Abstract: The greenhouse gas nitrous oxide (N$_2$O) has strong potential to drive climate change. Soils are a major source of N$_2$O, with microbial nitrification and denitrification being the primary processes involved in such emissions. The soybean endosymbiont *Bradyrhizobium diazoefficiens* is a model microorganism to study denitrification, a process that depends on a set of reductases, encoded by the *napEDABC*, *nirK*, *norCBQD*, and *nosRZDYFLX* genes, which sequentially reduce nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), N$_2$O, and dinitrogen (N$_2$). In this bacterium, the regulatory network and environmental cues governing the expression of denitrification genes rely on the FixK and NnrR transcriptional regulators. To understand the role of FixK and NnrR proteins in N$_2$O turnover, we monitored real-time kinetics of NO$_3^-$, NO$_2^-$, NO, N$_2$, N$_2$O, and oxygen (O$_2$) in a fixK and nnrR mutant using a robotized incubation system. We confirmed that FixK and NnrR are regulatory determinants essential for NO$_3^-$ respiration and N$_2$O reduction. Furthermore, we demonstrated that NO$_2^-$ reduction by *B. diazoefficiens* is independent of canonical inducers of denitrification, such as the nitrogen oxide NO$_2^-$, and it is negatively affected by acidic and alkaline conditions. These findings advance the understanding of how specific environmental conditions and two single regulators modulate N$_2$O turnover in *B. diazoefficiens*.

Keywords: denitrification; dinitrogen; gene expression; nitric oxide; nitrous oxide reductase

1. Introduction

Under shortage of oxygen, bacteria can adapt and thrive by two ATP-generating mechanisms: (i) induction of dedicated high-affinity terminal oxidases that permit bacteria to respire oxygen at very low concentrations or (ii) making use of inorganic terminal electron acceptors such as nitrate (NO$_3^-$), which can be reduced through the denitrification pathway to dinitrogen (N$_2$) or through dissimilatory nitrate reduction to ammonium (DNRA). Although such anaerobic respiration generates less ATP per mol electron than aerobic respiration, it allows bacteria to grow and persist in the absence of oxygen (O$_2$) [1]. Denitrification has been defined as the sequential reduction of NO$_3^-$ or nitrite (NO$_2^-$) to nitric oxide (NO), nitrous oxide (N$_2$O), and N$_2$: [2]. This process is catalyzed by the periplasmic (Nap) or membrane-bound (Nar) nitrate reductase, nitrite reductases (NirK/NirS), nitric oxide reductases (cNor, qNor, or Cu$_2$Nor), and nitrous oxide reductase (N$_2$OR) encoded by *nap/nar*, *nirK/nirS*, *nor*, and *nos* genes, respectively [2–4]. In addition
to denitrification, multiple pathways for N\textsubscript{2}O generation have been reported, including nitrification, nitrifier denitrification, nitrite oxidation, ammonia oxidation, heterotrophic denitrification, anaerobic ammonium oxidation (anammox), and DNRA \cite{3,5}. N\textsubscript{2}OR is the only known enzyme catalyzing the reduction of N\textsubscript{2}O to N\textsubscript{2} \cite{6}. Accordingly, expression and activity of N\textsubscript{2}OR are considered natural targets to mitigate N\textsubscript{2}O emissions from agricultural soils \cite{7-9}.

Given the impact of N\textsubscript{2}O as a powerful greenhouse gas in global warming and in depletion of the ozone layer \cite{5,7}, understanding its dynamics of production/reduction in soils and aquatic environments has become a priority. In fact, the application of synthetic nitrogen (N) fertilizers to agricultural soils, as well as local oxygen concentrations, water content, carbon availability, and pH, greatly affect N\textsubscript{2}O emissions from soils and aquatic ecosystems \cite{7-9}.

Many legumes establish symbiotic associations with soil bacteria, collectively termed “rhizobia”, which fix nitrogen in so-called root nodules on legume roots and on the stems of some aquatic legumes. Following invasion of the plant cells via a complex signaling pathway between bacteria and plant, rhizobia stop dividing and undergo differentiation into nitrogen-fixing bacteroids, at which point the nitrogenase complex reduces atmospheric N\textsubscript{2} into biologically useful forms in a process called “Biological N\textsubscript{2} Fixation”. Consequently, cultivation of legumes can reduce the need for environmentally polluting synthetic nitrogen fertilizers, thus decreasing N\textsubscript{2}O emissions as well as protecting ground water from toxicity while improving soil fertility. However, legume crops can also contribute to N\textsubscript{2}O emissions in several ways: (i) by biologically fixed N\textsubscript{2} being converted to NO and N\textsubscript{2}O through nitrification and denitrification \cite{10}; (ii) by providing N-rich residues for decomposition \cite{11}, and (iii) directly by some rhizobia that can denitrify under free-living conditions or in symbiotic association with legumes \cite{12,13}. In this context, one strategy to reduce N\textsubscript{2}O emissions from legume crops is to use as inoculants rhizobia strains with high N\textsubscript{2}OR activity. In fact, it has been shown that N\textsubscript{2}O emissions from soybean crops can be reduced by inoculating legumes with strains of the soybean endosymbiont Bradyrhizobium diazoefficiens that overexpress N\textsubscript{2}OR \cite{14,15}.

\textit{B. diazoefficiens} is considered a model bacterium to study denitrification in rhizobia, since it is the only rhizobial species that, in addition to fixing N\textsubscript{2}, has the ability to grow under anoxic conditions by reducing NO\textsuperscript{-} through the complete denitrification pathway, a process widely studied in this bacterium both in free-living conditions and in symbiosis with soybeans \cite{12,13,16}. \textit{B. diazoefficiens} possesses the complete set of \textit{napEDABC}, \textit{nirK}, \textit{norCBQD}, and \textit{nosRZDFYLX} denitrification genes \cite{12}, which encode the periplasmic nitrate reductase (Nap), copper-containing nitrite reductase (NirK), nitric oxide reductase type c (cNor), and nitrous oxide reductase (N\textsubscript{2}OR), respectively (Figure 1). Like many other denitrifiers, expression of denitrification genes in \textit{B. diazoefficiens} requires both oxygen limitation and the presence of NO\textsuperscript{-} or a nitrogen oxide (NOx) derived from its reduction. The response to low oxygen (≤0.5% O\textsubscript{2} in the gas phase, i.e., ≤5–10 µM O\textsubscript{2}) is mediated by the FixLJ-FixK\textgamma-NnrR regulatory network \cite{3}, in which the response regulator FixJ in its active phosphorylated form induces the expression of several genes, including \textit{fixK\textgamma}, which encodes the transcriptional regulator FixK\textgamma (Figure 1). This protein induces the expression of more than 300 genes, including genes associated with microoxic metabolism (\textit{fixNOQP}), denitrification genes (\textit{napEDABC}, \textit{nirK}, \textit{norCBQD}, and \textit{nosRZDFYLX}), and regulatory genes (\textit{rpoN}, \textit{fixK\textgamma}, and \textit{nnrR}) \cite{12,17,18}. It has also been demonstrated that expression of \textit{napEDABC}, \textit{nirK}, and \textit{nosRZDFYLX} genes requires microoxic conditions and directly depends on FixK\textgamma, while expression of \textit{norCBQD} genes relies on NO, being the transcriptional regulator NnrR the candidate that directly interacts with \textit{norCBQD} promoter \cite{19,20} (Figure 1).

Although much is known about the role of FixK\textgamma and NnrR in the regulation of denitrification in \textit{B. diazoefficiens}, this knowledge needs to be extended to include relevant physiological conditions that this bacterium is expected to meet in nature. In the present work, we investigated the dynamics of N\textsubscript{2}O balance by FixK\textgamma and NnrR as well as the
influence of specific environmental conditions such as the presence of nitrogen oxides, O$_2$ concentration, pH, and the redox state of C-sources.

Figure 1. Schematic representation of the denitrification process and its regulation in Bradyrhizobium diazoefficiens. B. diazoefficiens can reduce nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and dinitrogen (N$_2$) by the periplasmic nitrate reductase (Nap), copper-containing nitrite reductase (NirK), nitric oxide reductase type c (cNor), and nitrous oxide reductase (N$_2$OR) enzymes, respectively. In B. diazoefficiens, expression of denitrification enzymes is tightly regulated by the FixLJ, FixK$_2$, and NnrR regulatory proteins (see Introduction for further details). However, despite the coordinated activation of each reductase, environmental unfriendly gases such as NO and N$_2$O can leak from denitrification and be released to the atmosphere.

2. Results

2.1. N$_2$O Emissions by B. diazoefficiens 110spc4 Depend on the FixK$_2$ and NnrR Regulatory Proteins

B. diazoefficiens strains were raised oxically under vigorous stirring, and aliquots were inoculated into culture vials to an initial OD$_{600}$ of 0.01 ($8 \times 10^6$ cells mL$^{-1}$). Next, 2% O$_2$ and 10 mM NO$_3^-$ were added as oxic and anoxic electron acceptors, respectively. Figures 2 and S1 show the O$_2$, NO$_3^-$, NO$_2^-$, NO, N$_2$O, and N$_2$ concentrations throughout the 120 h incubation of B. diazoefficiens 110spc4 (wild type) (Figures 2A and S1A) and fixK$_2$ (Figure 2B) and nnrR (Figures 2C and S1B) mutant strains. B. diazoefficiens wild type consumed O$_2$ within 28 h, and bacterial OD$_{600}$ increased following O$_2$ depletion.

Rates of O$_2$ consumption for each time increment between two samplings were taken to calculate electron ($e^-$) flow rates to oxygen ($V_{e^-O_2}$). As shown in Figure 2D, $V_{e^-O_2}$ increased exponentially in the wild type during the first 16 h and declined gradually in response to diminishing O$_2$ concentrations. The increase in electron flow can be taken as an indirect measure of growth ($\mu_{ox}$) [21]. Thus, the apparent $\mu_{ox}$ estimated by linear regression of ln ($V_{e^-O_2}$) against time was 0.10 ($\pm$0.03) h$^{-1}$ (Figure 2D, Table 1A). Final OD$_{600}$ resulting from the consumption of 2% O$_2$ was 0.080 ($\pm$0.005) ($6.40 \times 10^7$ cells mL$^{-1}$, Table 1B), equivalent to a yield of 13.3 ($\pm$1.1) cells pmol$^{-1}e^-$ to O$_2$ (Table 1A). Remarkably, in contrast to the fast depletion of O$_2$ observed in the parental strain, the capacity to consume O$_2$ in the fixK$_2$ and nnrR mutant strains was slightly reduced (Figure 2A–C). In the case of the fixK$_2$ mutant, $V_{e^-O_2}$ increased exponentially throughout the first 19 h and then declined gradually (Figure 2E). As shown in Table 1A, the apparent $\mu_{ox}$ was 0.055 ($\pm$0.008) (Figure 2E, Table 1A). The final OD$_{600}$ from O$_2$ respiration was 0.044 ($\pm$0.003) (Table 1B), resulting in a yield of 6.6 ($\pm$0.3) cells pmol$^{-1}e^-$ to O$_2$ (Table 1A). In the nnrR mutant, electron flow to O$_2$
increased exponentially throughout the first 19 h with an apparent $\mu_{ox}$ of 0.090 ($\pm$0.004) and then decreased slowly (Figure 2F). The final OD$_{600}$ during oxic phase was 0.079 ($\pm$0.001) (Table 1B), with a subsequent yield of 13.1 ($\pm$0.6) cells pmol$^{-1}$e$^{-}$ to O$_2$ (Table 1A).

Initiation of denitrification in the parental strain, hallmarked by the reduction of NO$\\text{\textsuperscript{3}}$ and transient emissions of NO and N$_2$O (Figures 2A and S1A), was observed at O$_2$ concentrations of $\leq$5 ($\pm$0.3) µM O$_2$ (Figures 2A and S1A and Table 1B) after 17 h of incubation. Rapidly, N$_2$ production was detected as individual final product from NO$\\text{\textsuperscript{3}}$-denitrification, with 100% of NO$\\text{\textsuperscript{3}}$ being converted to N$_2$ within 80 h of growth. NO$\\text{\textsuperscript{3}}$ accumulated for a longer period than NO and N$_2$O; however, its concentration was maintained at low levels until it was totally reduced to its depletion (Figure S1A).

**Figure 2.** Denitrification phenotypes of the parental strain *B. diazoefficiens* 110spc4 (A) and the two mutant strains $\Delta$fixK (B) and $\Delta$nnrR (C). (A–C) measurement of O$_2$ and NO$\\text{\textsuperscript{3}}$-respiration, concentrations of denitrifying intermediaries (NO$\\text{\textsuperscript{2}}$, NO, N$_2$O, N$_2$), and bacterial growth (OD$_{600}$) yielded from such dynamics. (D–F) electron flow rates to O$_2$ and nitrogen oxides (NOx). Cells were incubated with 2% O$_2$ and 10 mM NO$\\text{\textsuperscript{3}}$- as oxic and anoxic respiratory substrates, respectively. O$_2$, NOx concentrations, and bacterial growth were monitored by automatic sampling from headspace and liquid phase. See Figure S1 to visualize individual gases’ dynamics from (A,C). Data are the means and standard deviations of at least three different cultures.
Table 1. Summary of growth parameters from O₂ (oxic growth phase) and NO₃⁻ respiration (anoxic growth phase) in the B. diazoefficiens 110spc4 parental and fixK: and nmrR mutant strains (A) and other parameters observed through anoxic NO₃⁻ respiration (B).

| Genotype   | Oxic Growth Phase | Anoxic Growth Phase |
|------------|-------------------|---------------------|
|            | µₒₒ (h⁻¹)        | Yieldₒₒ (cell pmol⁻¹e⁻) | µₐₐₐₐ (h⁻¹) | Yieldₐₐₐₐ (cell pmol⁻¹e⁻) |
| 110spc4    | 0.10 (±0.03) a    | 13.3 (±1.1) a        | 0.049 (±0.004) | 5.1 (±0.8) |
| ΔfixK:     | 0.055 (±0.008) b  | 6.6 (±0.3) b         | 0.00         | -         |
| ΔnmrR      | 0.090 (±0.004) a  | 13.1 (±0.6) a        | 0.00         | -         |

| Genotype   | [O₂] at Onset of NO₃⁻ Reduction (µM O₂) | Max [NO] in Liquid (mM) | Fraction of NO₃⁻ Reduced to N₂ (%) | Final OD₆₀₀ (oxic) | Final OD₆₀₀ (anoxic) |
|------------|-----------------------------------------|------------------------|-----------------------------------|-------------------|---------------------|
| 110spc4    | 5 (±0.3) a                              | 600 (±400) a           | 100                               | 0.080 (±0.005) a  | 0.40 (±0.05) a     |
| ΔfixK:     | -                                       | -                      | -                                 | 0.044 (±0.003) b  | 0.042 (±0.002) b  |
| ΔnmrR      | 3.3 (±2.3) b                            | 10797 (±1700) b        | -                                 | 0.079 (±0.001) a  | 0.061 (±0.004) b  |

All the experimental vials contained an initial O₂ concentration of 2% at headspace and 10 mM NO₃⁻ in the growth medium. Data are means with standard error (in parenthesis) from at least three independent cultures. Values in a column followed by the same lower-case letter are not significantly different according to One-Way ANOVA and the Tukey HSD test at p ≤ 0.05. Apparent oxic growth (µₒₒ, h⁻¹) and anoxic growth (µₐₐₐₐ, h⁻¹) rates based on O₂ consumption during the oxic phase or reduction of NO₃⁻, NO₂⁻, or N₂O during the anoxic phase. Yield (cells per mole electron) based on increase in OD vs. cumulated consumption of O₂ or reduction of NO₃⁻, NO₂⁻, or N₂O, not detected.

As shown in Figure 2A, growth of B. diazoefficiens increased proportionally with NO₃⁻ respiration. Interestingly, the parental strain was able to derive electrons to NO₃⁻ reduction during the oxic phase before O₂ was depleted, thus securing the transition from aerobic to anaerobic respiration and avoiding anaerobic entrapment (Figure 2D). Electron flow to NO₃⁻ increased exponentially during the anoxic phase, with an estimated growth rate (µₐₐₐₐ) of 0.049 (±0.004) h⁻¹ (Figure 2D; Table 1A). The final OD₆₀₀ was 0.40 (±0.05) (Table 1B) and cell yield resulting from NO₃⁻ respiration (5.1 (±0.8) cells pmol⁻¹e⁻ to NO₃⁻) (Table 1A) was around 2.6-fold lower than that observed during oxic respiration.

In contrast to the competent transition from aerobic to anaerobic NO₃⁻ respiration by the parental strain, the fixK: mutant strain was unable to shift to anaerobic respiration (Figure 2B), and following the oxygen depletion, the electron flow dropped drastically to zero (Figure 2E). Remarkably, ΔnmrR was able to initiate denitrification at O₂ concentrations of ≤3.3 µM (±2.3) after 31 h incubation but was unable to consume NO derived from NO₃⁻ reduction, and consequently, NO accumulated in the headspace of the incubation medium (Figures 2C and S1B and Table 1B). This accumulation of NO probably inhibited NO₃⁻ reduction and concomitant growth.

2.2. N₂O Reduction by B. diazoefficiens 110spc4 Relies on the FixK: and NmrR Regulatory Proteins in a Nitrogen-Oxides-Independent Manner

Transient detection of N₂O in B. diazoefficiens wild type and inhibition of the denitrification process in ΔfixK: and ΔnmrR strains precluded comparison of their N₂O reduction capacities. Thus, to specifically assess the capacity of B. diazoefficiens wild type and fixK: and nmrR mutant strains to consume N₂O, we undertook a complementary approach. We supplied B. diazoefficiens bacterial cells with artificial N₂O and analyzed their capacity to consume it. N₂O reduction and subsequent N₂ production were monitored in vials containing 5% N₂O injected into the headspace. In addition, to study the impact that the presence of nitrogen oxides (NOₓ) might exert on N₂O reduction, we also examined B. diazoefficiens’ capacity to consume N₂O in the absence (Figure 3A,C,E) and in the presence (Figure 3B,D,F) of NO₃⁻. In addition to N₂O, 0.5% O₂ was also added to the headspace as
aerobic respiratory substrate due to the incapacity of *B. diazoefficiens* to initiate growth in the total absence of O₂ (data not shown). Regardless of the presence of NO⁻, externally supplied N₂O was rapidly reduced to N₂ by the parental strain until its complete depletion (Figure 3A,B). The final OD₆₀₀(Table 2B) and yield (Table 2A) of *B. diazoefficiens* parental cells were also monitored upon N₂O consumption, and we found that both growth parameters were significantly enhanced when the bacterium was simultaneously incubated with both alternative electron acceptors, N₂O and NO⁻ (Table 2A,B).

In the absence of NO⁻, N₂O reduction was initiated at O₂ concentrations of ≤0.66 (±0.05) μM in the parental strain (Figure 3A; Table 2B). Under these conditions, electron flow to N₂O increased with an apparent growth rate (µN₂O) of 0.028 (±0.002) h⁻¹ estimated by linear regression of In (Vₑ-N₂O) against time (Figure S2A, Table 2A). Electron flow rates to N₂O remained unnoticeable during the first 5 h of oxic respiration; however, they increased exponentially after 8 h when electron flow to O₂ was high. Similar to that which was previously observed during anaerobic NO⁻ respiration, this premature induction of the N₂OR in the presence of O₂ might be a mechanism to elude anoxia entrapment during the transition from oxic to anoxic conditions.

When NO⁻ was present, initiation of denitrification, hallmarked by a transient emission of NO (113 nM ± 30), took place after 7 h incubation under nearly anaerobic conditions (O₂ concentrations of 1.5 (± 0.15) μM) (Table 2B and Figure 3B, respectively), and it preceded induction of N₂O consumption. In fact, N₂O reduction was initiated at lower O₂ concentrations of ≤0.15 (±0.05) μM (Figure 3B; Table 2B), indicating the *B. diazoefficiens* preference for NO⁻ as terminal electron acceptor. Estimated anaerobic growth rate supported by N₂O in the presence of NO⁻ was µN₂O = 0.046 (±0.003) h⁻¹ (Figure S2B, Table 2A). Equivalently to that which was observed in the absence of NO⁻, electron flow to N₂O reduction occurred during active O₂ respiration after 5 h incubation in the presence of NO⁻ (Figure S2B).

Strikingly, *B. diazoefficiens* strains lacking the regulatory transcriptional factors FixK or NnrR were severely impaired in N₂O consumption capacity and growth (Figure 3C–F, Table 2A,B). Despite ΔfixK: or ΔnnrR defective in the *nrrR* gene was significantly defective in its capacity to reduce N₂O when incubated without NO⁻ (only 8 (±0.5)% of N₂O was reduced to N₂), likely due to its incapacity to detoxify NO, which permanently accumulated in the medium up to 32.2 (±8.8) nM (Figure 3E, Table 2B). The presence of NO⁻ slightly induced N₂O reduction by the *nrrR* mutant (12.5 (±2.1)% of N₂O was reduced to N₂) at O₂ concentrations of 0.8 (±0.3) μM after 13 h incubation (Figure 3F, Table 2B). However, under these conditions, NO⁻ in the medium was further reduced to NO, which was accumulated after 20 h incubation reaching levels up to ~2 μM after 50 h incubation (Figure 3F, insert, Table 2B).

Our results explain that *B. diazoefficiens* can co-respire NO⁻ and N₂O and that activation of the N₂O reductase relies on the FixK and NnrR regulatory proteins, independently of the presence of nitrogen oxides. Lastly, we also found that N₂O reductase activity in *B. diazoefficiens* is highly sensitive to accumulation of endogenous NO derived from NO⁻ respiration, further supporting the importance of coordinated activation of denitrifying reductases by the FixK and NnrR regulators.
Figure 3. Impact of FixK² and NnrR inactivation on N₂O consumption. Measurement of O₂ and N₂O respiration and concentrations of NO and N₂. B. diazoefficiens 110spc4 parental strain (A,B) and fixK² (C,D) and nnrR (E,F) mutant strains were incubated in vials containing 0.5% O₂ and 5% N₂O as oxic respiratory and anoxic respiratory substrates, respectively. In addition, a second set of vials were also supplemented with 10 mM NO₃⁻ (B,D,F) as anoxic respiratory substrate. The gradual decline in N₂O concentration in (C,D) and (E) corresponds to dilution of headspace gases due to sampling. Data are the means and standard deviations of at least three different cultures.

Table 2. Summary of growth parameters from N₂O consumption in the B. diazoefficiens 110spc4 parental and fixK² and nnrR mutant strains (A) and other parameters observed through the incubations, depending on the presence or absence of NO₃⁻ (B).
### Table 1

| Genotype | \( [O_2] \) at Onset of N\textsubscript{2}O Reduction (\( \mu M \) O\textsubscript{2}) | Max [NO] in Liquid (nM) | \% N\textsubscript{2}O Reduced to N\textsubscript{2} | Final OD\textsubscript{600} |
|----------|---------------------------------|------------------------|------------------------|------------------------|
| 110spc4  | 0.15 (±0.05) \textsuperscript{a} | 113 (±30) \textsuperscript{a} | 100 \textsuperscript{a} | 0.2 (±0.05) \textsuperscript{a} |
| \( \Delta \text{fix}K_2 \)   | -                               | 35 (±6.9) \textsuperscript{b} | -                      | 0.05 (±0.01) \textsuperscript{b} |
| \( \Delta \text{nnrR} \)     | 0.8 (±0.3) \textsuperscript{b} | 2250 (±85) \textsuperscript{c} | 12.5 (±2.1) \textsuperscript{b} | 0.07 (±0.01) \textsuperscript{b} |

All the experimental vials contained an initial O\textsubscript{2} concentration of 0.5% at headspace, 5% N\textsubscript{2}O, and 10 mM NO\textsuperscript{3}\textsuperscript{-} in the growth medium when indicated. Values in a column followed by the same lower-case letter are not significantly different according to One-Way ANOVA and the Tukey HSD test at \( p \leq 0.05 \). Data are means with standard error (in parenthesis) from at least three independent cultures.

### 2.3. Acidic and Alkaline pHs Impair N\textsubscript{2}O Reduction by B. diazoefficiens 110spc4

To further elucidate how environmental cues prevailing in \( B. \) diazoefficiens niches might modulate N\textsubscript{2}O reduction, we monitored the expression of nosRZDFYLX genes and the capacity of \( B. \) diazoefficiens to reduce N\textsubscript{2}O in the presence of C-substrates commonly encountered in a plant’s rhizosphere [22], such as succinate, which generates 2 mol e\textsuperscript{-} per C-mol oxidized, and butyrate, which generates 5 mol e\textsuperscript{-} per C-mol oxidized. Interestingly, such C-sources did not affect expression of the nos operon (Figure S3A). Next, we analyzed N\textsubscript{2}O consumption by \( B. \) diazoefficiens determined as changes in N\textsubscript{2}O concentration in the headspace of vials containing 0.5% O\textsubscript{2} plus 5% N\textsubscript{2}O inoculated with aerobically raised bacterial cells. Monitoring O\textsubscript{2} uptake by \( B. \) diazoefficiens during the oxic phase also allowed us to evaluate any effect of C-source on bacterial metabolism/energetic that could subsequently alter N\textsubscript{2}O respiration. Despite N\textsubscript{2}O consumption was delayed around 20 h in the presence of butyrate compared to succinate (Figure 4A,B), such impairment could be attributed to a general metabolic defect, as oxygen consumption during the first hours of growth also was attenuated in that C-source. Further metabolic analyses are required to shed light on this respiratory inhibition induced by reduced C-sources.

To understand if local changes in soil pH might affect N\textsubscript{2}O emissions from \( B. \) diazoefficiens, we also examined N\textsubscript{2}OR gene expression and N\textsubscript{2}O consumption in cells incubated at different pHs. As shown in Figure S3B, nos expression levels after 20 and 30 h incubation were not affected by different pH levels. Interestingly, while O\textsubscript{2} consumption was similar at different pH levels, N\textsubscript{2}O reduction was strongly diminished at pH 6 and 8 (Figure 4C–F). These findings imply that, in addition to the impact of FixK\textsubscript{2} and NnrR regulatory proteins on N\textsubscript{2}O reduction, relevant environmental factors such as pH importantly influence dynamics of N\textsubscript{2}O reduction by \( B. \) diazoefficiens.
Figure 4. Impact of C-source and pH on N₂O consumption. Measurement of O₂ and N₂O respiration and concentrations of NO and N₂. B. diazoefficiens 110 sport 4 parental strain was incubated in vials containing 0.5% O₂, 5% N₂O, and 10 mM NO₃⁻ as substrates for aerobic and anaerobic respiration, respectively. C-sources (A, B) and pH (C–F) of the growth medium were modified as shown on the graphs (see Material and Methods for further details). O₂ and NOx concentrations were monitored by automatic sampling from headspace phase. Data are the means and standard deviations of at least three different cultures.

3. Discussion

Given the damaging effect of N₂O on climate, strategies to mitigate N₂O emissions arising from intensive agricultural practices must be developed. These strategies include: (i) management of soil chemistry and microbiology to ensure that bacterial denitrification proceeds to completion, forming N₂; (ii) promotion of sustainable agriculture, i.e., obtaining higher output from the same cultivated area of land; (iii) a better understanding of the
environmental and regulatory factors that contribute to the generation and consumption of biological N₂O; and (iv) reducing the dependence on fertilizers by using engineered crops that fix dinitrogen themselves or, alternatively, through application of nitrogen-fixing bacteria to legume crops. Despite the latter being one of the most promising alternatives to reduce N₂O emissions, denitrification within endosymbiotic and free-living rhizobia released from nodules also contributes to the emission of N₂O [10,13,16,23,24]; therefore, a better knowledge of the environmental and cellular factors controlling rhizobial denitrification is required.

Environmental cues (oxygen tensions and nitrogen oxides) and regulatory proteins (FixK and NnrR) governing denitrification in *B. diazoefficiens* are well-known [19,20,25] (Figure 1). In this work, we have validated, under physiological conditions, the importance of the FixK and NnrR transcription factors in real-time N₂O dynamics using a robotized incubation system. Hence, we were able to simultaneously monitor changes in O₂, NO⁻, NO₂⁻, NO, N₂O, and N₂ concentration during the transition from aerobic to anaerobic respiration in *B. diazoefficiens* wild type and fixK and nnrR regulatory mutants. In addition, we also performed precise estimations of growth parameters (i.e., μ, yield) and defined accurately the O₂ concentrations in which each step of the denitrification process is triggered. Therefore, we were able to determine that the denitrification process in *B. diazoefficiens* occurs at O₂ concentrations of ≤5 (±0.3) μM. This concomitant induction of the denitrifying machinery with oxic respiration ensures a smooth and efficient transition from aerobic to anaerobic respiration, avoiding depression of electron flow when O₂ is scarce (Figure 2A,D). A similar scenario was previously observed in the plant pathogen *Agrobacterium tumefaciens* [26]. In contrast to the early induction of the denitrification process found in these plant-interacting bacteria, *Paracoccus denitrificans* initiates transcription of nitrite reductase very late, resulting in entrapment of the majority of cells in anoxia [27].

We have also demonstrated, for the first time, that *B. diazoefficiens* 110sp4 is an efficient denitrifier, as it is able to transform 100% of NO⁻ to N₂ (Figures 2A and S1A). Interestingly, emission of N₂O was detected at an early peak in O₂ concentration of ≤5 μM (Figures 2A and S1A and Table 2) during the transition from aerobic to anaerobic respiration, but the bacteria rapidly reduced its concentration, keeping it under very low levels (~2 ppm in headspace; 40 nM in the liquid). Collectively, these results reveal that denitrification in *B. diazoefficiens* 110sp4 emits marginal amounts of N₂O, implying, as demonstrated by Mania et al., (2020) [28] and by Gao et al., (2021) [29], that bradyrhizobia can constitute a strong sink of the N₂O released by neighboring organisms in the soil. Such denitrifying activity depends on coordinated activities of FixK and NnrR regulatory proteins. The tight control on emission of N₂O and other denitrifying gases has been previously described in diverse bacterial species [26–29].

Although NO is a key signal molecule for the regulation of many processes, at high concentrations it exerts toxicity at different cellular levels [30–32]. Consequently, bacteria employ dedicated regulatory systems to keep NO at very low concentrations. Strikingly, we found that NO levels in *B. diazoefficiens* cultures reached very high concentrations (~600 nM) (Table 1B). Similarly, *A. tumefaciens* also accumulates large amounts of NO; however, those NO concentrations were not detrimental for this closely related rhizobium [26]. Conversely, *P. denitrificans*, *Pseudomonas aerofaciens*, and strains from the genus *Thauera* present a relatively tight control of NO production, maintaining NO concentration lower than 10–50 nM [21,27,33]. Although the reason for these differences in control of and tolerance to NO concentrations is unknown, it might arise from differential selective pressures exhibited by their ecological niches. Hence, while *P. denitrificans*, *P. aerofaciens*, and *Thauera* genus comprise bacteria that exist under free-living conditions, *A. tumefaciens* and *B. diazoefficiens* are bacteria that can interact with plants establishing pathogenic and symbiotic relationship, respectively. During its interaction with plants, *A. tumefaciens* might face diverse host defense systems such as NO production. Thus, a high tolerance to NO might confer a certain fitness advantage in respect to other soil competitors. NO is also known to be produced by plants in early stages during its interaction with nitrogen-fixing

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**Table 1** Environmental conditions and NO concentrations in cultures of *B. diazoefficiens* 110sp4 and wild type (WT) during the transition from aerobic to anaerobic respira

bacteria, as well as within the mature nodule [34–37]. In this context, symbiotic bacteria might require higher tolerance to NO to establish a productive symbiotic interaction with the plant.

In contrast to the efficient denitrifying capacity of *B. diazoefficiens* wild type, we found that the *fixK* mutant was unable to initiate NO\(^\text{2-}\) reduction. On the contrary, *nnrR* mutant cells were able to initiate the reduction of NO\(^\text{2-}\) to NO\(_\text{2}\) and to NO\(_\text{3}\); however, they were entrapped into anoxia due to accumulation of toxic concentrations of NO (Figure 2B,C). This disparate response of *fixK*; and *nnrR* mutants confirms previous results in vitro, where we demonstrated that FixK directly controls the expression of *napEDABC*, *nnrK*, and *nosRZDFYFLX* genes in response to microoxic conditions and NnrR is the regulator that directly interacts with *norCBQD* promoter in response to NO [19,20]. Similar denitrification phenotypes were observed in *P. denitrificans* mutants deficient in the O\(_2\) and NO sensors FnrP and NNR, respectively [38].

Since NO\(^\text{2-}\) reduction in Δ*fixK* and Δ*nnrR* was abrogated, we could not valuate their capacity to produce or consume N\(_2\text{O}\) resulting from NO\(^\text{2-}\) reduction. To achieve this goal, we incubated the cells in the presence of N\(_2\text{O}\), and we analyzed N\(_2\text{O}\) and N\(_2\) fluxes. In the parental strain *B. diazoefficiens* 110spec4, N\(_2\text{O}\) reduction was initiated at O\(_2\) concentrations of 0.15 (±0.05) and 0.66 (±0.05) µM in the presence and in the absence of NO\(^\text{2-}\), respectively. In contrast to the low O\(_2\) concentration required to trigger N\(_2\text{O}\) consumption in *B. diazoefficiens*, in other rhizobia species such as *Ensifer meliloti* strain 1021, N\(_2\text{O}\) consumption was initiated at O\(_2\) concentrations of 8 µM [39], indicating that *B. diazoefficiens* presents a N\(_2\text{O}\)R more sensitive to O\(_2\) than other closely related rhizobial species.

In addition to microoxia, the nitrogen oxide N\(_2\text{O}\) and its reduction products NO\(^\text{2-}\) or NO are considered essential inducers of denitrification in *B. diazoefficiens* [3,20]. Remarkably, we demonstrated in this work that N\(_2\text{O}\) reduction in this bacterium was triggered in the absence of NO\(^\text{2-}\). Supporting our observations, it has been previously reported that microoxia is the main signal of expression of *B. diazoefficiens* *nosRZDYFLX* genes and N\(_2\text{O}\)R activity [19]. This independence from NO\(^\text{2-}\) was also reported in *E. meliloti* [39] and *P. denitrificans* [33].

When N\(_2\text{O}\) was externally supplied, the parental strain reduced 100% of N\(_2\text{O}\) to N\(_2\). In contrast, the N\(_2\text{O}\)-reducing capacity of the *fixK* mutant was totally abolished in a medium without or with NO\(^\text{2-}\). However, *nnrR* mutant cells were able to reduce some N\(_2\text{O}\) to N\(_2\) in the absence or in the presence of NO\(^\text{2-}\) (8 (±0.5)% and 12.5 (±2.1)%, respectively) (Table 2B). These results confirm previous reports that propose FixK but not NnrR as the main transcriptional activator of the *nosRZDYFLX* genes [19]. In contrast to the disparate contribution of FixK and NnrR observed in our studies, it has been proposed that the homologous regulators of *P. denitrificans* FnrP and NNR contribute equally to N\(_2\text{O}\)R induction [38,40,41]. Interestingly, cultures from Δ*nnrR*, with or without nitrate, showed a weak N\(_2\text{O}\)R activity. In contrast to the transient accumulation of NO detected in cultures from the WT strain with NO\(^\text{2-}\) (Figure 3B), the Δ*nnrR* mutant seems to be unable to detoxify NO, which remains permanently in the medium throughout the incubation (Figure 3F, insert). This long-lasting accumulation of NO was also observed when the medium was not supplemented with nitrate (Figure 3E). This NO may arise from traces of nitrate present in this medium (~50–100 µM, data not shown). The permanent accumulation of NO (32 nM) in Δ*nnrR* cells incubated without nitrate or when they were incubated with nitrate (~2 µM) might impair N\(_2\text{O}\) reduction of the *B. diazoefficiens* Δ*nnrR* mutant.

An optimal management of soils is crucial to induce N\(_2\text{O}\)R activity. In this context, it has been reported that maintaining soil pH at high ranges promotes N\(_2\text{O}\)R activity. This strategy is based on the reported sensitivity of the N\(_2\text{O}\) reductase activity to low pH in denitrifying bacteria [33,39], in bacterial communities extracted from soils and in intact soils [42]. Carbon availability also has an important role in N\(_2\text{O}\) emissions from soils [43]. However, how specific forms of reductants might affect expression and activity of N\(_2\text{O}\)R is largely unexplored. To study ecologically relevant environmental factors that could influence *B. diazoefficiens* N\(_2\text{O}\)R expression and activity, we analyzed the expression of a
nosR-lacZ transcriptional fusion as well as N\textsubscript{2}OR activity by monitoring N\textsubscript{2}O consumption, in the presence of reduced or oxidized C-sources such as butyrate or succinate and at different pH values. Despite the fact that expression of the nos genes was not affected by any of the conditions tested, N\textsubscript{2}OR activity was significantly attenuated when B. diazoefficiens cells were incubated under acidic and alkaline pHs (i.e., pH 6 and pH 8). Moreover, N\textsubscript{2}OR activity was also negatively affected when cells were incubated with reduced C-sources. However, reduced C-sources also affected oxygen consumption, which may indicate a general defect in bacterial metabolism when using such a C-source.

Confirming these observations, low pH had little effect on the transcription of the nosZ gene in P. denitrificans [33]. Instead, the enzymatic rate of N\textsubscript{2}O reduction was significantly attenuated at low pH levels, suggesting that environmental pH may have a direct posttranslational effect on the assembly and/or activity of the N\textsubscript{2}OR holoenzyme. Consistent with these findings, pH did not affect gene expression of Marinobacter hydrocarbonoclasticus N\textsubscript{2}OR genes; however, the amount of N\textsubscript{2}O reductase isolated from cells grown at pH 6.5 was lower than that at pH 7.5 and 8.5, pointing to a post-transcriptional regulation [44]. Indeed, biochemical studies of the M. hydrocarbonoclasticus N\textsubscript{2}OR revealed that redox properties of its catalytic site are significantly altered by changes in pH values ranging from 6.5 to 8.5 [44]. Similarly, as observed in B. diazoefficiens, an inhibitory effect of reduced carbon sources such as butyrate or low pH on N\textsubscript{2}O activity was already observed in E. meliloti [39]. In contrast to E. meliloti [39] and M. hydrocarbonoclasticus [44], in our work, B. diazoefficiens N\textsubscript{2}O was also inhibited at a high pH, buttressing the importance of controlling soils pH regarding N\textsubscript{2}O emissions. Such sensitivity of B. diazoefficiens N\textsubscript{2}O to high pH is currently under investigation.

Altogether, these observations expand the knowledge of the regulatory and environmental factors that control N\textsubscript{2}O emissions by bacterial species associated with legumes. This information should be taken into consideration when developing new programs to manage N\textsubscript{2}O emissions from legume crops.

4. Materials and Methods

4.1. Bacterial Strains and Growth Conditions

Bradyrhizobium diazoefficiens 110spc4 [45] and ΔfixK::Ω and ΔnnrR::aphI II mutant strains [20] were used in this work. To analyze expression of the nosRZDYFLX genes, a B. diazoefficiens strain (110spc4-BG0306) containing a chromosomally integrated transcriptional fusion within the nosRZDYFLX genes promoter and the lacZ reporter gene was used [19]. B. diazoefficiens strains were firstly grown aerobically in 120 mL serum vials each containing a magnetic stirring bar and 50 mL of Peptone-Salts-Yeast extract (PSY) complete medium [46] at 30 °C. To analyze anaerobic growth from B. diazoefficiens, aliquots from aerobic cultures raised under vigorous stirring to avoid anoxic microzones by cells aggregation were transferred to vials with minimal defined Bergersen’s medium [47]. Oxygen from vials was removed by 6 cycles of air evacuation for 360 s and helium (He) filling for 40 s. Influence of pH on N\textsubscript{2}O consumption was analyzed by cultivating B. diazoefficiens under N\textsubscript{2}O respiring conditions in minimally defined medium buffered with 50 mM phosphate buffer at pH 6, 7, 7.5, and 8. In all the treatments, the headspace was filled with an initial concentration of O\textsubscript{2}: of 0.5 or 2% (6 or 24 µM dissolved O\textsubscript{2} at 30 °C, respectively). To study the N\textsubscript{2}O consumption by the bacterium, vials were also supplemented with N\textsubscript{2}O 5% (1.2 mM). A concentration of 10 mM KNO\textsubscript{3} was also added to the cultures as alternative respiratory substrate as indicated in the text. When needed, antibiotics were used at the following concentrations (in µg/mL): kanamycin, 30; spectinomycin, 25; streptomycin, 25; tetracycline, 10.

4.2. Gas Measurements

After transferring aerobically grown bacteria into anaerobic vials, they were placed together with blanks and gas standards in a thermostatic water incubator at 30 °C. Cells
dispersion and equal distribution of gases throughout the vial liquid and headspace was achieved by continuous stirring at 700 rpm. Emission of gases (O2, NO, N2O, and N2) resulting from bacterial aerobic and anaerobic metabolism were monitored by automatic gas sampling. Gas measurements were analyzed as described by Bueno et al., 2015, and Molstad et al., 2007 [39,48]. Briefly, the gas samples were drawn from each bacterial culture, and with each sampling an equal volume of He was pumped back into the vials to maintain gas pressure at 1 atm. Sampling and gases’ measurements were performed as previously described in detail [39].

4.3. Determination of Bacterial Growth and NO− and NO2− Concentrations

To measure bacterial growth and NO− and NO2− concentrations, aliquots from the liquid phase of vials were withdrawn manually by using sterile syringes. Bacterial growth was determined by measuring cell density at 600 nm (OD600). Concentrations of NO− and NO2− were determined as described by Bueno et al., 2015 [39].

4.4. Kinetic Analysis from Aerobic and Anaerobic Respiration

Aerobic and anaerobic respiration kinetics were determined as described by Bueno at al., 2015 [39]. To determine O2 and NO concentrations in the liquid, we considered the pressure of the gases, their solubilities, and their transport coefficients among headspace and liquid. O2 dissolved in liquid was also calculated considering O2 respiration rate during bacterial growth (see Molstad et al., 2007 for details). We analyzed N2O concentrations as µmol N2O vial−1, while N2 was estimated as net production of N2. Growth rates (µmax) and reduction of NOx during the anoxic phase (µanox) were determined by regression [ln (V−)] against time for the phases with exponentially increasing rates. Determination of cells yield (cells pmol−1 e−) was estimated considering the number of biomass produced per pmol electron consumed by the transport electron chain to reduce O2 to H2O in the oxic phase (Yieldox) or by the denitrifying machinery during the anoxic phase (Yieldanox). Vmax tells us about the specific efficiency for O2 and NO respiration per cell. For further details regarding these calculations, see Molstad et al. (2007) [48] and Nadeem et al. (2013) [27].

4.5. Determination β-galactosidase Activity

β-galactosidase activity to investigate gene expression was analyzed as previously described [49]. In brief, 5 mL of cells incubated for 20 and 30 h under the conditions detailed in the text were collected, centrifuged, and resuspended in 500 µL of growth medium. In total, 25 µL of this culture was mixed with 20 µL of freshly prepared SDS 0.1%, 25 µL chloroform, and 100 µL of Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4 and 50 mM β-mercaptoethanol). Next, 20 µL of ONPG (4 mg/mL) was added to initiate the reaction. Reaction mix was incubated at room temperature before the reaction was terminated by addition of 75 µL of 1 M Na2CO3. Supernatant was collected and absorbance at OD420 and OD550 used to determine β-galactosidase specific activity in Miller units.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/ijms23031486/s1.

Author Contributions: Conceptualization, E.B., A.F., L.R.B., and M.J.D.; methodology, E.B., D.M., A.F., L.R.B., and M.J.D.; validation, E.B., A.F., L.R.B., and M.J.D.; formal analysis, E.B., A.F., L.R.B., and M.J.D.; investigation, E.B.; resources, A.F., L.R.B., S.M., and M.J.D.; writing—original draft preparation, E.B., writing—review and editing, E.B., A.F., L.R.B., E.J.B., S.M., and M.J.D.; visualization, E.B., A.F., L.R.B., and M.J.D.; supervision, E.B., A.F., L.R.B., and M.J.D.; project administration, E.B., A.F., L.R.B., and M.J.D.; funding acquisition, E.B., A.F., L.R.B., S.M., and M.J.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by MCIN/AEI/10.13039/501100011033, “ERDF A way of making Europe”, grant AGL2017-85676-R to María J Delgado, grants AGL2015-63651-P and PID2020-
114330GB-100 to Socorro Mesa, and also Junta de Andalucía, grant P18-RT-1401 to María J Delgado and Socorro Mesa. EB was supported by a personal visiting researcher grant–IS-MOBIL (Oslo University, Norway) and the CSIC JAE-DOC Program co-financed by ESF.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Acknowledgments:** We acknowledge the continuous support from Junta de Andalucía. We also acknowledge the support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

**Conflicts of Interest:** The authors declare no conflict of interest

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