of the Cu treatment (Figure 1A). These results suggest that Cu was not a limiting factor for *B. diazoefficiens* growth by oxygen respiration (Figure 1A).
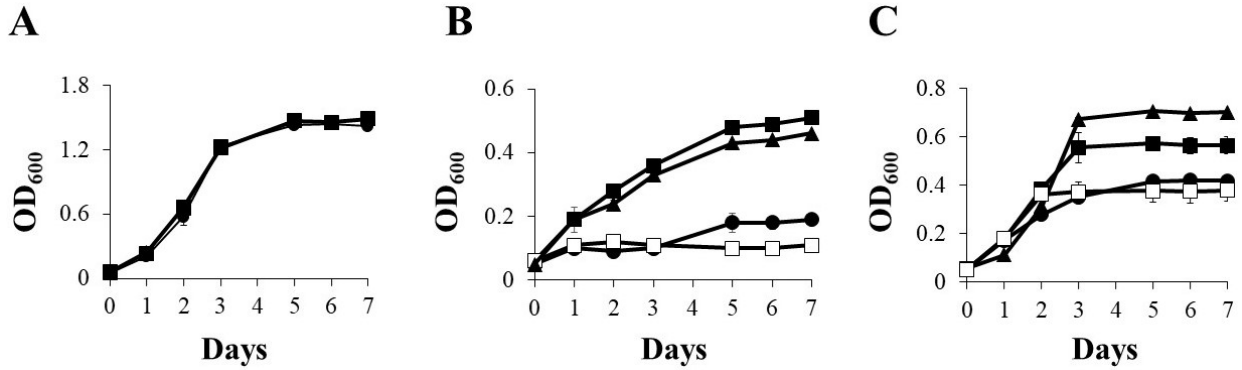
Figure 1: Growth of *B. diazoefficiens* 110spc4 in Cu limitation (Cu-L, black circle), Cu standard (Cu-S, black square) and high Cu (Cu-H, black triangle) BVMN media under oxic (A), anoxic (B) and microoxic (C) conditions. In (B,C), growth in the Cu-S BVM medium was also included (white square). Error bars represent standard error between triplicates, and where not visible, these were smaller than the symbols.

When *B. diazoefficiens* 110spc4 cells were cultured in BVMN medium under anoxic conditions (Figure 1B), Cu-L cultures reached a turbidity (OD_{600}) of about 0.2 after 7 days of incubation, while Cu-S and Cu-H cultures reached an OD_{600} of about 0.5, indicating that growth was severely affected in the Cu-L medium compared with Cu-S or Cu-H media (Figure 1B). This result indicates that Cu was essential for nitrate-dependent anaerobic growth of *B. diazoefficiens*. In fact, the growth profile displayed in BVMN Cu-L cultures with nitrate was similar to that observed in Cu-S cultures incubated without nitrate (BVM medium) (Figure 1B), indicating that Cu and nitrate were both indispensable for nitrate respiration under anoxic conditions. Finally, *B. diazoefficiens* 110spc4 cells were incubated under microoxic conditions in Cu-L, Cu-S and Cu-H BVMN media. As shown in Figure 1C, microaerobic growth under Cu-L conditions decreased compared with that reached under Cu-S conditions (about 0.4 and 0.6 OD_{600}, respectively, after 7 days of incubation). In contrast, cells grown in the Cu-H medium showed a significant increase in growth rates compared with those cultured in the Cu-S medium (about 0.7 and 0.6 OD_{600}, respectively, after 7 days of incubation). Interestingly, when cells were grown microaerobically in the Cu-S medium, but in the absence of nitrate, they displayed similar growth rates to those cultured in Cu-L conditions with nitrate as the respiratory substrate (Figure 1C). These results suggest that nitrate and Cu were necessary for *B. diazoefficiens* to grow from nitrate respiration under microoxic conditions, as it was observed under anoxic conditions (Figure 1B).

3.2 Disparate response of denitrification gene expression to Cu

Taking the previous results into consideration, we decided to perform a study into the Cu effect on denitrification gene expression by incubating cells for 3 days under different Cu concentrations. For this purpose, we analysed β-galactosidase activity from the *napE-lacZ*, *nirK-lacZ*, *norC-lacZ* and *nosR-lacZ* transcriptional fusions in *B. diazoefficiens* 110spc4 cells grown for 3 days in Cu-L, Cu-S and Cu-H BVMN media under microoxic conditions (Figure 2). Cultures grown aerobically were included as a control in the experiments. As shown in Figure 2A, *napE-lacZ* microaerobic expression was not significantly affected by Cu concentration in the medium, demonstrating similar β-galactosidase activity values under Cu-L, Cu-S or Cu-H conditions. These results indicate that Cu is not involved in the transcriptional control of *napEDABC* genes. In contrast, Cu limitation drastically lowered β-galactosidase activity from the *nirK-lacZ*, *norC-lacZ* and *nosR-lacZ* fusions (about 3-, 6- and 4-fold, respectively) compared with the values obtained in the Cu-S medium (Figure 2B–D). These results suggest that Cu availability is essential for *nirK*, *norCBQD* and *nosRZDFYLX* maximal expression.

The negative effect of Cu limitation on *nirK*, *nor* and *nos* transcriptional expression was also confirmed

5

by qRT-PCR analyses. When cells were cultured microaerobically in the Cu-L BVMN medium, expression of *nirK*, *norC* and *nosR* genes was reduced to 10.73, 33.25 and 6.79, respectively, compared with that observed in cells cultured in the Cu-S medium (Figure 2E). In contrast, Cu limitation did not affect *napE* expression compared with Cu-S conditions, similar to the results obtained when we analysed the *napE-lacZ* transcriptional fusion (Figure 2A,E). Taken together, these results confirm the negative effect of Cu limitation on *nirK*, *nor* and *nos* but not on *nap* gene expression.
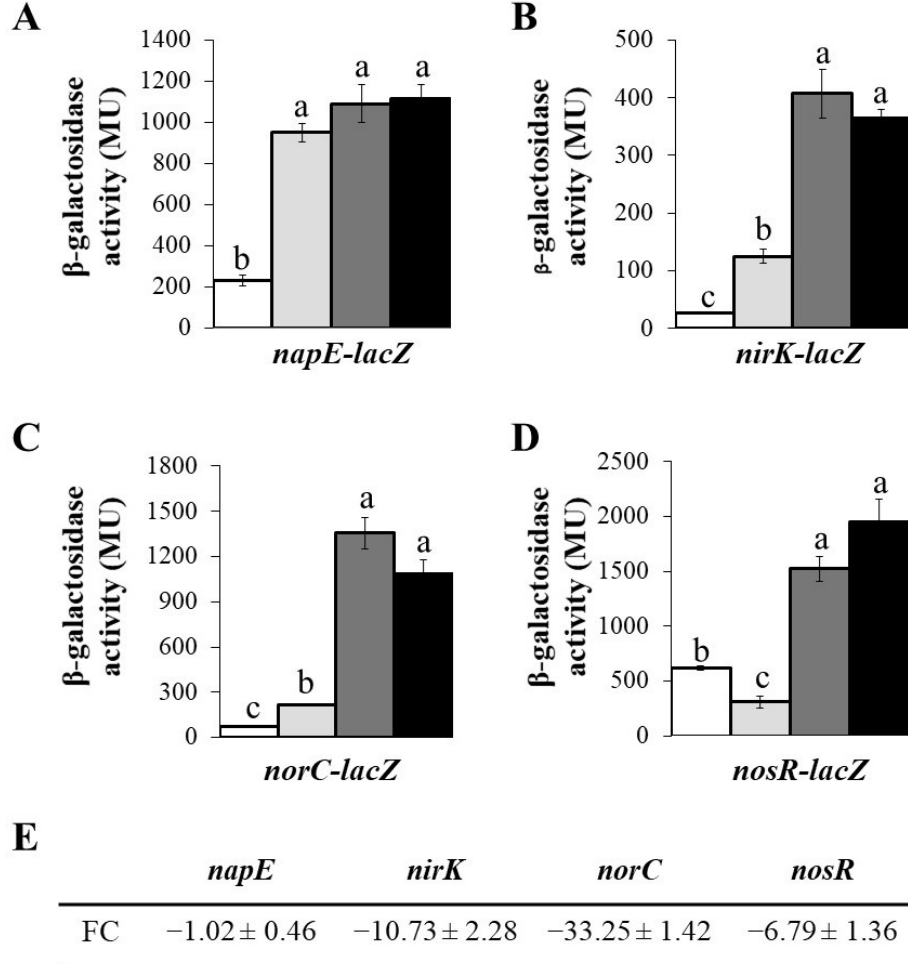

Figure 2: Transcriptional expression of denitrification genes monitored as β-galactosidase activity from *napE-lacZ* (A), *nirK-lacZ* (B), *norC-lacZ* (C) and *nosR-lacZ* (D) fusions chromosomally integrated in *B. diazoefficiens* 110spc4 grown aerobically in Cu-S (white bars) and microaerobically in Cu-L (light grey bars), Cu-S (dark grey bars) and Cu-H (black bars) BVMN media for 3 days. A *post-hoc* Tukey HSD test at $p < 0.05$ was applied in (A–D); same lower-case letters in each figure indicate that values are not statistically different. (E) Expression changes of *napE*, *nirK*, *norC* and *nosR* genes in *B. diazoefficiens* 110spc4 grown microaerobically in Cu-L compared with Cu-S measured by qRT-PCR. Data expressed as Miller Units (MU) and Fold Change (FC) are means with standard deviation from at least three independent cultures assayed in triplicate.

4 Discussion

The main focus of the present work was to contribute to a better understanding of the role of Cu in *B. diazoefficiens* denitrification. To achieve this goal, we undertook a study of the expression of the *nap*,

nirK, *nor* and *nos* genes involved in the denitrification pathway. Firstly, we analysed the capacity of *B. diazoefficiens* 110spc4 to grow under different Cu conditions: Cu limitation (Cu-L), Cu standard (Cu-S) or high Cu (Cu-H). Aerobic growth was not affected by Cu limitation indicating that Cu-independent terminal oxidases can function under these conditions. In fact, like other aerobic facultative bacteria, *B. diazoefficiens* adapts its metabolism to different oxygen conditions through the expression of multiple terminal oxidases with a distinct affinity for oxygen[43]. Eight terminal oxidases have been identified in *B. diazoefficiens*, of which two are Cu-independent *bd*-type oxidases, and the remaining six are heme-Cu oxidases, which use Cu as cofactor[44]. It might be possible that *bd*-type oxidases are responsible for the aerobic growth under Cu limitation. As reviewed by Jünemann (1997)[45], the expression of cytochrome *bd* increased concomitantly with oxygen depletion in *E. coli*; however, major expression levels of this type of cytochrome were observed with the increase in oxygen concentration in *Azotobacter vinelandii*. In the Gram-positive human pathogenic bacterium *Mycobacterium tuberculosis*, Cu-independent cytochrome *bd* oxidases were induced under hypoxia, decreasing Cu requirement as a consequence, which is beneficial for the bacterium because Cu toxicity rises under these conditions[46]. Therefore, an adaptation to oxic Cu-depleted conditions through the synthesis of Cu-independent oxidases in *B. diazoefficiens* would be a plausible hypothesis for the observed phenotype.

In contrast to oxic conditions, Cu limitation negatively affected anaerobic and microaerobic nitrate-dependent growth. However, other denitrifiers, such as *P. denitrificans* or *A. xylosoxidans* did not show significant growth differences between Cu-L and Cu-H media under anaerobiosis with nitrate as the respiratory substrate[32]. The presence of the Cu-independent NirS in *P. denitrificans* could explain the growth differences under Cu-L conditions between this bacterium and *B. diazoefficiens*. However, *A. xylosoxidans* that, similarly to *B. diazoefficiens*, possesses a Cu-dependent NirK, was also able to grow anaerobically in a Cu-limiting medium[32]. These different results could be explained by the different growth conditions used in our work and in the aforementioned study, where both *A. xylosoxidans* and *P. denitrificans* were grown as continuous cultures in a chemostat. Another possible explanation for these differences could be given by the fact that both *A. xylosoxidans* and *P. denitrificans* are rapid-growing microorganisms[32], while *B. diazoefficiens* is a slow-growing bacterium. The slow growth rate of *B. diazoefficiens* might have contributed to the negative effect of Cu limitation on the expression and activation of the complete denitrification machinery, and this could be a possible reason for the growth defects observed in Cu-L compared with Cu-S and Cu-H. Contrary to the results observed in this work, Sullivan et al. (2013)[33] did not observe any growth difference between Cu-L and Cu-H in *P. denitrificans* batch cultures. The lack of a growth defect observed in *P. denitrificans* under Cu-L conditions[33] compared with *B. diazoefficiens* could also be explained by the fact that the denitrification process starts by a Nar enzyme in *P. denitrificans*, and by a Nap enzyme in *B. diazoefficiens*. Recent evidence supporting this idea was reported in *P. stutzeri*, another rapid-growing microorganism in which the denitrification process also begins with the reduction of nitrate to nitrite by a Nar[30]. In the latter study, no significant differences in growth were observed in anaerobic cultures of *P. stutzeri* over a 7-day period throughout a Cu concentration range between 0 and 1 mM Cu. Therefore, our work provides evidence that Cu limitation could affect growth of slow-growing microorganisms provided with both a Nap as the first enzyme of the denitrification process and a Cu-dependent NirK.

To better understand the effect of Cu on *B. diazoefficiens* denitrification, we investigated gene expression in microaerobic Cu-L, Cu-S and Cu-H cultures. By using transcriptional fusions to the reporter gene *lacZ*, we demonstrated the Cu dependence of *nirK*, *nor* and *nos* denitrification gene induction under microoxic conditions. These observations were confirmed by performing qRT-PCR analyses of the *napE*, *nirK*, *norC* and *nosR* genes. Similarly to our observations, adequate bioavailable Cu concentrations (0.15 mM) resulted in the greatest transcription levels of *P. stutzeri nirS*, *norB* and *nosZ* denitrification genes compared with low Cu[30]. Moreover, low levels of *nosZ* expression during Cu limitation were also reported in *P. denitrificans*[33].

Gene expression results clearly indicated that the induction of *nirK*, *nor* and *nos* genes by low oxygen and nitrate was significantly reduced under Cu limitation, suggesting that this control might be modulated by a specific repressor. In this context, CsoR and CopY are considered as Cu-sensing transcriptional ATPase repressors in bacteria such as *M. tuberculosis*[47], *E. coli* and *Enterococcus hirae* (for a review, see Rademacher and Masepohl, 2012[48]). Nevertheless, a *csoR-cueA* divergon encoding a CsoR-like repressor and a heavy

metal transporting P-type ATPase (CueA) was recently reported in the Gram-negative bacterium *Bradyrhizobium liaoningense* CCNWSX0360[47]. These authors attributed a crucial role in Cu homeostasis to this system, as well as in zinc/cadmium resistance. A search of the *B. diazoefficiens* genome in the KEGG database (https://www.genome.jp/kegg/) revealed the presence of a putative gene encoding a repressor from the CsoR family whose annotation is *bsr0701*. Apart from the probable role of this repressor in metal tolerance, a putative role of the predicted CsoR protein in the low transcription levels of *B. diazoefficiens* *nirK*, *nor* and *nos* under Cu limitation requires further investigation.

5   Conclusion

The main goal of the present work was to investigate the influence of Cu on denitrification in the soybean endosymbiont *B. diazoefficiens*. Taken together, our results suggest that Cu may act as an essential factor in the regulation of the denitrification gene expression. Therefore, Cu could be involved in the denitrification regulatory network and not only acts as a mere enzymatic cofactor of the Cu-dependent enzymes, but also as an important regulatory signal of this process.

References

[1] AR Ravishankara, John S Daniel, and Robert W Portmann. Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. *Science*, 326(5949):123–125, 2009.

[2] Priyadarshi R Shukla, J Skeg, E Calvo Buendia, Valérie Masson-Delmotte, H-O Pörtner, DC Roberts, Panmao Zhai, Raphael Slade, Sarah Connors, S van Diemen, et al. Climate change and land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems. 2019.

[3] James N Galloway, John D Aber, Jan Willem Erisman, Sybil P Seitzinger, Robert W Howarth, Ellis B Cowling, and B Jack Cosby. The nitrogen cascade. *Bioscience*, 53(4):341–356, 2003.

[4] David Richardson, Heather Felgate, Nick Watmough, Andrew Thomson, and Elizabeth Baggs. Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle–could enzymic regulation hold the key? *Trends in biotechnology*, 27(7):388–397, 2009.

[5] Philip G Taylor and Alan R Townsend. Stoichiometric control of organic carbon–nitrate relationships from soils to the sea. *Nature*, 464(7292):1178–1181, 2010.

[6] Andrew J Thomson, Georgios Giannopoulos, Jules Pretty, Elizabeth M Baggs, and David J Richardson. Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367(1593):1157–1168, 2012.

[7] Walter G Zumft. Cell biology and molecular basis of denitrification. *Microbiology and molecular biology reviews*, 61(4):533–616, 1997.

[8] RJM van Spanning. The nitrogen cycle: denitrification and its relationship to N_2 fixation. Technical report.

[9] Rob JM van Spanning, David J Richardson, and Stuart J Ferguson. Introduction to the biochemistry and molecular biology of denitrification. In *Biology of the nitrogen cycle*, pages 3–20. Elsevier, 2007.

[10] Beate Kraft, Marc Strous, and Halina E Tegetmeyer. Microbial nitrate respiration–genes, enzymes and environmental distribution. *Journal of biotechnology*, 155(1):104–117, 2011.

[11] Emilio Bueno, Socorro Mesa, Eulogio J Bedmar, David J Richardson, and Maria J Delgado. Bacterial adaptation of respiration from oxic to microoxic and anoxic conditions: redox control. *Antioxidants and redox signaling*, 16(8):819–852, 2012.

[12] MJ Torres, Jorg Simon, Gary Rowley, EJ Bedmar, DJ Richardson, AJ Gates, and MJ Delgado. Nitrous oxide metabolism in nitrate reducing bacteria: physiology and regulatory mechanisms. *Advances in microbial physiology*, 68:353–432, 2016.

[13] EJ Bedmar, EF Robles, and MJ Delgado. The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium Bradyrhizobium japonicum, 2005.

[14] EJ Bedmar, E Bueno, D Correa, MJ Torres, MJ Delgado, and S Mesa. Ecology of denitrification in soils and plant-associated bacteria. *Beneficial plant-microbial interactions: Ecology and applications*, pages 164–182, 2013.

[15] Ana Salas, Juan J Cabrera, Andrea Jiménez-Leiva, Socorro Mesa, Eulogio J Bedmar, David J Richardson, Andrew J Gates, and María J Delgado. Bacterial nitric oxide metabolism: Recent insights in rhizobia. *Advances in microbial physiology*, 78:259–315, 2021.

[16] Philip Poole, Vinoy Ramachandran, and Jason Terpolilli. Rhizobia: from saprophytes to endosymbionts. *Nature Reviews Microbiology*, 16(5):291–303, 2018.

[17] María J Delgado, Nathalie Bonnard, Alvaro Tresierra-Ayala, Eulogio J Bedmar, and Peter Müller. The Bradyrhizobium japonicum napEDABC genes encoding the periplasmic nitrate reductase are essential for nitrate respiration. *Microbiology*, 149(12):3395–3403, 2003.

[18] Leonardo Velasco, Socorro Mesa, Marıa J Delgado, and Eulogio J Bedmar. Characterization of the nirK gene encoding the respiratory, Cu-containing nitrite reductase of Bradyrhizobium japonicum. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1521(1-3):130–134, 2001.

[19] Socorro Mesa, Leonardo Velasco, Maximino E Manzanera, Marıa J Delgado, and Eulogio J Bedmar. Characterization of the norCBQD genes, encoding nitric oxide reductase, in the nitrogen fixing bacterium Bradyrhizobium japonicum. *Microbiology*, 148(11):3553–3560, 2002.

[20] Leonardo Velasco, Socorro Mesa, Chang-ai Xu, María J Delgado, and Eulogio J Bedmar. Molecular characterization of nosRZDFYLX genes coding for denitrifying nitrous oxide reductase of Bradyrhizobium japonicum. *Antonie Van Leeuwenhoek*, 85(3):229–235, 2004.

[21] Socorro Mesa, Eulogio J Bedmar, Astrid Chanfon, Hauke Hennecke, and Hans-Martin Fischer. *Bradyrhizobium japonicum* NnrR, a denitrification regulator, expands the FixLJ-$FixK_2$ regulatory cascade. *Journal of bacteriology*, 185(13):3978–3982, 2003.

[22] Socorro Mesa, Felix Hauser, Markus Friberg, Emmanuelle Malaguti, Hans-Martin Fischer, and Hauke Hennecke. Comprehensive assessment of the regulons controlled by the FixLJ-$FixK_2$-$FixK_1$ cascade in Bradyrhizobium japonicum. *Journal of bacteriology*, 190(20):6568–6579, 2008.

[23] Emilio Bueno, Eloy F Robles, María J Torres, Tino Krell, Eulogio J Bedmar, María J Delgado, and Socorro Mesa. Disparate response to microoxia and nitrogen oxides of the Bradyrhizobium japonicum napEDABC, nirK and norCBQD denitrification genes. *Nitric Oxide*, 68:137–149, 2017.

[24] María J Torres, Emilio Bueno, Andrea Jiménez-Leiva, Juan J Cabrera, Eulogio J Bedmar, Socorro Mesa, and María J Delgado. $FixK_2$ is the main transcriptional activator of Bradyrhizobium diazoefficiens nosRZDYFLX genes in response to low oxygen. *Frontiers in microbiology*, 8:1621, 2017.

[25] Andrea Jiménez-Leiva, Juan J Cabrera, Emilio Bueno, María J Torres, Sergio Salazar, Eulogio J Bedmar, María J Delgado, and Socorro Mesa. Expanding the regulon of the Bradyrhizobium diazoefficiens NnrR transcription factor: new insights into the denitrification pathway. *Frontiers in microbiology*, page 1926, 2019.

[26] Juan J Cabrera, Andrea Jiménez-Leiva, Laura Tomás-Gallardo, Sergio Parejo, Sara Casado, María J Torres, Eulogio J Bedmar, María J Delgado, and Socorro Mesa. Dissection of $FixK_2$ protein-DNA interaction unveils new insights into Bradyrhizobium diazoefficiens lifestyles control. *Environmental Microbiology*, 23(10):6194–6209, 2021.

[27] Lars R Bakken and Åsa Frostegård. Sources and sinks for N_2O, can microbiologist help to mitigate N_2O emissions?, 2017.

[28] Cíntia Carreira, Rute F Nunes, Olga Mestre, Isabel Moura, and Sofia R Pauleta. The effect of pH on Marinobacter hydrocarbonoclasticus denitrification pathway and nitrous oxide reductase. *JBIC Journal of Biological Inorganic Chemistry*, 25(7):927–940, 2020.

[29] Alfonso Olaya-Abril, Jesús Hidalgo-Carrillo, Víctor M Luque-Almagro, Carlos Fuentes-Almagro, Francisco J Urbano, Conrado Moreno-Vivián, David J Richardson, and María Dolores Roldán. Effect of pH on the denitrification proteome of the soil bacterium Paracoccus denitrificans PD1222. *Scientific reports*, 11(1):1–12, 2021.

[30] Amanda Black, Pei-Chun L Hsu, Kelly E Hamonts, Tim J Clough, and Leo M Condron. Influence of copper on expression of nirS, norB and nosZ and the transcription and activity of NIR, NOR and N_2OR in the denitrifying soil bacteria Pseudomonas stutzeri. *Microbial biotechnology*, 9(3):381–388, 2016.

[31] T Matsubara, K Frunzke, and WG Zumft. Modulation by copper of the products of nitrite respiration in Pseudomonas perfectomarinus. *Journal of Bacteriology*, 149(3):816–823, 1982.

[32] Heather Felgate, Georgios Giannopoulos, Matthew J Sullivan, Andrew J Gates, Thomas A Clarke, Elizabeth Baggs, Gary Rowley, and David J Richardson. The impact of copper, nitrate and carbon status on the emission of nitrous oxide by two species of bacteria with biochemically distinct denitrification pathways. *Environmental microbiology*, 14(7):1788–1800, 2012.

[33] Matthew J Sullivan, Andrew J Gates, Corinne Appia-Ayme, Gary Rowley, and David J Richardson. Copper control of bacterial nitrous oxide emission and its impact on vitamin B_{12}-dependent metabolism. *Proceedings of the National Academy of Sciences*, 110(49):19926–19931, 2013.

[34] Fabio Serventi, Zeb Andrew Youard, Valérie Murset, Simona Huwiler, Doris Bühler, Miriam Richter, Ronny Luchsinger, Hans-Martin Fischer, Robert Brogioli, Martina Niederer, et al. Copper starvation-inducible protein for cytochrome oxidase biogenesis in Bradyrhizobium japonicum. *Journal of Biological Chemistry*, 287(46):38812–38823, 2012.

[35] Brigitte Regensburger and Hauke Hennecke. RNA polymerase from Rhizobium japonicum. *Archives of microbiology*, 135(2):103–109, 1983.

[36] EF Robles, C Sanchez, N Bonnard, MJ Delgado, and EJ Bedmar. The Bradyrhizobium japonicum napEDABC genes are controlled by the FixLJ-$FixK_2$-NnrR regulatory cascade, 2006.

[37] JM Vincent et al. A manual for the root-nodule bacteria. *A manual for the root-nodule bacteria.*, 1970.

[38] Anke Becker, Hélène Bergès, Elizaveta Krol, Claude Bruand, Silvia Rüberg, Delphine Capela, Emmanuelle Lauber, Eliane Meilhoc, Frédéric Ampe, Frans J de Bruijn, et al. Global changes in gene expression in Sinorhizobium meliloti 1021 under microoxic and symbiotic conditions. *Molecular Plant-Microbe Interactions*, 17(3):292–303, 2004.

[39] Paul E Bishop, Juan G Guevara, Jean A Engelke, and Harold J Evans. Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between Rhizobium japonicum and Glycine max. *Plant Physiology*, 57(4):542–546, 1976.

[40] Juan J Cabrera, Ana Salas, María J Torres, Eulogio J Bedmar, David J Richardson, Andrew J Gates, and María J Delgado. An integrated biochemical system for nitrate assimilation and nitric oxide detoxification in Bradyrhizobium japonicum. *Biochemical Journal*, 473(3):297–309, 2016.

[41] JH Miller. Experiments in Molecular Genetics, 1972.

[42] Michael Pfaffl, Graham Horgan, and Leo Dempfle. Relative expression software tool for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic acids research*, 30(9), 2002.

[43] Maria J Delgado, Eulogio J Bedmar, and J Allan Downie. Genes involved in the formation and assembly of rhizobial cytochromes and their role in symbiotic nitrogen fixation. *Advances in microbial physiology*, 40:191–231, 1998.

[44] Doris Bühler, Reinhild Rossmann, Sarah Landolt, Sylvia Balsiger, Hans-Martin Fischer, and Hauke Hennecke. Disparate pathways for the biogenesis of cytochrome oxidases in Bradyrhizobium japonicum. *Journal of Biological Chemistry*, 285(21):15704–15713, 2010.

[45] Susanne Jünemann. Cytochrome *bd* terminal oxidase. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1321(2):107–127, 1997.

[46] Sarah A Marcus, Sarah W Sidiropoulos, Howard Steinberg, and Adel M Talaat. CsoR is essential for maintaining copper homeostasis in Mycobacterium tuberculosis. *PloS one*, 11(3):e0151816, 2016.

[47] Tong Liu, Arati Ramesh, Zhen Ma, Sarah K Ward, Limei Zhang, Graham N George, Adel M Talaat, James C Sacchettini, and David P Giedroc. CsoR is a novel Mycobacterium tuberculosis copper-sensing transcriptional regulator. *Nature chemical biology*, 3(1):60–68, 2007.

[48] Corinna Rademacher and Bernd Masepohl. Copper-responsive gene regulation in bacteria. *Microbiology*, 158(10):2451–2464, 2012.