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Genetic basis for denitrification in *Ensifer meliloti*

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Abstract

Background: Denitrification is defined as the dissimilatory reduction of nitrate or nitrite to nitric oxide (NO), nitrous oxide (N_2O), or dinitrogen gas (N_2). N_2O is a powerful atmospheric greenhouse gas and cause of ozone layer depletion. Legume crops might contribute to N_2O production by providing nitrogen-rich residues for decomposition or by associating with rhizobia that are able to denitrify under free-living and symbiotic conditions. However, there are limited direct empirical data concerning N_2O production by endosymbiotic bacteria associated with legume crops. Analysis of the *Ensifer meliloti* 1021 genome sequence revealed the presence of the *napEFDABC*, *nirK*, *norECBQD* and *nosRZDFYLX* denitrification genes. It was recently reported that this bacterium is able to grow using nitrate respiration when cells are incubated with an initial O_2 concentration of 2%; however, these cells were unable to use nitrate respiration when initially incubated anoxically. The involvement of the *nap*, *nirK*, *nor* and *nos* genes in *E. meliloti* denitrification has not been reported.

Results: *E. meliloti nap*, *nirK* and *norC* mutant strains exhibited defects in their ability to grow using nitrate as a respiratory substrate. However, *E meliloti nosZ* was not essential for growth under these conditions. The *E. meliloti napA*, *nirK, norC* and *nosZ* genes encode corresponding nitrate, nitrite, nitric oxide and nitrous oxide reductases, respectively. The NorC component of the *E. meliloti* nitric oxide reductase has been identified as a *c*-type cytochrome that is 16 kDa in size. Herein, we also show that maximal expression of the *E. meliloti napA*, *nirK, norC* and *nosZ* genes occurred when cells were initially incubated anoxically with nitrate.

Conclusion: The *E. meliloti napA*, *nirK, norC* and *nosZ* genes are involved in nitrate respiration and in the expression of denitrification enzymes in this bacterium. Our findings expand the short list of rhizobia for which denitrification gene function has been demonstrated. The inability of *E. meliloti* to grow when cells are initially subjected to anoxic conditions is not attributable to defects in the expression of the *napA*, *nirK, norC* and *nosZ* denitrification genes.

Keywords: Cu-containing nitrite reductase, Nitrate respiration, Nitric oxide reductase, Nitrous oxide reductase, Periplasmic nitrate reductase

Background

Denitrification is the respiratory reduction of nitrate or nitrite to the gaseous products nitric oxide (NO), nitrous oxide (N_2O), or dinitrogen (N_2). N_2O is a powerful greenhouse gas (GHG) that has a 300-fold greater global warming potential than CO_2 based on its radiative capacity and could persist for up to 150 years in the atmosphere [IPCC 2007, [1]]. In bacteria, the denitrification process requires four separate enzymatically catalysed reactions. The first reaction in denitrification is the reduction of nitrate to nitrite, which is catalysed by a membrane-bound nitrate reductase (Nar) or a periplasmic nitrate reductase (Nap) (reviewed in [2-6]). In denitrifying bacteria, the reduction of nitrite to nitric oxide is catalysed by two types of respiratory Nir: the NirS cd_1 nitrite reductase, a homodimeric enzyme with haems *c* and d_1, and NirK, a copper-containing Nir [7-11]. Then, nitric oxide is reduced to nitrous oxide by three types of nitric oxide reductase (Nor), which are classified based on the nature of their electron donor as cNor, qNor or qCuANor (reviewed in [4,9,10,12]). The final step in denitrification consists of the two-electron reduction of nitrous oxide to dinitrogen gas. This reaction is performed by nitrous oxide reductase

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(Nos), a copper-containing homodimeric soluble protein located in the periplasmic space (reviewed in [9-11,13-15]). Bacteria of the order *Rhizobiales*, collectively referred to as rhizobia, are best known for their ability to establish N_2-fixing symbiosis on legume roots and on the stems of some aquatic leguminous plants. In addition to fixing N_2, many rhizobia species have enzyme-encoding genes for some or all of the four reductase reactions in denitrification. Several studies have reported that legume crops contribute to N_2O production by providing N-rich residues for decomposition [16] and by associating with some rhizobia that are able to denitrify under free-living and under symbiotic conditions, producing N_2O [17-19]. However, soybean endosymbiont *Bradyrhizobium japonicum* is the only rhizobia species for which it has been demonstrated that the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes are involved in complete denitrification [17,19,20].

Ensifer (formerly *Sinorhizobium*) *meliloti* is a rhizobial species that establishes symbiotic N_2-fixing associations with plants of the genera *Medicago*, *Melilotus* and *Trigonella*. Genes for the complete denitrification pathway are present in the *E. meliloti* pSymA megaplasmid [21,22]. Transcriptomic analyses have shown that the *E. meliloti nap, nir, nor* and *nos* genes are induced in response to O_2 limitation [23]. Under these conditions, the expression of denitrification genes is coordinated via a two-component regulatory system, FixLJ, and via a transcriptional regulator, FixK [24]. Recent transcriptomic studies demonstrated that denitrification genes (*nirK* and *norC*) and other genes related to denitrification (*azu1*, *hemN*, *nnrU* and *nnrS*) are also induced in response to NO and that the regulatory protein NnrR is involved in the control of this process [25]. In symbiotic association with *M. truncatula* plants, recent findings have demonstrated that the *E. meliloti napA* and *nirK* denitrification genes contribute to nitric oxide production in root nodules [26]. Although the regulation and symbiotic characterisation of *E. meliloti* denitrification genes is well understood, the roles of these genes in nitrate reduction through denitrification and in the emission of N_2O are not known.

Recent results from our group [21] reported the capability of *E. meliloti* to use nitrate or nitrite as respiratory substrates when cells were incubated with an initial oxygen concentration of 2%; however, nitrate and nitrite could not be used as respiratory substrates when the cells were initially incubated anoxically. In the present work, functional analyses of the *E. meliloti napA*, *nirK*, *norC* and *nosZ* genes reveal their involvement in the ability of *E. meliloti* to grow using nitrate as a respiratory substrate and in the expression of denitrification enzymes.

Results

Nitrate-dependent growth of *E. meliloti napA, nirK, norC* and *nosZ* mutants

To investigate the involvement of denitrification genes in the ability of *E. meliloti* to grow using nitrate as an electron acceptor, the wild-type strains 1021 and 2011 and *napA*, *nirK*, *norC* and *nosZ* mutant strains (Table 1) were incubated in minimal medium (MM) supplemented with 10 mM KNO_3 (MMN) with an initial O_2 concentration of 2%, and the growth was determined by monitoring the optical density at 600 nm (OD_{600}) (Figure 1). Under these conditions, *E. meliloti* 1021 cells consumed the oxygen present in the atmosphere after incubation for 6 h and reached anoxic conditions (Figure 1A, insert). Similar oxygen consumption rates were observed for strain 2011 and the *napA*, *nirK*, *norC* and *nosZ* mutants (data not shown). Confirming the previous results [21], *E. meliloti* 1021 exhibited a cell density of approximately 1 after 48 h of incubation in MMN (Figure 1A). A similar growth rate was observed after incubation of the wild-type strain 2011 (data not shown). As shown in Figure 1A, the *napA*, *nirK* and *norC* mutant strains exhibited growth defects compared with the WT cells, reaching a turbidity of approximately 0.6, 0.7 and 0.35, respectively, after incubation in MMN for 48 h (Figure 1A). *E. meliloti nosZ* mutant cells demonstrated similar growth to WT cells (Figure 1A), suggesting that *nosZ* was not essential for growth under these conditions. As previously reported for *E. meliloti* 1021 [21], none of the *E. meliloti* denitrification mutants were

Table 1 Bacterial strains

Strain	Relevant characteristics	Reference
Ensifer meliloti		
1021	Wild type; Sm^r	Meade *et al.*, 1982 [27]
2011	Wild type	Casse *et al.*, 1979 [28]
2011mTn5STM.3.02.F08	*napA*::mini-Tn5 Sm^r, Km^r	Pobigaylo *et al.*, 2006 [29]
2011mTn5STM.3.13.D09	*napC*::mini-Tn5; Sm^r, Km^r	Pobigaylo *et al.*, [29]
2011mTn5STM.1.13.B08	*nirK*::mini-Tn5; Sm^r, Km^r	Pobigaylo *et al.*, [29]
SmPl.1021.G1PELR32E8	*norC*::Pl.G1PELR32E8; Sm^r, Km^r	Becker *et al.*, 2009 [30]
2011mTn5STM.5.07.B03	*nosZ*::mini-Tn5; Sm^r, Km^r	Pobigaylo *et al.*, [29]

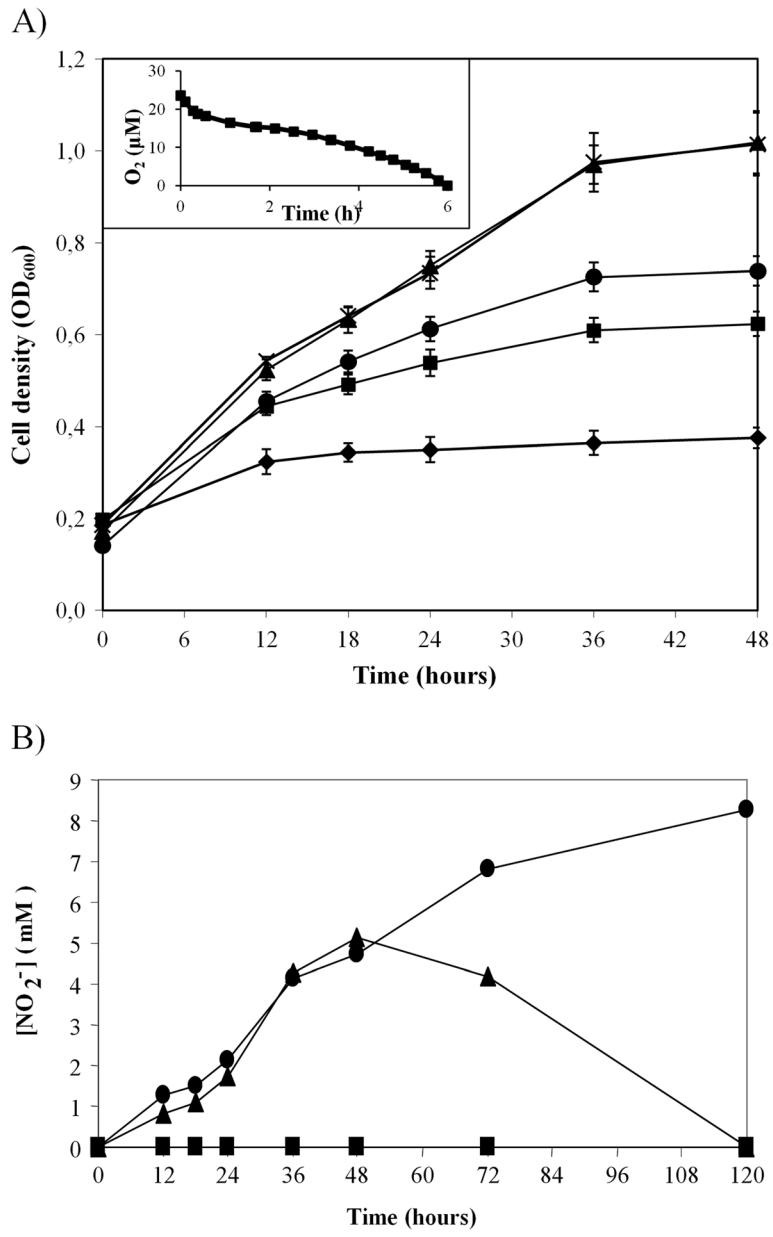

Figure 1 Growth of *E. meliloti* strains with nitrate. (A) Growth of *E. meliloti* 1021 (▲) and the *napA* (■), *nirK* (●), *norC* (♦) and *nosZ* (*) mutant strains in MMN under 2% initial O_2 conditions. The oxygen consumption by the WT cells is also shown (insert). **(B)** The extracellular nitrite concentrations of *E. meliloti* 1021 (▲), *napA* (■) and *nirK* (●) mutant strains. Representative curves of three independent experiments run in triplicate are shown.

able to grow in MMN when they were subjected to anoxic conditions starting at the beginning of the incubation period (data not shown). As shown in Figure 1B, after incubation in MMN with an initial O_2 concentration of 2%, nitrite was not observed in the growth medium of *napA*. However, in the *nirK* mutant, the nitrite concentration increased over the course of the incubation period, reaching a final concentration of 8.3 mM. The WT strains demonstrated a similar rate of nitrite accumulation during the first 48 h; however, this nitrite was depleted over the subsequent 70 h of incubation (Figure 1B).

E. meliloti napA, *nirK*, *norC* and *nosZ* genes encode functional reductases

The functions of the *E. meliloti* denitrification genes were also investigated by analysing the activities of the denitrification enzymes in WT and *napA*, *nirK*, *norC* and *nosZ* mutants incubated under oxygen-limiting conditions.

Cells of the *napA* mutant demonstrated an approximately 11-fold decrease in methyl viologen-dependent nitrate reductase (MV^+-NR) activity compared with the WT cells after incubation for 18 h in MMN with an initial O_2 concentration of 2% (Table 2). As observed for the NR activity in *napA* cells, the methyl viologen-dependent nitrite reductase (MV^+-Nir) activity levels in the *nirK* mutant cells were 10-fold lower than the levels detected in the parental strain when the cells were incubated in MMN with an initial O_2 concentration of 2% (Table 2). As shown in Table 2, the MV^+-NR and MV^+-Nir activities were detected in WT cells incubated under anoxic conditions from the start of the incubation period. Under these conditions, the NR activity levels in *napA* cells and the Nir activity levels in *nirK* cells were undetectable (Table 2).

We also investigated the ability of the *E. meliloti nirK* and *norC* mutants to produce nitric oxide. After incubation for 18 h with an initial O_2 concentration of 2%, NO production rates were determined in an NO-electrode chamber after adding nitrite to the reaction mixture. A significant decrease in NO production was observed in the *nirK* mutant compared with the WT strain (0.57 ± 0.19 *vs.* 202 ± 15 nmol $NO \cdot mg\ protein^{-1} \cdot min^{-1}$, respectively), whereas the *norC* mutant produced 4.6-fold more NO than the WT cells (943 ± 4.52 *vs.* 202 ± 15 nmol $NO \cdot mg\ protein^{-1} \cdot min^{-1}$, respectively). The high levels of NO produced by the *norC* mutant are most likely due to its defect in NO consumption activity. After 18 h of incubation in MMN under an initial O_2 concentration of 2%, the *norC* mutant cells demonstrated NO consumption activity that was practically abolished compared with the activity of WT cells (Table 2); the same results were observed when the *norC* mutant cells were incubated under initially anoxic conditions.

Figure 2 shows that *E. meliloti* 1021 is able to produce N_2O after incubation in MMN under an initial O_2 concentration of 2% and under anoxic conditions. Under both conditions, the *nosZ* mutant cells achieved N_2O accumulation values of approximately 8- and 2-fold higher than the values produced by WT cells after 18 h and 36 h of incubation in MMN, respectively (Figure 2).

Identification of *E. meliloti* NorC

As previously reported by Torres and colleagues [31], four haem-stained bands of 40, 33, 32 and 27 kDa were detected in *E. meliloti* 1021 cells grown in minimal media (MM) with an initial O_2 concentration of 2% in the headspace (Figure 3, lane 1). Although the identities of the 40 kDa and 33 kDa proteins are unknown, the 32 kDa and 27 kDa *c*-type cytochromes were identified as the *E. meliloti* FixP and FixO proteins, respectively, which are subunits of the cbb_3-type high-affinity cytochrome *c* oxidase encoded by the *fixNOQP* operon [31]. The addition of nitrate to the growth medium revealed a haem-stainable band of approximately 16 kDa in the membranes of the WT cells (Figure 3, lane 2). This protein was absent in the *norC* mutant when it was incubated with a 2% initial oxygen concentration in MMN (Figure 3, lane 3), which identifies this *c*-type cytochrome as the NorC component of the *E. meliloti* 1021 nitric oxide reductase. As shown in Figure 3 (lane 4), membranes from the *napC* mutant presented a similar band pattern to that of membranes from the WT cells incubated under an initial O_2 concentration of 2% with nitrate (Figure 3, lanes 2 and 4). These results did not permit us to identify the *E. meliloti* NapC protein, which has a predicted size of 25 kDa. In contrast, in other rhizobia species, such as *B. japonicum*, NapC has been detected via haem-staining analyses and identified as a protein approximately 25 kDa in size [32].

When the cells were subjected to anoxic conditions starting at the beginning of the incubation period, a strong defect in FixP and FixO expression was observed compared with the expression levels detected in cells incubated with an initial O_2 concentration of 2% (Figure 3, lanes 1 and 5). Only proteins approximately 40 and 33 kDa in size could be detected in the anoxically incubated cells. These 40 kDa and 33 kDa proteins were also

Table 2 The methyl viologen-dependent (MV^+) nitrate reductase (MV^+-NR), nitrite reductase (MV^+-Nir) and nitric oxide reductase (Nor) activities of *E. meliloti* 1021 (WT) and the *napA, nirK*, and *norC* mutant strains incubated in MMN under 2% initial O_2 or anoxic conditions

Strain	Genotype	Oxygen conditions					
		2% O_2			Anoxia		
		MV^+-NR[a]	MV^+-NiR[b]	Nor[c]	MV^+-NR	MV^+-NiR	Nor
1021	WT	210.93 (10.33)	32.57 (1.42)	563.33 (21.81)	62.96 (5.70)	10.522 (1.465)	335.88 (32.12)
STM.3.02.F08	*napA*	18.86 (3.79)	-	-	n.d.	-	-
STM.1.13.B08	*nirK*	-	3.34 (0.26)	528.26 (20.86)	-	n.d.	308.19 (23.18)
G1PELR32E8	*norC*	-	-	1.11 (0.01)	-	-	2.84 (0.78)

[a]MV^+-NR and [b]MV^+-Nir activities are expressed as nmol NO_2^- produced or consumed · mg protein^{-1} · min^{-1}. Nor activity is expressed as nmol NO consumed · mg protein^{-1} · min^{-1}. All of the activities were determined after incubation for 18 h. The data are expressed as the means with the standard error in parentheses from at least two different cultures assayed in triplicate. -, not determined; n.d., not detectable.

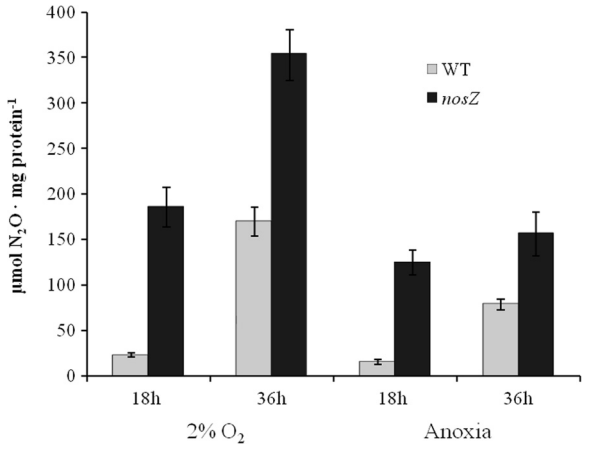

Figure 2 N_2O accumulation in *E. meliloti* 1021 (WT) and the *nosZ* mutant incubated in MMN under 2% initial O_2 or anoxic conditions. N_2O was measured in the headspace of the cultures after 18 and 36 h of incubation. The data represent the means with the standard deviations from at least two different cultures assayed in triplicate.

present in cells grown under oxic conditions [31]. These proteins might remain in the membranes of cells that are grown aerobically prior to the anoxic incubation period. As shown in Figure 3 (lanes 2 and 6), nitrate-dependent NorC expression decreased under anoxic conditions compared with cells incubated with an initial O_2 concentration of 2%. As observed for NorC, the expression of FixP and FixO was weak in the membranes from the anoxically incubated cells in the presence of nitrate (Figure 4, lanes 2 and 6).

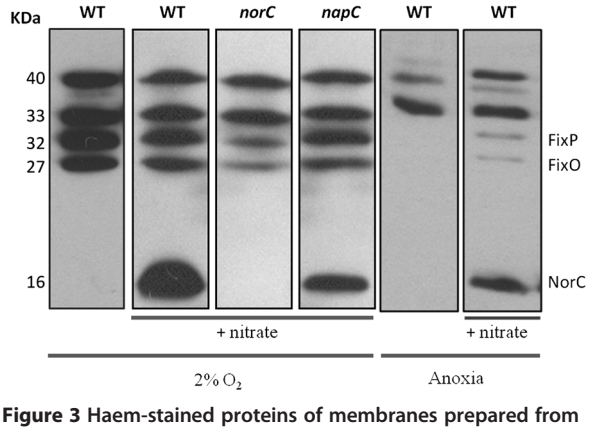

Figure 3 Haem-stained proteins of membranes prepared from *E. meliloti* 1021 (WT) and the *norC* and *napC* mutants incubated in MM or MMN for 24 h under 2% initial O_2 or anoxic conditions. Each lane contains 25 μg of membrane proteins. Haem-stained *c*-type cytochromes identified previously (FixP and FixO) and in this work (NorC) are specified in the right margin. Apparent protein molecular masses (kDa) are shown in the left margin.

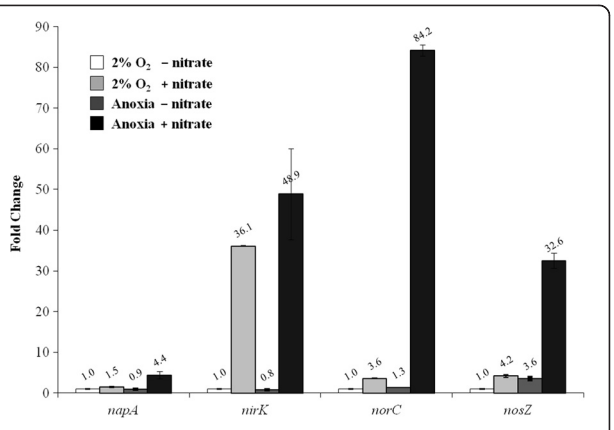

Figure 4 Expression of *E. meliloti* 1021 *napA, nirK, norC* and *nosZ* denitrification genes in cells incubated for 12 h in MM or MMN under an initial oxygen concentration of 2% or under anoxic conditions. The transcription levels were quantified using qRT-PCR with total RNA samples as the templates. The data were analysed using the standard curve method (*nirK* data were analysed with the comparative C_T method), and the expression levels were normalised against the *E. meliloti smc00128* gene as an internal standard. The values expressed relative to the values of cells incubated under 2% initial O_2 in the absence of nitrate are the means and standard deviations of three independent experiments run in triplicate.

Expression of *E. meliloti* denitrification genes

We analysed the expression of the *E. meliloti napA*, *nirK*, *norC* and *nosZ* genes using qRT-PCR analyses. With the exception of *nirK* expression, which was induced 36-fold by nitrate, the presence of nitrate in the growth medium of cells incubated under an initial O_2 concentration of 2% provoked the induction of *napA*, *norC* and *nosZ* expression by 1.5-, 3.6- and 4.2-fold, respectively, compared with the expression observed in the absence of nitrate (Figure 4). When the cells were incubated anoxically from the beginning of culture, the *napA, nirK, norC* and *nosZ* genes were induced approximately 4-, 48-, 84- and 32-fold by nitrate compared with the expression levels observed after a 12 h incubation in MM at an initial O_2 concentration of 2% (Figure 4). These results indicate that the maximal expression of the *E. meliloti napA*, *nirK*, *norC* and *nosZ* denitrification genes occurs when the cells are initially incubated anoxically and when nitrate is present in the growth medium.

Discussion

E. meliloti has been considered a partial denitrifier because of its traditionally reported inability to use nitrate as an electron acceptor for ATP generation and growth under anoxic conditions [18,33]. Recent results from our group confirmed the inability of *E. meliloti* to grow via nitrate respiration when cells were initially incubated under anoxic conditions [21]; however, *E. meliloti* 1021 was able to use nitrate as a respiratory substrate when

cells were initially incubated with 2% O_2 in the headspace [21]. Under these conditions, O_2 was consumed after 6 h of incubation, as we demonstrated in the present manuscript. In this work, we demonstrated that *E. meliloti nap* genes are involved in *E. meliloti* nitrate-dependent growth and that nitrite derived from nitrate reduction is produced by Nap. The low levels of NR activity observed in the *napA* mutant explain the growth defect and the inability of this strain to produce nitrite in cells incubated in MMN with 2% initial O_2. The majority of the most well-characterised denitrifying bacteria use the membrane-bound nitrate reductase (Nar) to catalyse the first step of denitrification. In contrast to Nar, which has a respiratory function, Nap systems demonstrate a range of physiological functions, including the disposal of reducing equivalents during aerobic growth on reduced carbon substrates or anaerobic nitrate respiration [2-6]. Our results support the proposed role of Nap in nitrate respiration. Some rhizobial species, such as *Pseudomonas* sp. G179 (*Rhizobium galegae*) and *Bradyrhizobium japonicum*, could express *nap* genes under anaerobic conditions, and the disruption of these genes is lethal for growth under denitrifying conditions [32,34].

Whereas the deletion of *nosZ* did not have a significant effect on the ability of *E. meliloti* to respire nitrate and increase growth yield, the *nirK* and *norC* mutants exhibited clear defects in nitrate-dependent growth, most likely because of the toxicity of the intermediates nitrite and nitric oxide, respectively. Nitrite or NO were accumulated by the *nirK* and *norC* mutants, respectively, because of the strong defects in Nir and Nor activities observed in these mutants compared with WT levels. Similar phenotypes for *nirK* and *norC* mutants were reported for *B. japonicum* [35,36] and *Rhizobium etli* [37]. The increased levels of N_2O accumulated by the *nosZ* mutant relative to the WT cells indicated that this gene is involved in nitrous oxide reduction in *E. meliloti*. Similar observations were noted with a *B. japonicum nosZ* mutant [38]. In addition to demonstrate the involvement of the *E. meliloti napA*, *nirK*, *norC* and *nosZ* genes in nitrate, nitrite, nitric oxide and nitrous oxide reduction, respectively, we have identified the NorC subunit of nitric oxide reductase as a cytochrome c that is approximately 16 kDa in size.

Growth experiments in this study and in previous studies [21] clearly demonstrated that *E. meliloti* utilises nitrate-dependent growth when transitioning to anoxic conditions occurs when cells are incubated under an initial O_2 concentration of 2%; however, nitrate-dependent growth does not occur when cells are subjected to anoxic conditions starting at the beginning of the incubation period. To understand the differential responses of *E. meliloti* denitrification capability to these different anoxically induced conditions, we investigated the ability of *E. meliloti* to express the denitrification genes in cells incubated under 2% initial O_2 compared with cells initially subjected to anoxic conditions. Despite the inability of *E. meliloti* to grow, we demonstrated that the *napA*, *nirK, norC* and *nosZ* denitrification genes were fully induced in cells initially subjected to anoxia and in the presence of nitrate. Furthermore, denitrification enzymes are active in cells initially incubated under anoxic conditions; we were able to detect significant MV^+-NR, MV^+-NiR and Nor activity levels and N_2O production under these conditions. In contrast, the protein levels corresponding to NorC and the FixP and FixO components of the high affinity cbb_3 oxidase were very weak after incubation of the cells under anoxic conditions starting at the beginning of the incubation period. The latter observations might explain the limited nitrate-dependent growth capacity of *E. meliloti* when anoxic conditions are induced starting at the beginning of the growth period. Under these conditions, cells would be trapped, without energy, and they would be unable to produce the proteins required to cope with the oxygen-limiting conditions, most likely because of the lack of energy. Supporting this hypothesis, it was reported in *Pseudomonas* sp. G59 that the formation of nitrate reductase and nitrous oxide reductase did not occur under aerobic or anaerobic conditions; however, nitrate reductase and nitrous oxide reductase were produced under microaerobic incubation [39]. The latter study suggests that dependence on microaerobiosis for the formation of these reductases was attributable to an inability to produce energy anaerobically until these anaerobic respiratory enzymes formed [39]. Recent studies have shown that the soil bacterium *Agrobacterium tumefaciens* is unable to maintain balanced expression of denitrification genes if oxygen depletion occurs too quickly [40,41]. Similarly, the soil bacterium *P. denitrificans* appears unable to effectively switch from oxic to anoxic respiration, leaving a large fraction of the cell population in anoxia without a chance to express the denitrification proteome [41]. As suggested by Nadeem and co-workers [42], "microaerobic" denitrification is an essential trait for securing an efficient transition to anaerobic denitrification. Considering that *B. japonicum,* which is able to grow under anoxic nitrate-respiring conditions, is a slow-growth bacterium and *E. meliloti* is a fast-growth bacterium, the transition from oxic to anoxic metabolism might be different in these species. Supporting this suggestion, we observed that *B. japonicum* cells are able to express the FixO and FixP subunits of the cbb_3 oxidase under anoxic conditions (E. Bueno, personal communication). However, as shown in this work, *E. meliloti* does not express the FixO and FixP proteins under anoxic conditions. A lack of the energy necessary for protein synthesis might contribute to the inability of *E. meliloti* to grow via nitrate respiration when cells are initially incubated anoxically.

Conclusion

The potential impact of denitrification by plant endosymbiotic bacteria on the emission of the greenhouse gas N_2O has been poorly investigated. The results of this work demonstrate the involvement of the *napA*, *nirK*, *norC* and *nosZ* genes in the previously reported ability of *E. meliloti* to grow via nitrate respiration when cells are subjected initially to 2% O_2. Furthermore, the roles of the reductases encoded by *napA*, *nirK*, *norC* and *nosZ* in nitrite, nitric oxide, N_2O production and N_2O reduction, respectively, were demonstrated. Thus, our results contribute to the investigation of the unexplored genetic basis for denitrification in the alfalfa endosymbiont *E. meliloti*. This knowledge will be instrumental in the development of agricultural strategies and management practices for mitigating the release of N_2O from legume crops.

Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *E. meliloti* strains were routinely grown aerobically at 30°C in tryptone yeast (TY) complete medium [43]. These cultures were then used as the inocula for subsequent incubation experiments, which were performed in minimal medium (MM) [44] or in MM medium supplemented with 10 mM KNO_3 (MMN); the cells were subjected to two experimental oxygen-limiting conditions. In the first set of experiments, 17-ml serum tubes or 500-ml flasks containing 5 or 200 ml medium, respectively, were sealed with rubber septa, and the headspace atmospheres were replaced with a gas mixture (2% oxygen, 98% argon) at the starting point of the incubation. In the second experiment, the cells were incubated in completely filled 200-ml bottles or 17-ml tubes without added oxygen; these conditions are referred to throughout the manuscript as "anoxic conditions". Antibiotics were added to the cultures at the following concentrations ($\mu g \cdot ml^{-1}$): streptomycin, 200; and kanamycin, 200.

Headspace O_2 measurements

After inoculation at an OD_{600} of 0.2, 1 ml of each culture was placed in a 3-ml thermostatted and magnetically stirred reaction chamber with an O_2 electrode (Hansatech, Norkfolk, England). The headspace atmosphere in the chamber was replaced with a gas mixture (2% oxygen, 98% argon) at the starting point of the incubation. The kinetics of oxygen depletion in the chamber were monitored.

Determination of nitrate reductase and nitrite reductase activity

E. meliloti cells were incubated (initial OD_{600} of approximately 0.15-0.2) under 2% initial oxygen or under anoxic conditions for 18 h in MMN medium. The cells were harvested by centrifugation at 8000 *g* for 10 min at 4°C, washed with 50 mM Tris/HCl buffer (pH 7.5) until no nitrite was detected and then resuspended in 0.5 ml of the same buffer. The methyl viologen-dependent nitrate reductase (MV^+-NR) activity was analysed essentially as described by Delgado and colleagues (2003) [32]. To determine the methyl viologen-dependent nitrite reductase (MV^+-Nir) activity, the reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 200 μM $NaNO_2$, 400 μM methyl viologen (MV) and 100 μl of cell suspension (0.02–0.04 mg of protein). The reaction was started by the addition of 50 μl of freshly prepared sodium dithionite solution (30 $mg \cdot ml^{-1}$ in 300 mM $NaHCO_3$). After incubation for 20 min at 30°C, the reaction was stopped by vigorous shaking until the samples lost their blue colour.

Haem-staining analysis

E. meliloti cells grown aerobically in 150 ml of TY medium were harvested by centrifugation at 8000 *g* for 5 min, washed twice with MM, resuspended in 200 ml of MM or MMN at an OD_{600} of 0.15-0.2 and incubated under 2% initial O_2 or anoxic (filled bottles) conditions for 24 h. The cell pellets were resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7) containing 100 μM 4-(2-aminoethyl) benzene-sulfonyl fluoride hydrochloride (ABSF), RNAse (20 $\mu g \cdot ml^{-1}$) and DNAse I (20 $\mu g \cdot ml^{-1}$). The cells were disrupted using a French pressure cell at a constant pressure of approximately 1000 psi (SLM Aminco, Jessup, MD, USA). The cell extract was centrifuged at 10,000 *g* for 20 min to remove the unbroken cells, and the supernatant was centrifuged at 140,000 *g* for 1 h. The membrane pellet was resuspended in 100 μl of the same buffer. The membrane protein aliquots were diluted in sample buffer [124 mM Tris–HCl, pH 7.0, 20% glycerol, 4.6% sodium dodecyl sulphate (SDS) and 50 mM 2-mercaptoethanol] and incubated at room temperature for 10 min. The membrane proteins were separated at 4°C using 12% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and stained for haem-dependent peroxidase activity, as described previously [45], using the SuperSignal chemiluminescence detection kit (Pierce, Thermo Fisher Scientific, IL, USA).

Analytical methods

The nitrite concentration was estimated after diazotisation by adding the sulphanilamide/naphthylethylene diamine dihydrochloride reagent [46]. The protein concentration was estimated using the Bradford method (Bio-Rad Laboratories, Richmond, CA) with a standard curve constructed with varying bovine serum albumin concentrations.

Nitric oxide determination

E. meliloti cells were incubated at an OD_{600} of 0.15-0.2 in MMN under 2% initial O_2 or anoxic conditions, harvested and washed similar to the NR or Nir activity assays. Nitric oxide was measured amperometrically with a 2-mm ISONOP electrode APOLO 4000® (World Precision Inst., Sarasota, FL, USA) in a 3-ml thermostatted and magnetically stirred reaction chamber [47]. The membrane-covered electrode was situated at the bottom of the chamber above the stirrer, and the reactants were injected using a Hamilton syringe through a port in the glass stopper. To determine the net production of NO, the 3-ml cuvette was filled with 1.410 ml of 25 mM phosphate buffer (pH 7.4), 250 μl (0.1-0.2 mg protein) of a cellular solution, 100 μl of an enzymatic mix containing glucose oxidase (*Aspergillus niger*) (80 units/2 ml) and catalase (bovine liver) (500 units/2 ml), 90 μl of 1 M sodium succinate and 100 μl of 320 mM glucose. When oxygen was consumed and a steady base line was observed, 50 μl of 1 M $NaNO_2$ was added to the cuvette to begin the reaction. Each assay was continued until NO was detected. To determine the NO consumption rates, the electrode chamber was filled with 1.655 ml of 25 mM phosphate buffer (pH 7.4), 5 μl (0.02-0.04 mg protein) of a cellular solution, 100 μl of an enzymatic mix containing glucose oxidase (*Aspergillus niger*) (80 units/2 ml) and catalase (bovine liver) (500 units/2 ml), 90 μl of 1 M sodium succinate and 100 μl of 320 mM glucose. Once a steady base line was observed, 50 μl of a saturated NO solution (1.91 mM at 20°C) was added to the cuvette to start the reaction. Each assay was continued until NO detection dropped to zero (when all of the NO was consumed).

Nitrous oxide determination

E. meliloti cells were incubated in MMN with an initial O_2 concentration of 2% in the headspace or anoxically. After 18 or 36 h of incubation, 500-μl gaseous aliquots were taken from the culture headspaces to determine the N_2O level. In anoxic cultures (filled tubes), headspace was created by transferring 10 ml of liquid culture into a 20-ml headspace vial (Supelco®). Gas–liquid phase equilibration was performed by incubating the vials for 2 h at 30°C and at 185 rpm. To stop cell growth, 200 μl of 1 $mg \cdot ml^{-1}$ $HgCl_2$ was added to each vial. The N_2O production in liquid cultures was corrected using the dissolved N_2O Bunsen solubility coefficient (47.2% at 30°C). Then, N_2O was measured with a gas chromatograph type HP 4890D equipped with an electron capture detector (ECD). The column was packed with Porapak Q 80/100 MESH (6 ft), and the carrier gas was N_2 at a flow rate of 23 ml/min. The injector, column and detector temperatures were 125, 60 and 375°C, respectively. The N_2O peaks were integrated using GC ChemStation Software (Agilent Technologies© 1990–2003). The samples were injected manually through a Hamilton® Gastight syringe. The concentrations of N_2O in each sample were calculated from pure nitrous oxide standards (Air Liquid, France).

Quantitative real-time PCR analysis

For immediate stabilisation of the bacterial RNA, the RNAprotect Bacteria Reagent (Qiagen Valencia, CA, USA) was added directly to cells incubated for 12 h in MM or MMN with an initial headspace O_2 concentration of 2% or anoxically. Bacterial lysis was performed by resuspension and incubation of the cell pellet in 1 mg/ml lysozyme from chicken egg whites (Sigma-Aldrich) in Tris-EDTA buffer, pH 8.0. The total RNA was isolated using the RNeasy Mini kit (Qiagen). The isolated RNA was subjected to DNase (Qiagen) treatment. The RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA), and intactness was verified by the visual inspection of rRNA bands in electrophoretically separated total RNA [48]. Reverse transcription reactions were performed with 0.8 μg of total RNA per reaction using the First Strand cDNA Synthesis kit for RT-PCR (Roche) with random hexamers. The cDNA synthesis reaction mixture was diluted 50 times with distilled water before use in real-time PCR analysis.

The primers for the PCR reactions were designed using Primer Express v3.0 software (PE Applied Biosystems, Foster City, CA, USA) to have a melting temperature of approximately 57°C to 62°C and to produce a PCR product of approximately 50 to 100 bp. The primer sequences were as follows: *napA* (forward, 5′-CCGGCTATCGTGG CAAGA-3′; reverse, 5′-CGGGAAGCTGTCGACATTG-3′); *nirK* (forward, 5′-CCGCGCGACGCAAA-3′; reverse, 5′-TCGAGCGTATCGGCATAGG-3′); *norC* (forward, 5′-AGCTCACAGAGCAGGAACTGAAC-3′; reverse, 5′-TG ATGCGGCTCGTCCATT-3′); and *nosZ* (forward, 5′-CG AGGATCTCACGCATGGAT-3′; reverse, 5′-GCGGTGC AACCTCCATGT-3′). *sMC00128* was used as an internal standard [49,50] (forward, 5′-ACGAGATCGAGATCG CCATT-3′; reverse, 5′-CGAACGAGGTCTTCAGCAT GA-3′).

Each PCR reaction contained 7.5 μl of SYBR Green PCR master mix (PE Applied Biosystems), 5 μl of cDNA and various final concentrations of each primer depending on the studied gene. This concentration was 0.2 μM for *norC* and *sMC00128* and 0.4 μM for *napA*, *nosZ* and *nirK*. The final volume of the PCR reactions was 15 μl. The real-time PCR reactions were performed on a 7300 Real Time PCR System (PE Applied Biosystems). The initial denaturing time of 10 min was followed by 40 PCR cycles consisting of 95°C for 15 s and 60°C for 60 s. A melting curve was run after the PCR cycles. During real-time PCR, the efficiency of *nirK* gene amplification was

approximately equal to that of the housekeeping (internal standard) gene; in this case, the comparative C_T method (also called $\Delta\Delta C_T$ method) was applied for relative quantification. For the other genes, the amplification efficiencies were different from that of the housekeeping gene; the comparative C_T method could not be applied, and it was necessary to use the standard curve method. The data were analysed using the 7300 System Software (PE Applied Biosystems). The gene expression values under different conditions were expressed relative to the values of cells incubated under an initial O_2 concentration of 2% in the absence of nitrate.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MJT and MJD conceived of the study. MJT and MIR carried out the phenotypic analyses of the *E. meliloti* denitrification mutants. TC and JJP participated in the gene expression experiments. MJD and EJB supported the research. MJT and MJD wrote the manuscript. EJB coordinated and critically revised the manuscript. All of the authors read and approved the manuscript.

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