Bacterial nitric oxide metabolism: recent insights in Rhizobia

Ana Salas[1†], Juan J. Cabrera[1,2†], Andrea Jiménez-Leiva[1], Socorro Mesa[1], Eulogio J. Bedmar[1], David J. Richardson[2], Andrew J. Gates[2] and María J. Delgado[1*]

[1]Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain

[2]School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, U.K.

†These authors share first authorship

*Corresponding author: María J. Delgado (mariajesus.delgado@eez.csic.es)

Running title: Nitric oxide metabolism in Rhizobia

Key words: bacteroids, denitrification, haemoglobins, nitrate assimilation, nitrate reductase, nitric oxide reductase, regulation, rhizobia-legume symbiosis, root nodules

Contents

Abstract

Nitric oxide (NO) is a reactive gaseous molecule that has several functions in biological systems depending on its concentration. At low concentrations, NO acts as a signaling molecule, while at high concentrations, it becomes very toxic due to its ability to react with multiple cellular targets**.** Soil bacteria, commonly known as rhizobia, have the capacity to establish a N_2-fixing symbiosis with legumes inducing the formation of nodules in their roots. Several reports have shown NO production in the nodules where this gas acts either as a signaling molecule which regulates gene expression, or as a potent inhibitor of nitrogenase and other plant and bacteria enzymes. A better understanding of the sinks and sources of NO in rhizobia is essential to protect symbiotic nitrogen fixation from nitrosative stress. In nodules, both the plant and the microsymbiont contribute to the production of NO. From the bacterial perspective, the main source of NO reported in rhizobia is the denitrification pathway that varies significantly depending on the species. In addition to denitrification, nitrate assimilation is emerging as a new source of NO in rhizobia. To control NO accumulation in the nodules, in addition to plant haemoglobins, bacteroids also contribute to NO detoxification through the expression of a NorBC-type nitric oxide reductase as well as rhizobial haemoglobins**.** In the present review, updated knowledge about the NO metabolism in legume-associated endosymbiotic bacteria is summarized.

List of abbreviations

AOB	ammonia oxidizing bacteria
bNOS	bacterial nitric oxide synthase
CRP/FNR	cyclic AMP receptor protein/fumarate and nitrate reductase
DMSOR	dimethyl sulfoxide reductase
DNIC	dinitrosyl iron complex
DNRA	dissimilatory nitrate reduction to ammonium
FAD	flavin adenine dinucleotide
fHbs	flavohaemoglobins
Hbs	haemoglobins
HCO	haem-copper oxidases superfamily
ITC	isothermal titration calorimetry
IVT	*in vitro* transcription
Q/QH_2	quinone/quinol
Lb	leghaemoglobin
LbNO	nitrosyl-leghaemoglobin
Mb	myoglobin
MFS	major facilitator superfamily
MK/MKH_2	menaquinone/menaquinol
$Mo[MGD]_2$	molybdenum *bis* molybdopterin guanine dinucleotide

83	Nap	periplasmic nitrate reductase
84	Nar	membrane-associated nitrate reductase
85	Nas	cytosolic assimilatory nitrate reductase
86	NDH	NADH dehydrogenase
87	Nir	nitrite reductase
88	NOD	nitric oxide dioxygenation
89	Nor	nitric oxide reductase
90	NOS	nitric oxide synthase
91	Nos	nitrous oxide reductase
92	NR	nitrate reductase
93	ns-Hbs	non-symbiotic haemoglobins
94	ORF	open reading frame
95	RNAP	RNA polymerase
96	RNS	reactive nitrogen species
97	SNF	symbiotic nitrogen fixation
98	SDH	succinate dehydrogenase
99	sdHbs	single domain haemoglobins
100	SNO	S-nitrosothiol
101	UQ/UQH_2	ubiquinone/ubiquinol
102	trHbs	truncated haemoglobins

1. Introduction to the nitric oxide (NO) molecule

Nitric oxide (NO), or nitrogen monoxide, is a heteronuclear diatomic gas with a single unpaired electron that makes it a highly reactive radical represented as NO·. NO is slightly soluble in water and may diffuse through the hydrophilic parts of the cell, such as the cytoplasm, but it is more soluble in organic solvents and so can move freely through the membrane lipids and diffuse to neighboring cells. Being a reactive free radical, it has a relatively short biological half-life of a few seconds. Therefore, its effects are mostly limited to the cell in which it is produced, or the nearest neighboring cells. NO is present in all living organisms where it acts as signalling molecule at low concentrations (nmolar levels), while at higher concentrations (µmolar levels) it functions as a pathological or cytotoxic agent (Toledo & Augusto, 2012).

In biological systems, NO can react with several oxygen species, including superoxide anion ($O_2\cdot^-$) and molecular oxygen (O_2), to form so-called reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($NO_2\cdot$), dinitrogen trioxide (N_2O_3), nitrosonium cation (NO^+), or nitroxyl anion (NO^-) (Bartberger et al., 2002; Hughes, 2008; Möller et al., 2019) (Fig. 1). In addition to RNS, NO can induce formation of other reactive species, such as hydroxyl and carbonate radicals (OH· and $CO_3\cdot^-$, respectively) (Fig. 1). Some of these species are powerful oxidants that can directly oxidize and damage DNA. For example, a high concentration of NO causes cytosine deamination and drives G:C→A:T transition mutation in DNA through conversion of cytosine (C) to thymine (T) (Wink et al., 1991). Peroxynitrite can also directly oxidize guanosine residues transforming them to 8-oxo-2'-deoxyguanosine that causes DNA strand breaks (Burney, Niles, Dedon, & Tannenbaum, 1999; Salgo, Stone, Squadrito, Battista, & Pryor, 1995). In addition to the formation of powerful oxidants, RNS cause formation of stable modifications in macromolecules, such as

dinitrosyl iron complexes (DNICs), S-nitrosylation of Cys residues to form S-nitrosothiols (SNOs), and tyrosine nitration (NO_2-Tyr) (Fig. 1). These modifications alter protein structure and function and, consequently, also gene regulation and cell physiology (Stern & Zhu, 2014). The mechanism by which NO induces the formation of SNO groups is unclear since NO does not react directly with thiol groups under physiological conditions (Stern & Zhu, 2014). In contrast to other posttranslational protein modifications, S-nitrosylation is generally considered to be non-enzymatic, involving multiple chemical routes. However, it has been recently reported that protein S-nitrosylation by NO in *Escherichia coli* is essentially enzymatic (Seth et al., 2018). Similarly to S-nitrosylation, NO does not react directly with tyrosines but it can indirectly through the formation of peroxynitrite (Souza, Peluffo, & Radi, 2008). Tyrosine nitration has less capacity for enzymatic inhibition than S-nitrosylations and, additionally, requires the presence of reactive oxygen species. The current knowledge on the biochemistry of peroxynitrite and tyrosine nitration has been recently reviewed by (Bartesaghi & Radi, 2018).

NO also reacts readily with transition metals, such as iron (Fe), including both haem and non-heam Fe as well as Fe-sulphur (Fe-S) cofactors, and has greater affinity for ferrous compared with ferric forms. NO binds to Fe in a similar way as O_2 does, because both molecules have an unpaired electron capable of forming a covalent bond coordinated with the *d*- orbital of the Fe atom. The capacity for binding both NO and O_2 makes Fe paradoxically a key element for sensitivity, for example blocking respiratory chains, but also promoting tolerance to NO given the capacity of haemoglobins to detoxify NO (Gell, 2018).

A large number of bacterial proteins are affected by NO (Brandes, Rinck, Leichert, & Jakob, 2007; Rhee, Erdjument-Bromage, Tempst, & Nathan, 2005; Seth,

153 Hausladen, Wang, & Stamler, 2012; Seth et al., 2018). These include transcription
154 factors, such as NorR, NnrR, NsrR, HcpR, and DNR (Stern & Zhu, 2014 and references
155 therein). NorR was the first NO-responsive transcriptional regulator to be described that
156 contains a non-heam Fe that, in the presence of NO, forms a mononitrosyl-Fe complex.
157 NsrR is a repressor that contains an Fe-S cluster in its sensory domain. DNR and NnrR
158 are members of the cyclic AMP receptor protein/fumarate and nitrate reductase
159 (CRP/FNR) family of transcription factors. The crystal structures of DNR suggest the
160 involvement of haem in NO sensing rather than Fe-S or non-haem Fe. In the case of
161 NnrR, no specific mechanism of NO sensing has been proposed.

162 Finally, NO also plays an important role in atmospheric chemistry and
163 influences the production of ground-level ozone and acid rain (Pilegaard, 2013).

164

165 [Insert Figure 1 here]

166 **2. NO sources**

167 Denitrification and the dissimilatory nitrate reduction to ammonium (DNRA) are
168 currently considered the main respiratory sources of NO. NO is a key intermediate of
169 the denitrification process by which nitrate (NO_3^-) is reduced to molecular nitrogen (N_2)
170 for respiratory purposes (Zumft, 1997). DNRA consists of the respiratory reduction of
171 NO_3^- to nitrite (NO_2^-) and, finally, to ammonium (NH_4^+), whereby NO is produced
172 either chemically and/or enzymatically from NO_2^- (Simon & Klotz, 2013). For some
173 time, NO formation in prokaryotes was thought to occur only through denitrification,
174 DNRA, and other related respiratory pathways, such as anaerobic NH_4^+ oxidation also
175 named anammox (Kartal & Keltjens, 2016; Kuenen, 2020). In these processes, NO is a
176 common and necessary intermediate, working also as a signal molecule regulating the

177 genes necessary for its own metabolism. Recently, it has been demonstrated that
178 ammonia oxidizing bacteria (AOB) also emit substantial amounts of NO. The accepted
179 model for AOB metabolism involves NH_4^+ oxidation to NO_2^- via a single obligate
180 intermediate, hydroxylamine (NH_2OH). However, recent studies have demonstrated that
181 aerobic NH_4^+ oxidation occurs via two obligate intermediates: NH_2OH and NO
182 (Caranto & Lancaster, 2017).

183 At present, it is widely recognized that NO is also produced by non-respiratory
184 routes, including oxidative stress cytoprotection (Gusarov, Shatalin, Starodubtseva, &
185 Nudler, 2009; Mukhopadhyay, Zheng, Bedzyk, LaRossa, & Storz, 2004; Nakano, 2002;
186 Shatalin et al., 2008), recovery from damage caused by radiation (Patel et al., 2009) or
187 the biosynthesis of secondary metabolites, such as tryptophan nitration (Buddha, Tao,
188 Parry, & Crane, 2004). However, the major non-respiratory source of NO in bacteria is
189 nitric oxide synthase (bNOS), which is homologous to mammalian NOS that catalyzes
190 the aerobic formation of NO from arginine using cellular reducing equivalents (Crane,
191 Sudhamsu, & Patel, 2010; Santolini, 2019). NO production by bNOS is involved in the
192 protection of pathogenic bacteria against the immune system oxidative attack from their
193 hosts (Hutfless, Chaudhari, & Thomas, 2018). Consequently, bNOS has a revelant role
194 on virulence and also during the infection of plants.

195

196 **2.1 Denitrification**

197 Denitrification is defined as the reductive conversion of soluble nitrogen
198 oxyanions to gaseous nitrogen oxides and N_2. These substrates are ubiquitous in diverse
199 aquatic and terrestrial ecosystems. The complete denitrification process consists of four
200 enzymatic steps. First, NO_3^- is reduced to NO_2^-, which is subsequently reduced to NO,
201 nitrous oxide (N_2O) and, finally, N_2. Each nitrogen oxyanion and nitrogen oxide acts

individually as a final electron acceptor in a respiratory chain that functions under O_2 limited conditions and allows the survival and replication of denitrifying microorganisms during its anaerobic life-style (Zumft, 1997).

$$2NO_3^- \xrightarrow{4e^-} 2NO_2^- \xrightarrow{2e^-} 2NO \xrightarrow{2e^-} N_2O \xrightarrow{2e^-} N_2$$

Denitrification is widely distributed in Bacteria and appears to be dominant within Proteobacteria (Shapleigh, 2006). In addition, some fungi (Prendergast-Miller, Baggs, & Johnson, 2011; Shoun & Fushinobu, 2017; Takaya, 2002) and Archaea (Treusch et al., 2005) can also denitrify, and some nitrifying organisms may also encode genes involved in denitrification (Cébron & Garnier, 2005). Most studies on denitrification have focused on Gram-negative bacteria that inhabit terrestrial niches. The α-proteobacterium *Paracoccus denitrificans* and the γ-proteobacteria *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* are the first model organisms where denitrification has been widley studied (Kraft, Strous, & Tegetmeyer, 2011; Richardson, 2011; Torres et al., 2016; van Spanning, Richardson, & Ferguson, 2007; Zumft, 1997). The denitrification reactions are catalyzed by the periplasmic nitrate reductase (Nap) or membrane-associated nitrate reductase (Nar), nitrite reductases (NirK or NirS), nitric oxide reductases (cNor, qNor or Cu_ANor) and nitrous oxide reductase (NosZ) (Fig. 2). These reductases consist of multisubunit metalloprotein complexes coupled to electron transport chains. Many components of these complexes require transport across or insertion into the cytoplasmic membrane, as well as assembly of the complexes and cofactors incorporation. In addition to hard-wired complexes, these processes can also involve transient protein-protein interactions. Recently, an interactomic approach was

used to determine the exact protein-protein interactions involved in the assembly of the denitrification apparatus of *P. aeruginosa* (Borrero-de Acuña, Timmis, Jahn, & Jahn, 2017).

Most denitrifiers express one or more of the three different types of nitrate reductase (NR) that catalyze the reduction of NO_3^- to NO_2^-, and belong to the molybdopterin oxidoreductases superfamily. There are: (i) the cytosolic assimilatory NR (Nas) (see Section 2.2), the respiratory membrane-bound NR (Nar) and the periplasmic NR (Nap) (González, Rivas, & Moura, 2017; Potter, Angove, Richardson, & Cole, 2001; Richardson, 2011; Richardson, van Spanning, & Ferguson, 2007; Simon & Klotz, 2013; Torres et al., 2016). Depending on the species, Nap can be used for anaerobic NO_3^- respiration or as an electron sink to maintain redox homeostasis. This occurs in *P. denitrificans* and *Paracoccus pantotrophus* that express Nap in addition to Nar. In *P. pantotrophus*, Nap serves to dissipate the excess reducing equivalents formed during aerobic growth on highly reduced carbon substrates (Ellington, Bhakoo, Sawers, Richardson, & Ferguson, 2002).

Nap is a functional heterodimer comprising the catalytic subunit NapA (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 haem *c* groups and receives electrons from the membrane-bound NapC (25 kDa), which binds 4 haem *c* groups (Fig. 2). NapC is a quinol-oxidase that receives electrons from the ubiquinone pool. The *napEFDABC* genes encode the structural proteins (NapAB), as well as proteins necessary for the synthesis, maturation and enzymatic activity of Nap. In most Nap systems, the transfer of electrons from the quinone pool to the NapAB complex requires NapC. However, in *E. coli* and *Wolinella succinogenes* a second quinol oxidase NapGH has been identified and is encoded in the operon *napFDAGHBC* and *napAGHBFLD*,

respectively. Here, NapH is proposed to act as a membrane-bound quinol dehydrogenase, while NapG is a periplasmic protein that contains Fe-S centres and transfers electrons to NapB (Simon & Klotz, 2013). It is important to note that not all Nap enzymes form tight 'hard-wired' heterodimeric NapAB complexes. For example, the crystal structure of *E. coli* Nap is that of a monomeric NapA enzyme (Jepson et al., 2007). Whilst *E. coli* does have a NapB that is the likely redox partner of NapA, some bacteria such as *Desulfovibrio desulfuricans* have a Nap system that lacks NapB, suggesting that other redox proteins may partner this enzyme, including NapG for example (Marietou, Richardson, Cole, & Mohan, 2005). Sequence analysis has suggested that the monomeric class of Nap forms a structural clade and represents the evolutionary link between the monomeric Nas (see Section 2.2) and the heterodimeric Nap enzymes (Jepson et al., 2006).

Nar is a complex multi-subunit molybdoenzyme that has distinct biochemical properties to Nap. Nar is a large functional heterotrimeric enzyme (NarGHI) bound to the inner face of the cytoplasmic membrane and has been widely studied in *E. coli*. The NarG subunit (140 kDa) is located in the cytoplasm and binds a $Mo[MGD]_2$ and [4Fe-4S] cluster. NarH (60 kDa) forms a tight association with NarG and contains one [3Fe-4S] and also three [4Fe-4S] clusters. The cytoplasmic NarGH sub-complex associates, through NarH, with the integral membrane dihaem cytochrome *b* quinol oxidase NarI (25 kDa) (Fig. 2). Nar proteins are encoded by the *narGHJI* operon and while *narGHI* encode the structural subunits for the quinol/nitrate oxidoreductase, *narJ* codes for a cognate cytoplasmic chaperone required for the maturation of the functional Nar holoenzyme. Recent studies have shown in *P. denitrificans* that NarJ serves not only as chaperone of Nar, but also for the assimilatory nitrate reductase (NasC) (Pinchbeck et al., 2019). In *P. denitrificans*, NarGHI is encoded by the *narK1-K2GHIJ* genes. The

narK1-K2 gene encodes a functional fused transmembrane transporter that belongs to the major facilitator superfamily (MFS) of solute-transport proteins. The NarK1-like domain of NarK1-NarK2 functions primarily as a NO_3^- transporter, while the NarK2-like domain is more specialized in NO_3^-/NO_2^- antiport (Goddard et al., 2017; Goddard, Moir, Richardson, & Ferguson, 2008).

In contrast to proteobacterial Nar systems, the active site subunit of the archaeal Nar systems and some bacterial systems have a twin arginine ('RR') motif in the N-terminal sequence of NarG. Examples include the Haloarchaea *Haloferax mediterranei* and the anammox bacterium *Candidatus Kuenia stuttgartensis* (Martínez-Espinosa, Richardson, Butt, & Bonete, 2006). This indicates the translocation of NarGH to the periplasm. Whilst these NarGH proteins are similar to those of proteobacterial Nar systems, there is no NarI equivalent such that the electron transfer system coupling the acitve NarGH module to the quinol pool will be distinct (Martínez-Espinosa et al., 2006).

[Insert Figure 2 here]

NO production occurs in the second stage of denitrification and is catalyzed by respiratory nitrite reductases, of which two types have been described: NirS (cd_1 type) and NirK (Cu-type) (Rinaldo et al., 2008; van Spanning, 2011). These enzymes catalyze the reduction of NO_2^- to NO in the periplasmic compartment and receive electrons from cytochromes *c* or from the blue copper protein, pseudoazurin, via the cytochrome bc_1 complex (Fig. 2). Most denitrifiers endcode either NirK or NirS. However, it has recently been shown that *Bacillus nitroreducens* sp. Nov (Jang et al., 2018) and *Bradyrhizobium oligotrophicum* (Sánchez & Minamisawa, 2018) contain genes for both nitrite reductase systems.

NirS is a functional homodimer with a small haem *c* domain and a larger haem d_1 domain per monomer. Electrons are transferred from the electron donor via haem *c* to haem d_1, where NO_2^- is bound and reduced to NO (Rinaldo et al., 2008). The genes involved in NirS synthesis have been characterized in *P. aeruginosa* (*nirSMCFDLGHJEN*), *P. denitrificans* (*nirXISECFDLGHJN*) and *P. stutzeri* (*nirSTBMCFDLGH* and *nirJEN*). The *nirS* gene encodes the functional NirS homodimer, and the rest of the genes from the operon are needed for the haem d_1 cofactor synthesis, assembly and insertion to the active centre. The biosynthesis of haem d_1 cofactor has been the subject of numerous investigations (Bali, Palmer, Schroeder, Ferguson, & Warren, 2014; Rinaldo, Giardina, & Cutruzzolà, 2017).

NirK enzymes are homotrimers that contain three type 1 Cu (T1Cu) centres and three type 2 Cu (T2Cu) centres. NO_2^- binds to T2Cu, replacing an exogenous ligand (water or chloride ion), and there it is reduced to NO by electron transfer from the T1Cu site. The structural properties of NirK have been recently reviewed by (Horrell, Kekilli, Strange, & Hough, 2017; Nojiri, 2017). Most of the two-domain NirKs are named as classes I and II depending on the colour of their (T1Cu) centre, where class I is blue and class II is green. Three-domain NirKs (Class III) have been recently identified that comprise an extra T1Cu site. This is the case of *Thermus scotoductus* SA-01 NirK, a homotrimer with subunits of 451 residues. The N-terminal region possesses a T2Cu and a T1CuN, while the C-terminus contains an extra T1CuC bound within a cupredoxin motif (Opperman, Murgida, Dalosto, Brondino, & Ferroni, 2019). In contrast to the complex organization of genes that encode NirS protein, NirK is encoded by a single gene, *nirK*. Sometimes next to *nirK* another gene, *nirV* is present and encodes the NirV protein, which is related to desulphurases and may thus be necessary for correct insertion of Cu into the catalytic centre (van Spanning, 2011).

The next step of denitrification is NO reduction to N_2O performed by the Nor enzyme at the outer face of the cytoplasmic membrane (de Vries, Suharti, & Pouvreau, 2007; Richardson, 2011; Tosha & Shiro, 2017). Nor enzymes are categorised into three different groups depending on the electron donor, i.e. cNor, qNor and Cu_ANor, where they receive electrons from *c*-type cytochromes and/or quinones depending on the enzyme sub-class (Figs 2 and 7) (a detailed description of the Nor enzymes is given in Section 3.1).

In the last stage of denitrification, N_2O produced by Nor is reduced to N_2, in a reaction catalyzed by the soluble enzyme nitrous oxide reductase (NosZ). NosZ (120-160 kDa) is a homodimer Cu-containing enzyme with two distinct Cu centres: a binuclear Cu_A and a tetranuclear copper sulfide Cu_Z centre (Fig. 2). Cu_A transfers electrons to Cu_Z, the catalytic centre. There are two different forms of Cu_Z: a Cu_Z* (4Cu1S) inactive form, and Cu_Z (4Cu2S) active form (for details about the structural and spectroscopic properties see reviews by Carreira, Pauleta, & Moura, 2017; Eady, Antonyuk, & Hasnain, 2016; Pauleta, Carepo, & Moura, 2019; Pauleta, Carreira, & Moura, 2017). Two different clades of NosZ that are differentially distributed by microbial taxa are known. NosZ clade I, also known as the typical NosZ, is predominantly present in denitrifying organisms and NosZ clade II or atypical, that curiously can be found in non-denitrifying bacteria, possibly to eliminate N_2O generated by NO detoxification systems (Hallin, Philippot, Löffler, Sanford, & Jones, 2018; Hein & Simon, 2019).

The expression, maturation and maintenance of the NosZ holoenzyme require several auxiliary proteins, all of which are encoded together by a group of six genes (*nosRZDFYL*) in Nos from clade I. This group of genes is associated, in some cases, with *nosX* and *nosC* genes. The *nosDFY* genes encode an ABC-type transporter

(NosFY) and a periplasmic interacting protein, NosD that is thought to be required to supply a sulphur species for the assembly of the CuZ centre. In fact, it has been recently demonstrated that NosFY and NosD are essential for the formation of the [4Cu:2S] site Cu_Z but not for the electron transfer site Cu_A (Zhang, Wüst, Prasser, Müller, & Einsle, 2019). Recent genetic and biochemical studies in *P. denitrificans* have shown that NosL is a Cu (I) binding protein that is also required for efficient assembly of the Cu_Z site (Bennett et al., 2019). NosR and NosX do not participate in the biogenesis of Cu_Z, but play a role in the reduction of N_2O *in vivo* contributing to the maintenance of the reduced state of the Cu_Z centre (Wunsch & Zumft, 2005). In *P. stutzeri*, where the *nosX* gene is absent, the ApbE protein is a monomeric FAD-binding domain that acts as the flavin donor for NosR maturation (Zhang, Trncik, Andrade, & Einsle, 2017).

The ε-proteobacterium *W. succinogenes* is an example of a clade II NosZ microorganism. The genes *nosZ, -B, -D, -G, -C1, -C2, -H, -F, -Y and -L* are responsible for the synthesis of this enzyme. The presence of *nosB, -G, -H, -C1 and -C2*, as well as the absence of *nosR* and *nosX*, is characteristic of this group. The proteins NosG, -C1, -C2 and -H constitute an electron transport pathway from the menaquinol pool to NosZ. Specifically, NosGH is a menaquinol dehydrogenase complex and NosC1, C2 two cytochromes *c* (Hein, Witt, & Simon, 2017).

Nowadays, it is very well known the regulation of denitrification. The key molecules that act as signals for the expression of the denitrification genes are; O_2, the presence of a nitrogen oxide (NO_3^-, NO_2^-, or NO), and the redox state of the cell. These signals are perceived by a number of transcriptional regulators that participate in complex regulatory networks (for a review, see Torres et al., 2016). In the recent years, new environmental factors such as pH, and metals (Mo, Fe or Cu) are emerging to be involved in the control of denitrification. In particular, Cu is a key cofactor of NirK and

NosZ. There is evidence that Cu plays a regulatory role in *nos* gene expression in *P. denitrificans*, as the *nosZDFYL* genes were found to be downregulated under conditions of Cu deficiency, thereby leading to N_2O as the end product of denitrification. However, the regulatory mechanism underlying Cu control remains unknown. A putative candidate for such regulation is NosR, since it is involved in the transcriptional regulation of *nosZ* in *P. stutzeri* and *P. denitrificans*, and also the Cu-dependent regulation did not appear in a *P. denitrificans nosR* mutant (Honisch & Zumft, 2003; Sullivan, Gates, Appia-Ayme, Rowley, & Richardson, 2013).

Whilst N_2O is widely known to be a potent greenhouse gas, its cytotoxic properties are much less well-recognised than those of NO. In this context, it was demonstrated that N_2O inactivates Vitamin B12 and so disrupts Vitamin B12 metabolism. Thus, accumulation of N_2O leads to a switch from vitamin B12-dependent to vitamin B12-independent biosynthetic pathways, through the transcriptional modulation of genes controlled by vitamin B12 riboswitches (Sullivan et al., 2013).

2.1.1 Diverse denitrification pathways in rhizobia. **Bradyrhizobium diazoefficiens, Ensifer meliloti *and* Rhizobium etli *as models***

During the last years, several reports about denitrification in plant endosymbiotic bacteria have emerged. 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 fertilization. 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 *Leguminosae* family is established. Most of the rhizobia belong to the α-Proteobacteria subclass which includes members of the genera *Rhizobium*, *Ensifer*,

Allorhizobium, *Pararhizobium*, *Neorhizobium*, *Shinella*, *Mesorhizobium*, *Aminobacter*, *Phyllobacterium*, *Ochrobactrum*, *Methylobacterium*, *Microvirga*, *Bradyrhizobium*, *Azorhizobium* and *Devosia*. Another three symbiotic N_2-fixing genera, *Paraburkholderia*, *Cupriavidus* and *Trinickia* belong to the family *Burkholderiaceae* of the β-Proteobacteria subclass (de Lajudie et al., 2019). They induce the formation of nodules in the roots of the plants and some in the stems, which are specialized structures where N_2 fixation takes place (more details in Section 4).

Bradyrhizobium diazoefficiens is the most widely used species in commercial inoculants for soybean crops. *B. diazoefficiens* occupies two distinct niches in soil: free-living and symbiotic association in root nodules with *Glycine max* (soybean), *Macroptilium atropurpureum* (siratro), *Vigna unguiculata* (cowpea) and *Vigna radiata* (mung bean) (Göttfert, Grob, & Hennecke, 1990). *B. diazoefficiens* USDA 110, which was originally isolated from soybean nodules in Florida (United States) in 1957, has been widely used for research into its molecular genetics, physiology and ecology. The genome of *B. diazoefficiens* USDA 110 is composed of a single circular chromosome about 9.1 million base pairs in length. This organism does not contain plasmids, but instead has a 410 kb region of groups of genes involved in biological N_2 fixation that has been called a symbiotic island (Kaneko et al., 2002). Until 2013, *B. diazoefficiens* USDA 110 was classified within the species *Bradyrhizobium japonicum*, whose type strain is USDA 6, however, morphophysiological, genetic and genomic differences between both resulted in the reclassification as *B. diazoefficiens* USDA 110 (Delamuta et al., 2013). Recently, it was published the genome of *B. diazoefficiens* 110*spc*4, a spontaneous resistant derivative of *B. diazoefficiens* USDA 110 (Regensburger & Hennecke, 1983), used as model strain for most of functional genomics studies (Fernández et al., 2019). Interestingly, it revealed a deletion of about 202 kb and several

425 additional differences in comparison to that of the USDA 110 strain, which do not affect
426 its symbiotic performance with several host plants (Fernández et al., 2019).
427 Remarkably, both USDA 110 and 110*spc*4 strains of *B. diazoefficiens* have been used
428 simultaneously for investigation.

429 *B. diazoefficiens* is considered a model for rhizobial denitrification studies since
430 it is the only rhizobia species that, in addition to fixing N_2, has the ability to grow under
431 anoxic conditions by reducing NO_3^- through the complete denitrification pathway, a
432 process widely studied in this bacterium both in free-living conditions and in symbiosis
433 (Bedmar et al., 2013; Bedmar, Robles, & Delgado, 2005). *B. diazoefficiens* possesses
434 the complete set of *napEDABC* (Delgado, Bonnard, Tresierra-Ayala, Bedmar, &
435 Muller, 2003), *nirK* (Velasco, Mesa, Delgado, & Bedmar, 2001), *norCBQD* (Mesa,
436 Velasco, Manzanera, Delgado, & Bedmar, 2002) and *nosRZDFYLX* (Velasco, Mesa,
437 Xu, Delgado, & Bedmar, 2004) denitrification genes (Fig. 3), which encode the
438 periplasmic nitrate reductase (Nap), copper nitrite reductase (NirK), nitric oxide
439 reductase type *c* (cNor) and nitrous oxide reductase (Nos), respectively (for a detailed
440 description of Nap, NirK, cNor and Nos enzymes see Sections 2.1 and 3.1).

441 Phenotypic characterization of a *B. diazoefficiens napA* mutant revealed its
442 inability to grow anaerobically with NO_3^- as respiratory electron acceptor and to express
443 periplasmic NR activity (Delgado et al., 2003). The sequencing of the *B. diazoefficiens*
444 genome (Fernández et al., 2019; Kaneko et al., 2002) has confirmed that it does not
445 possess *nar* genes responsible for the synthesis of the membrane-bound respiratory NR.
446 Therefore, Nap is the only enzyme responsible for NO_3^- respiration and initiates
447 denitrification in *B. diazoefficiens* (Delgado et al., 2003). A *B. diazoefficiens nirK*
448 deficient strain lacking respiratory nitrite reductase (Nir) activity was also unable to
449 grow in anoxia with NO_3^-, and accumuled NO_2^- in the medium (Velasco et al., 2001).

The *B. diazoefficiens* soluble cytochrome c_{550} is the intermediary in the electronic transport between the bc_1 complex and the respiratory NirK (Bueno, Bedmar, Richardson, & Delgado, 2008).

B. diazoefficiens mutant strains defective in the *norC* or *norB* genes are unable to grow under denitrifying conditions. Under these conditions, these *nor* mutants accumulate NO (Mesa et al., 2002). Finally, mutant strains of *B. diazoefficiens* for the *nosR* and *nosZ* genes were able to grow under denitrifying conditions, but they accumulated N_2O, rather than N_2 as the end product of denitrification (Velasco et al., 2004). The involvement of the periplasmic cyrochrome c_{550} (CycA) in the activity of Nos has been recently reported (Jiménez-Leiva et al., 2019).

Ensifer meliloti is a symbiotic N_2-fixing soil bacterium which associates with plants of the genera *Medicago*, *Melilotus* and *Trigonella*. *E. meliloti* 1021 is the model strain for investigating the interaction between the *E. meliloti-M. truncatula* symbiotic system, whose molecular, biochemical and genetic characterization has been widely studied (Jones, Kobayashi, Davies, Taga, & Walker, 2007; Young et al., 2011). The genome of *E. meliloti* 1021 is composed of three replicons: a 3.65 Mb chromosome and two megaplasmids, pSymA (1.35 Mb) and pSymB (1.68 Mb) (Galibert et al., 2001). Plasmid pSymA includes genes that are implicated in symbiosis and genes likely responsible for N and C metabolism, transport, stress and resistance responses that confer *E. meliloti* a benefit for the symbiotic interaction (Barnett et al., 2001). This plasmid also contains structural and accessory genes for denitrification (*napEFDABC*, *nirKV*, *norEFCBQD*, *nosRZDFYLX*, *azu1*, *hemN*) as well as regulatory genes (*nnrR*, *nnrU*, *nnrS1*, *fixKTL*), and genes required for cbb_3 cytochrome oxidase (*fixNOQP*) and flavohaemoglobin (*hmp*) synthesis (Barnett et al., 2001) (Fig. 3). The denitrification pathway in *E. meliloti* is induced under microoxic and symbiotic conditions (Becker et

al., 2004). Importantly, this bacterium is unable to grow under anaerobic conditions with NO_3^- as terminal electron acceptor, despite having and expressing the entire set of denitrification genes. However, *E. meliloti napA, nirK, norC* and *nosZ* structural genes are functional under specific growth conditions (initial O_2 concentrations of 2% and initial cell density of 0.20-0.25) (Torres, Rubia, Bedmar, & Delgado, 2011; Torres, Rubia, et al., 2014). By using a robotized incubation system, the inability of *E. meliloti* to reduce NO_3^- or NO_2^- to N_2O or N_2 under anaerobic conditions was confirmed. By contrast, *E. meliloti* was able to grow during anaerobic respiration by reducing externally provided N_2O to N_2 (Bueno et al., 2015). Recently, it has been shown that overexpression of *napEFDABC* genes (Nap+) confers to *E. meliloti* the capacity to grow anaerobically through NO_3^- respiration. These results suggest that the inability of *E. meliloti* to grow under anaerobic conditions using NO_3^- as electron acceptor can be attributed to a limitation in the expression of the periplasmic nitrate reductase (Torres, Ávila, Bedmar, & Delgado, 2018).

Rhizobium etli is an N_2-fixing soil bacterium that establishes symbiotic associations with *Phaseolus vulgaris* L., or common bean. The genome of *R. etli* CFN42 contains a chromosome and six large plasmids (pCFN42a to pCFN42f) whose sizes range from 184.4 to 642.45 kb (González et al., 2006). Plasmid d corresponds to the symbiotic plasmid (pSym) and includes several genes implicated in nodulation and N_2-fixation processes. Plasmid pCFN42f contains a gene cluster that includes the *nirK* and *norEFCBQD* denitrification genes and other related genes (*fixKf, hfixL, fxkR, nnrR, nnrS, nnrU, azuP, fixNOQ, hemN*) (Girard et al., 2000; González et al., 2006) (Fig. 3). This bacterium does not have *nap*, *nar* and *nos* denitrification genes and it is unable to respire NO_3^-. The presence of NirK and Nor-coding regions in *R. etli* suggests a NO_2^- and NO-detoxifying pathway that prevents accumulation of NO inside free-living cells.

In vivo assays showed that NirK is necessary for NO_2^- reduction to NO and that Nor is essential to detoxify NO under free-living conditions (Bueno, Gómez-Hernández, Girard, Bedmar, & Delgado, 2005; Gómez-Hernández et al., 2011).

[Insert Figure 3 here]

2.1.2 Nitric oxide signalling and transcriptional control of rhizobial denitrification

In *B. diazoefficiens,* expression of *napEDABC* genes is induced under microoxic conditions, regardless of the presence of a nitrogen oxide (NOx) (Bueno et al., 2017). This control is mediated by $FixK_2$, a transcriptional regulator that belongs to the CRP/FNR-family of bacterial transcriptional regulators that respond to a wide range of intracellular and environmental stimuli (Körner, Sofia, & Zumft, 2003), and it is subjected to a complex regulation at transcriptional, posttranscriptional and posttranslational levels (Fernández et al., 2016; Fernández et al., 2019; Mesa, Reutimann, Fischer, & Hennecke, 2009). In fact, *in vitro* transcription (IVT) experiments demonstrated direct control of the *napEDABC* genes by $FixK_2$ (Bueno et al., 2017). Similarly, to *napEDABC* genes, the *nirK* gene is also significantly induced under microoxic conditions; however, its maximum expression was only reached in the presence of a NOx. The *nirK* gene is also a direct target of the $FixK_2$ protein, since a specific transcript was detected in IVT assays in the presence of purified $FixK_2$ (Bueno et al., 2017) (Fig. 4). Unlike the *napEDABC* and *nirK* genes, *norCBQD* genes require the presence of both microoxia and a NOx for their expression, being NO the molecular signal necessary for *norCBQD* gene induction (Bueno et al., 2017). The NO-response of *norCBQD* expression is mediated by another transcriptional activator belonging to the

524 CRP/FNR-type family, NnrR (Fig. 4). Isothermal titration calorimetry (ITC)
525 experiments have shown that NnrR specifically binds to a conserved palindromic
526 regulatory sequence present in the *norCBQD* gene promoter (possible NnrR box) under
527 anoxic conditions (Bueno et al., 2017). However, in these studies, NnrR did not interact
528 with the *napEDABC* or *nirK* promoters in ITC experiments, and $FixK_2$ was unable to
529 activate transcription from the *norCBQD* promoter in IVT assays. With respect to the
530 *nosRZDFYLX* genes, similar to that observed for *napEDABC* or *nirK* genes, $FixK_2$ is the
531 main regulator which controls the expression of the *nosRZDFYLX* genes in response to
532 microoxia (Torres et al., 2017). Taken together, these findings suggested a disparate
533 response to NO of the *B. diazoefficiens* denitrification genes, where expression of
534 *napEDABC*, *nirK*, and *nosRZDFYLX* genes requires microoxic conditions and is
535 directly dependent on $FixK_2$, but NO is the key signal for the expression of *norCBQD*,
536 being NnrR the regulator which directly interacts with its promoter (Bueno et al., 2017;
537 Torres et al., 2017) (Fig. 4). Recent transcriptomic analyses expanded the NnrR regulon
538 in response to anoxic denitrifying conditions which comprises 175 genes, including
539 structural genes (*nirK*, *norCBQD*, *nosRZDFYLX*), accessory genes for denitrification
540 (*cycA*, *cy2*, $hemN_{1+2}$), and genes encoding regulatory factors ($rpoN_1$) (Jiménez-Leiva et
541 al., 2019).

542 $FixK_2$ and NnrR are part of the FixLJ-$FixK_2$-NnrR regulatory cascade (the
543 genomic context of *fixLJ*, $fixK_2$ and *nnrR* genes is shown in Fig. 3). A moderate
544 decrease in O_2 concentration (≤5%) is sufficient to activate the cascade at the level of
545 the two-component regulatory system FixLJ, where the response regulator FixJ in its
546 active phosphorylated form induces the expression of several genes, including $fixK_2$.
547 $FixK_2$ induces the expression of more than 300 genes, including genes associated with
548 microoxic metabolism (*fixNOQP*), the denitrification genes (*napEDABC*, *nirK*,

549 *norCBQD* and *nosRZDFYLX*) and regulatory genes (*rpoN*$_1$, *fixK*$_1$ and *nnrR*) (Bedmar et
550 al., 2005; Mesa, Bedmar, Chanfon, Hennecke, & Fischer, 2003; Mesa et al., 2008;
551 Mesa, Ucurum, Hennecke, & Fischer, 2005; Nellen-Anthamatten et al., 1998; Robles,
552 Sánchez, Bonnard, Delgado, & Bedmar, 2006). Thus, NnrR expands the cascade as
553 FixLJ-FixK$_2$-NnrR through an additional control in response to NO (Fig. 4). This
554 cascade has been confirmed at the molecular level by IVT assays that shown *nnrR* as a
555 direct target for FixK$_2$ (Jiménez-Leiva et al., 2019). The mechanism involved in NO-
556 sensing by NnrR is presently under investigation.

557 Studies based on the structure of the FixK$_2$-DNA complex (Bonnet et al., 2013),
558 as well as those deduced from alignment of the FixK$_2$ boxes present in close proximity
559 to genes controlled directly by this protein (Bueno et al., 2017; Mesa et al., 2008;
560 Reutimann, Mesa, & Hennecke, 2010) proposed a palindromic sequence of 14 bp,
561 TTG(A/C)-N$_6$-(T/G)CAA as the binding site for FixK$_2$ (FixK$_2$ box). Although the
562 promoter region of *napEDABC, nirK, norCBQD*, *nosRZDFYLX*, and *nnrR* comprise
563 palindromic sequences that resemble a 'genuine' FixK$_2$ box, only the boxes associated
564 with the *napEDABC, nosRZDFYLX* and *nnrR* genes conserve the specific molecular
565 determinants for FixK$_2$ binding identified in the consensus sequence. These findings
566 support the direct control of those genes by FixK$_2$ observed by using other approaches
567 (Bueno et al., 2017; Jiménez-Leiva et al., 2019; Torres et al., 2017). Currently, we are
568 investigating the molecular mechanism underlying differential control of *B.*
569 *diazoefficiens* denitrification gene expression mediated by FixK$_2$ and/or NnrR.

570 In addition to the FixLJ-FixK$_2$-NnrR cascade, *B. diazoefficiens* denitrification
571 genes are also under the control of the RegSR-NifA cascade (Fig. 4). RegSR-NifA
572 responds to a very low O_2 concentration (≤0.5%). NifA, the master regulator of nitrogen
573 fixation genes, is encoded by *nifA* located within the *fixRnifA* operon which is expressed

574 from two overlapping promoters, P_1 and P_2 (Barrios, Fischer, Hennecke, & Morett,
575 1995; Bauer, Kaspar, Fischer, & Hennecke, 1998). The response regulator for the two-
576 component RegSR system, RegR activates transcription from P_2 regardless of O_2
577 concentration. Under anoxic conditions, NifA in concert with RNA polymerase
578 (RNAP)-containing RpoN (σ54) enhances its own synthesis by activating the P_1
579 promoter of *fixRnifA*. RpoN from *B. diazoefficiens* is encoded by two very similar and
580 functionally equivalent genes (*rpoN*$_1$ and *rpoN*$_2$) (Kullik et al., 1991). The *rpoN*$_1$ gene is
581 under the control of $FixK_2$, representing the link between the two regulatory cascades
582 (Fig. 4). In addition to controlling the expression of *nifA*, RegR is also involved in the
583 maximal induction of the *norCBQD* genes, and it is able to interact directly with the
584 promoter region of the *norCBQD* and *nosRZDFYLX* genes (Torres, Argandoña, et al.,
585 2014). NifA has also been reported to drive maximal expression of *napEDABC, nirK*
586 and *norCBQD* genes (Bueno, Mesa, Sánchez, Bedmar, & Delgado, 2010). However, the
587 mechanism involved in the control of *B. diazoefficiens* denitrification by RegSR-NifA is
588 currently unknown.

589 Several studies have proposed the involvement of new regulatory proteins in
590 controlling expression of the *B. diazoefficiens* denitrification pathway. Particularly, in
591 the case of *nosRZDFYLX* genes, the NasST two-component system (more details in
592 Section 2.2) has been demonstrated to be an important regulator of *nos* genes
593 transcription in response to NO_3^- under both aerobic and anaerobic conditions (Sánchez
594 et al., 2014; Sánchez, Mitsui, & Minamisawa, 2017).

595 [Insert Figure 4 here]

596 Similar to that described for *B. diazoefficiens*, the induction of *E. meliloti*
597 denitrification genes in response to microoxic conditions is coordinated by the two-
598 component FixLJ regulatory system. In this system, the phosphorylated FixJ protein

activates the transcription of the *fixK* and *nifA* regulatory genes that induce the expression of the *fix* (respiration) and *nif* (N_2 fixation) genes, respectively (Becker et al., 2004; Bobik, Meilhoc, & Batut, 2006) (Fig. 5). In *E. meliloti*, FixLJ responds to both microoxic conditions and NO, however O_2 is the only molecule capable of suppressing the kinase activity of FixL (Gilles-González, González, Sousa, & Tuckerman, 2008). The regulatory protein NnrR is also involved in the NO response in *E. meliloti* (Fig. 5). Here, in the presence of NO, NnrR regulates the expression of *nirK* and *norC* as well as others genes required for denitrification, such as *azu*1, *hemN*, *nnrU* and *nnrS* (Meilhoc, Cam, Skapski, & Bruand, 2010). However, FixK and NnrR belong to two different regulatory cascades, unlike *R. etli* and *B. diazoefficiens*, where both proteins belong to the same regulatory cascade (Cabrera et al., 2011) (Fig. 5). The genomic organization of *E. meliloti fixLJ*, *fixK* and *nnrR* regulatory genes is shown in Fig. 3.

Finally, in *R. etli* the region encoding the *nor* and *nir* genes is present in plasmid pCFN42f which also includes genes for regulatory proteins, such as *fixK*, *hfixL*, *fxkR* and *nnrR* (Girard et al., 2000; Granados-Baeza et al., 2007) (Fig. 3). In this bacterium, the hFixL-FxkR two-component regulatory system detects the O_2 signal. The hFixL protein is a hybrid histidine kinase that unlike FixL from *E. meliloti* (and the related protein FixL from *B. diazoefficiens*) is not membrane-bound. FxkR is the regulator that allows the transduction of the microoxic signal for the activation of the FixKf regulator (Zamorano-Sánchez et al., 2012) (Fig. 5). The microoxic expression of *nirK* and *nor* genes requires a functional FixKf protein. Additionally, these genes also respond to NO through NnrR that is also controlled by FixKf in response to microoxia. In *R. etli*, expression of *nor* genes under microoxic conditions also depends on NO_3^- reduction to NO_2^- by the assimilatory nitrate reductase NarB (more details in Section 2.2) and NO_2^- reduction to NO by NirK (Hidalgo-García et al., 2019). These observations suggest that,

as reported in *B. diazoefficiens* (Bueno et al., 2017), NO is the signal molecule that activates expression of the *nor* genes in *R. etli* under conditions of low-O_2. In *R. etli* NifA decreases the transcription of the *nirK* gene whereas in *B. diazoefficiens*, NifA is involved in the maximum expression of the *nap, nirK* and *norC* denitrification genes (Bueno et al., 2010; Gómez-Hernández et al., 2011) (Fig. 5).

[Insert Figure 5 here]

2.2 Nitrate assimilation: an emerging source in rhizobia

For years, NO formation from NO_2^- was assumed to be produced by the respiratory Nir from denitrifiers (NirK and NirS) or from nitrate-ammonifying bacteria (NirDB and NrfA). Currently, a new class of NO-forming molybdoenzymes has been found to represent an important source of NO (Maia & Moura, 2015). Presently, more than 50 classes of Mo-containing enzymes are known, many of which have been biochemically and structurally characterised (Maia, Moura, & Moura, 2017). Importantly, the vast majority of the Mo-enzymes are prokaryotic, whereas only a restricted number are found in eukaryotes.

In prokaryotes, as discussed earlier (Section 2.1), Nar, Nap and Nas are Mo-enzymes, belonging to the dimethyl sulfoxide reductase (DMSOR) family, which catalyze the reduction of NO_3^- to NO_2^-. In spite of catalyzing the same reaction and having Mo in their active sites coordinated by four sulphur atoms from two pyranopterin cofactor molecules both present as a guanine dinucleotide (Mo $[MGD]_2$), the three NR types display significant differences in the remainder of the Mo coordination sphere. In addition to significant differences in the NO_3^--biding regions

around their active-site centres, these NRs have different biological function, subcellular location, different subunit composition and quaternary structure (see Sections 2.1 and 2.2). In terms of catalytic specificity, Nap has a high specificity for NO_3^- while Nar and Nas enzymes have broader substrate specificity which can lead to these being sources of NO produced from direct reduction of NO_2^-. In fact, several studies have suggested that NO generation in *E. coli* and *Salmonella enterica* serovar Typhimurium is due to NR-catalysed NO_2^- reduction, with the majority of NO being formed by NarG. While NO generation in *E. coli* is estimated to be < 1% of total NO_3^- reduced, in *S.* Typhimurium this can reach up to ~20% (Gilberthorpe & Poole, 2008; Rowley et al., 2012; Vine & Cole, 2011; Vine, Purewal, & Cole, 2011). NO production is higher under NO_3^--sufficient conditions where NarGHI is the predominant NR, rather than NO_3^--limited conditions where NapAB is the predominant NR present. In line with NarGHI being a source of NO, NO_3^--sufficient conditions also lead to increased expression of genes encoding a number of different nitrosative stress response systems (Rowley et al., 2012). Thus bacteria that only synthesise Nap are unlikely to produce NO as a by-product of NO_3^- metabolism in contrast to those that express Nar systems.

Two classes of Nas have been described in bacteria; the NADH-dependent Nas and the ferrodoxin- or flavodoxin-dependent Nas. NADH-dependent Nas are found in most proteobacteria and they are heterodimers consisting of a 45 kDa FAD-containing diaphorase and the 95 kDa catalytic subunit with a $Mo[MGD]_2$ cofactor and a N-terminal [4Fe-4S] centre (Moreno-Vivián, Cabello, Martínez-Luque, Blasco, & Castillo, 1999; Richardson, Berks, Russell, Spiro, & Taylor, 2001). A special case of an NADH-dependent Nas is NasC from *P. denitrificans* which lacks the FAD-containing NADH-diaphorase subunit. In this bacterium, the NADH-reductase activity is carried out by the nitrite reductase (NasB) that is associated with a small 12 kDa ferredoxin-type subunit

673 [2Fe-2S] called NasG that may transfer electrons to both NasB and NasC (Gates et al.,
674 2011). As reported for Nas, two types of assimilatory nitrite reductases have been
675 described in prokaryotes, according to their electron donor. The ferredoxin-dependent
676 Nir, such as NirA from the cyanobacterium *Synechococcus* sp. PCC7942, and the
677 NADH-dependent Nir described in heterotrophic bacteria (Moreno-Vivián, Luque-
678 Almagro, Cabello, Roldán, & Castillo, 2011). Both types of Nir contain [4Fe-4S] and a
679 sirohaem group, which is responsible for the reduction of NO_2^-. In the case of Nir
680 NADH-dependent, unlike ferredoxin-dependent, they have an N-terminal extension
681 where they host a FAD group, additional Fe-S sites and the NADH-binding domain, as
682 it is the case of NasB from *P. denitrificans*. The best known NADH-dependent Nir is
683 present in *E. coli* (NirBD) which it is not an assimilative Nir in strict sense, since it is
684 induced in anoxia and is involved in nitrite disassimilation, but it is also used to
685 assimilate NO_2^- (Cole, 1996; Lin & Stewart, 1998). This enzyme is composed of two
686 different subunits, a large 93 kDa (NirB) subunit containing the sirohemo group and a
687 [4Fe-4S], and a small 12kDa (NirD) subunit homologous to a ferredoxin group [2Fe-
688 2S]. This structure is homologous to the NasBG complex from *P. denitrificans* where
689 (as described above) NasB has a ferredoxin [2Fe-2S] type small subunit, NasG, which
690 can donate electrons for the reduction of NO_2^- by NasB as well as for the reduction of
691 NO_3^- by NasC (Gates et al., 2011).

692 In *B. diazoefficiens*, assimilatory NO_3^- reduction is catalysed by the NasC
693 enzyme which is similar to that present in *P. denitrificans* (Cabrera et al., 2016). In this
694 bacterium, a FAD-dependent NAD(P)H oxidoreductase (Flp) has been proposed as the
695 physiological electron donor to NasC (Fig. 6B). The *nasC* and *flp* genes belong to a
696 coordinated NO_3^- assimilation and NO detoxification system in *B. diazoefficiens*
697 encoded by the *narK-bjgb-flp-nasC* operon (Fig. 6A). In addition to NasC and Flp, this

cluster also codes for a MFS-type NO_3^-/NO_2^- transporter (NarK) that in *B. diazoefficiens* is involved in NO_2^- export (Fig. 6B). Upstream of the *narK-bjgb-flp-nasC* operon there are three genes that encode for proteins predicted as an NrtABC family transporter (Fig. 6A). However, deletion of *nrtABC* did not affect the ability of *B. diazoefficiens* to assimilate NO_3^- as sole N-source. In this context, other NrtABC-like candidates present on the chromosome (e.g. bll5732–34) might facilitate NO_3^- transport to the cytoplasm and the main route(s) for NO_3^- import in this rhizobium species remain to be established. *B. diazoefficiens* uses a ferredoxin-dependent assimilatory NO_2^--reductase (NirA) to reduce NO_2^- to NH_4^+ (Fig. 6B). The *nirA* gene is located at a distinct locus to *narK-bjgb-flp-nasC* operon and instead resides downstream of genes that code for a NO_3^-/NO_2^--responsive regulatory system (NasST) (Fig. 6A), which is similar to that characterized in *P. denitrificans* PD1222 (Luque-Almagro et al., 2013). In *P. denitrificans*, the RNA-binding protein NasT directly and positively regulates exresion of the *nasABGHC* gene cluster required for NO_3^-assimilation by interacting with the *nasA*-leader mRNA. NasS is a NO_3^-/NO_2^--binding sensor that controls NasT activity. In *B. diazoefficiens*, the NasST system is required for NO_3^--dependent expression of the *narK-bjgb-flp-nasC* transcriptional unit and the *nirA* gene (Cabrera et al., 2016). These genes are subjected to an additional control mediated by the general nitrogen-regulatory NtrBC system in response to nitrogen limitation (Franck, Qiu, Lee, Chang, & Stacey, 2015; López, Cabrera, Salas, Delgado, & López-García, 2017).

In *B. diazoefficiens*, reduction of NO_2^- by NirK during denitrification is the main NO-forming process. As mentioned in Section 2.1.2, NO is the signal molecule that activates transcription of *nor* genes and this control is mediated by the NnrR transcription factor. In fact, *B. diazoefficiens* cells lacking Nap, where NO synthesis from denitrification is blocked, show very low expression of NorCB (Bueno et al.,

723 2017; Cabrera et al., 2016). In addition to NapA, NasC is also responsible for
724 generating NO, as induction of NorCB is significantly decreased in a *B. diazoefficiens*
725 *nasC* mutant suggesting the importance of NasC not only in NO_3^--assimilation but also
726 in NO production (Cabrera et al., 2016).

727 As described above, *R. etli* lacks genes encoding the respiratory nitrate
728 reductases (Nap or Nar). Instead, it contains a gene encoding for a putative assimilatory
729 NR annotated as *narB.* Similar to other NADH-dependnet Nas systems like NasC from
730 *P. denitrificans* or *B. diazoefficiens*, the *R. etli* NarB enzyme, with a predicted
731 molecular weight of approximately 94.5 kDa, contains the typical Mo[MGD]$_2$ binding
732 domain and the consensus motifs for co-ordination of an N-terminal FeS cluster. An
733 additional FeS binding site may be present in the C-terminal domain. This gene is
734 clustered with other open reading frames (ORFs) predicted to encode the large and
735 small subunits of the NADH-dependent Nir (NirBD). As proposed for NasBG from *P.*
736 *denitrificans*, NirBD might also transfer electrons to NarB, but this hypothesis needs to
737 be demonstrated (Fig. 6C). Upstream of *nirB*, three genes are located in the
738 chromosome which have been annotated as a NO_3^-/NO_2^- transporter (NarK), a ATP-
739 binding protein from NO_3^- ABC transporter (NrtCch) and a two-component response
740 regulator protein (NasT), respectively (Fig. 6A). In plasmid pCFN42f, *nrtABC* genes
741 encoding an NrtABC-type transporter are also present. Phenotypic characterization of a
742 *R. etli narB* mutant has recently demonstrated the dual role of NarB in NO_3^-
743 assimilation and in NO production (Hidalgo-García et al., 2019). In contrast to *B.*
744 *diazoefficiens* where a direct role of NasC on NO production has been reported (Cabrera
745 et al., 2016), *E. meliloti* NarB would have an indirect role. In this context, it has been
746 shown that, under free-living microoxic conditions, NO_2^- produced in the cytoplasm by
747 NarB is reduced to NO by NirK in the periplasm. Thus, NO_2^- needs to be transported

748 through the membrane. The potential role of *R. etli* NarK transporting NO_2^- from the
749 cytoplasm to the periplasm is currently under investigation. In *R. etli*, the involvement
750 of NarB not only as NO source, but also in N_2O production (since NO in the periplasm
751 is further reduced to N_2O) by cNor has also been demonstrated (Hidalgo-García et al.,
752 2019). These results propose a new pathway in bacteria to produce NO and N_2O by
753 coupling nitrate assimilation with denitrification under microoxic conditions.

754 As reported in *R. etli*, recent studies have suggested that *E. meliloti* NO_3^-
755 assimilation participates indirectly to NO synthesis by allowing NO_2^- flux to the
756 periplasm such that it can enter the denitrification pathway (Ruiz et al., 2019). In *R.*
757 *meliloti*, *nirB nirD narB cysG, nasTS* and *narK* genes are located in plasmid PsymB and
758 *nrtABC* genes in plasmid PsymA (Galibert et al., 2001) (Fig. 6A). *E. meliloti* NarB is
759 similar to *R. etli* NarB and NirBD is predicted to be a sirohaem-dependent assimilatory
760 Nir similar to *R. etli* NirBD. *E. meliloti cysG* encodes a putative uroporphyrin-III C-
761 methyltransferase that might be involved in the synthesis of the sirohaem cofactor. The
762 involvement of *E. meliloti* NarB and NirBD enzymes in NO_3^- and NO_2^- reduction has
763 been demonstrated (Ruiz et al., 2019). However, the implication of NarK, NrtABC and
764 NasST in NO_3^-/NO_2^- transport and regulation is currently unknown. Interestingly, in *E.*
765 *meliloti*, NO produced through denitrification was reduced by 80% when *narB* was
766 deleted (Ruiz et al., 2019). Similarly to that suggested in *R. etli*, Ruiz and colleagues
767 proposed that NO_2^- produced in the cytoplasm by NarB could be used as substrate for
768 NirK in the periplasm. However, further experiments are needed to confirm this
769 hypothesis.

770

771 [Insert Figure 6]

772

3. NO sinks

3.1 Nitric oxide reductases

Under microoxic conditions, the denitrifying nitric oxide reductases are the main systems that drive NO detoxification. As core components of the denitrification pathway, these enzymes are considered to have a predominant physiological role in respiration, rather than in conferring resistance to nitrosative stress *per se*. However, in some cases they have a role in resistance to endogenous and exogenous nitrosative stress (Anjum, Stevanin, Read, & Moir, 2002; Wang et al., 2011). Bacterial Nor is a membrane-bound enzyme that catalyzes the reduction of NO to N_2O at the outer face of the cytoplasmic membrane. Currently, three Nor types have been characterized in bacteria: cNor, qNor and Cu_ANor (de Vries et al., 2007; Hendriks et al., 2000; Richardson, 2011; Tosha & Shiro, 2017). In the cNor type, the catalytic subunit NorB receives electrons from a cytochrome *c* (NorC subunit), however, the monomeric qNor directly reacts with quinones in the membrane (Fig. 7). In addition to denitrifiers, it has been recently demonstrated that the anammox bacterium *K. stuttgartiensis* is able to use NO as its terminal electron acceptor, and conserve energy and grow by coupling NO reduction to NH_4^+ oxidation in the absence of NO_2^-. Under these conditions, NO_3^- is not produced and the sole end product is N_2. Using comparative transcriptomics and proteomics, it has been demonstrated that, when growing on NO-dependent NH_4^+ oxidation, *K. stuttgartiensis* downregulates the transcription of proteins responsible for NO generation, as well as NO_2^- oxidation (Hu, Wessels, van Alen, Jetten, & Kartal, 2019).

The cNor-type enzymes from *P. denitrificans*, *P. stutzeri* and *P. aeruginosa* have been well characterized, and the cNor from *P. stutzeri* was the first to be isolated (Kastrau, Heiss, Kroneck, & Zumft, 1994). The structural subunits, NorCB, are encoded

by the *norCB* genes, which are co-transcribed together along with the *norD* and *norQ* accessory genes and with the *norE* and *norF*. In *Thermales* and *Aquificales* orders, the *norC* and *norB* genes are followed by a third gene (*norH*) that encodes a small membrane protein, important for an efficient denitrification (Bricio et al., 2014). The catalytic subunit of cNor enzymes, NorB, belongs to the haem-copper oxidases superfamily (HCOs). NorB has 12 transmembrane helices and contains haem *b* and a binuclear active centre (comprising haem b_3 and Fe_B) (Fig. 7A) (Daskalakis, Ohta, Kitagawa, & Varotsis, 2015). NorC is a membrane-anchored protein that contains haem *c* in a periplasmic hydrophilic domain and transfers electrons from the bc_1 complex to NorB through cytochromes *c* or pseudoazurins (the structural properties of cNor have been extensively reviewed by (Daskalakis et al., 2015; Hino et al., 2010; Mahinthichaichan, Gennis, & Tajkhorshid, 2018; Shiro, Sugimoto, Tosha, Nagano, & Hino, 2012; Tosha & Shiro, 2017). NorD and NorE are membrane-bound proteins involved in the correct assembly of NorCB complex (Butland, Spiro, Watmough, & Richardson, 2001). Mutation of the *norE* and *norF* genes decreases NO reductase activity in *P. denitrificans* and *Rhodobacter sphaeroides* (de Boer et al., 1996; Hartsock & Shapleigh, 2010). Although these proteins are not essential for Nor activity, they are important under conditions where endogenous Nir activity generates a prolonged exposure to NO (Bergaust, Hartsock, Liu, Bakken, & Shapleigh, 2014). Recently, it was shown that NorQ and NorD from *P. denitrificans* are implicated in non-haem Fe (Fe_B) insertion cofactor into NorB (Kahle, Ter Beek, Hosler, & Ädelroth, 2018). Crystallographic studies of the *P. aeruginosa* cNor demonstrated that NorB does not have any transmembrane proton channel, unlike proton translocating HCOs, so this enzyme does not act as a proton pump and, therefore, it is not directly involved in

822 energy conservation (Hino et al., 2010; Pisliakov, Hino, Shiro, & Sugita, 2012; Shiro et
823 al., 2012).

824 As discussed previously, the qNor type obtains electrons from ubiquinol and
825 menaquinol (Figure 7B). The qNor structure is similar to that of the NorB subunit of
826 cNor, differing in an N-terminal extension with homology to the NorC subunit, but
827 without a haem *c* binding motif. Crystallization of the *Geobacillus stearothermophilus*
828 qNor has shown an aqueous channel from the cytoplasm that could act to translocate
829 protons (Matsumoto et al., 2012). Thus, it is possible that quinol oxidation coupled to
830 NO reduction by qNor is electrogenic. The replacement of Fe_B by Zn_B in *G.*
831 *stearothermophilus* qNor structure might be the reason for the appearance of the water
832 channel from the cytoplasmic side to the active centre (Fig. 7B) (Terasaka et al., 2014).
833 However, these authors suggest that the water channel may also be present in the Fe_B-
834 qNOR. The qNor enzyme is also present in the haloarchaea class of Archaea. The
835 unique *nor* gene found in haloarchaea encodes a single subunit quinone-dependent
836 respiratory Nor homologous to bacterial qNor (Torregrosa-Crespo et al., 2017). An
837 unusual qNor subgroup, the qCu_ANor, present for example in *Bacillus azotoformans*,
838 contains a NorB subunit in complex with another subunit containing the characteristic
839 Cu_A site of HCOs, which makes this enzyme capable of receiving electrons from
840 cytochrome c_{550} (Fig. 7C) (de Vries et al., 2007). However, in the *Bacillus* genus it was
841 shown that qCu_ANor lacks menaquinone oxidase activity, so a nomenclature change
842 from qCu_ANor to Cu_ANor was suggested (Al-Attar & de Vries, 2015). Cu_ANor
843 reconstituted in liposomes generates a proton electrochemical gradient across the
844 membrane similar in magnitude to cytochrome aa_3, suggesting that Cu_ANor can produce
845 ATP from NO reduction compared to cNor (Al-Attar & de Vries, 2015).

In rhizobia the main enzyme involved in NO reduction by free-living cells is cNor. As mention in Section 2.1.1, *B. diazoefficiens* mutant strains defective in the *norC* or *norB* genes are unable to grow under denitrifying conditions and accumulate NO (Mesa et al., 2002). *E. meliloti* cells of a *norB* mutant showed more sensitivity to NO than wild type cultures confirming the involvement of cNor in NO reduction in this rhizobial species (Meilhoc et al., 2010). *R. etli* NorCB is also involved in NO reduction (Gómez-Hernández et al., 2011).

[Insert Figure 7 here]

3.2 Haemoglobins

Haemoglobins (Hbs) are haem-Fe containing proteins distributed across all three domains of life Bacteria, Archaea and Eukarya that, in addition to be essential for O_2 transport, are also well-characterized systems for NO detoxification (Gell, 2018). All Hbs share a common three-dimensional structure and haem cofactor. They have a conserved core topology, comprising 6-8 α-helices (labelled A-H) (Fig. 8). The myoglobin (Mb) from muscle of the sperm whale and the red blood cell (RBC) Hb from horse erythrocytes were the first Hbs whose structure was determined (Kendrew et al., 1960; Perutz et al., 1960). Today, many Hb structures (>200) have been published and they share a conserved haem-coordinating histidine residue (HisF8) where F8 refers to the eighth residue in helix F of sperm whale Mb (Freitas et al., 2004; Kapp et al., 1995) (Figure 8A). Residues in non-helical segments are referenced in relation to adjacent helices; thus, CD1 refers to the first residue of the linker joining α-helices C and D (Fig. 8). In addition to HisF8, sequence alignment of several Hbs revealed the presence of other conserved residues, such as Phe, but also Trp, which are common in the haem

pocket. Particularly, PheCD1, which makes π-stacking interactions with pyrrole ring C, is the second-most highly conserved globin residue after HisF8 (Gell, 2018).

Globins can fold in two different structural ways: 3-on-3 and 2-on-2 (Gell, 2018). The 3-on-3 structural class consists in the α-helical 'sandwich' composed by the A-G-H and B-E-F helices. The C and D helices are supporting structures and are not always present. The 3-on-3 fold is represented by Mb from muscle of the sperm whale (Fig. 8A). Whereas some Hbs function as monomers, other Hbs are assembled from multiple globin subunits. Each globin polypeptide binds a single molecule of Fe-protoporphyrin-IX (haem *b*) (Fig. 8E). The haem molecule contains two charged propionate groups that interact with water and/or polar amino acid side chains on the surface of the globin. The haem cofactor is largely hydrophobic and binds in the hydrophobic interior of the globin, surrounded by apolar side chains. Each haem has a central Fe atom that is coordinated by four equatorial N ligands, one from each of the four pyrrole rings of the porphyrin (Fig. 8E). Haem is bound to the protein through a covalent bond from an axial N ligand provided by the imidazole side chain of HisF8 as well as through non-covalent interactions between the porphyrin and globin. The Fe-coordinating HisF8 side chain occupies the proximal haem pocket, leaving diatomic ligands bind on the opposite face of the porphyrin, which is the distal haem pocket.

It has been known for long time that NO has extremely high affinity for Hbs (Gibson & Roughton, 1957, 1965). Under physiological conditions, the major Hb species are Hb^{2+} and O_2-Hb^{2+}, both of which undergo extremely rapid reaction with NO. NO induces oxidation of O_2-Hb^{2+} by an NO dioxygenation (NOD) mechanism that involves capture of NO in the distal pocket of O_2-Hb^{2+}, where it reacts with bound O_2 to form an Fe(III) cis-peroxynitrite transition state, ONOO-Hb^{3+}, that immediately isomerises to an Hb^{3+}+NO_3^- complex (Yukl, de Vries, & Moënne-Loccoz, 2009). An

alternative pathway by which non-coordinated O_2 reacts in the distal pocket of NO-Hb^{2+}, termed O_2 nitrosylation, has been proposed (Hausladen, Gow, & Stamler, 1998). However, the NOD mechanism is considered to be the general mechanism for NO reaction with Hb over a wide range of O_2 concentrations *in vivo* (Gardner, 2005, 2012).

[Insert Fig. 8 here]

In prokaryotes, three types of haemoglobins (Hbs) have been identified: flavohaemoglobins (fHbs), single domain haemoglobins (sdHbs) and truncated haemoglobins (trHbs) (Gell, 2018; Poole, 2005; Stern & Zhu, 2014). Similar to Mb, fHbs and sdHbs also possess a globin domain with a 3-on-3 α-helical fold (Tinajero-Trejo & Shepherd, 2013) (Figs. 8B and C). The second structural class corresponds to trHbs, also called 2-on-2 Hbs, based on the arrangement of the B-E and G-H helical pairs (Fig. 8D). In trHbs, the A, C, D, and F helices are much reduced or absent.

The best studied bacterial haemoglobins are fHbs, with *E. coli* Hmp as the main representative (for a recent review see Poole, 2020). The sequence of the *E. coli hmp* gene revealed that this fHb is a 44-kDa monomer which contains an N-terminal haem domain, and a C-terminal domain named ferredoxin reductase which has binding sites for NAD(P)H and FAD (Andrews et al., 1992). Residues of the haem pocket implicated in NOD activity are highly conserved across fHbs, as are residues surrounding the flavin cofactor that play roles in electron transfer from NADP (Bonamore & Boffi, 2008). The presence of haem *b* and FAD has been confirmed by crystal structures from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Ermler, Siddiqui, Cramm, & Friedrich, 1995) and *E. coli* (Ilari et al., 2002). (Fig. 8B). FHbs have also been identified in *S.* Typhimurium (Bang et al., 2006; McLean, Bowman, & Poole, 2010), *Staphylococcus aureus* (Richardson, Dunman, & Fang, 2006), *Bacillus subtilis*

(Nakano, 2006), and *Saccharomyces cerevisiae* (yeast) (Lewinska & Bartosz, 2006; Liu, Zeng, Hausladen, Heitman, & Stamler, 2000).

Regarding the molecular mechanism of fHbs for NO detoxification, two pathways have been proposed depending on the O_2 availability. As mention above, under aerobic conditions, they transform NO into NO_3^- by a NOD activity (Gardner, Gardner, Martin, & Salzman, 1998). Under anaerobic conditions, it has been shown that *E. coli* Hmp is able to reduce NO to N_2O. However, the rates of NO reduction are modest by comparison with better characterised NO reductases in fungi and bacteria (Kim, Orii, Lloyd, Hughes, & Poole, 1999). This led to the conclusion that Hmp does not provide physiologically relevant protection to anaerobic cultures. By performing gene knockout studies, it has been shown that fHbs protect their host organisms from nitrosative stress. In a number of human pathogenic bacteria such as *E. coli*, *Salmonella* or *Yersinia pestis*, resistance to nitrosative stress conferred by fHb contributes to pathogen virulence (Bang et al., 2006; Sebbane et al., 2006; Svensson, Marklund, Poljakovic, & Persson, 2006; Svensson et al., 2010). FHbs also confer tolerance to plant pathogens such as *Erwinia chrysanthemi* reducing the hyper-sensitive response by detoxifying NO produced by the plant (Boccara et al., 2005).

SdHbs resemble fHbs but lack the oxidoreductase and FAD domain and thus comprise the single globin domain that cordiantes a haem *b* group (Fig. 8C). The first haemoglobin of this type to be identified and sequenced was *Vitreoscilla stercoraria* haemoglobin (Vgb), whose presence increases under microoxic conditions. The physiological role of Vgb has not been conclusively demonstrated yet, but an NO detoxification function for this Vgb expressed in a heterologous organism conferred some protection against nitrosative stress (Wu, Wainwright, & Poole, 2003). Vgb fused with an fHb reductase domain has been extensively used in biotechnology applications

945 to protect against nitrosative stress (Kaur, Pathania, Sharma, Mande, & Dikshit, 2002).
946 In *Campylobacter jejuni*, a sdHb (Cgb) has been also demonstrated to be involved in the
947 response to nitrosative stress by performing gene deletion (Elvers, Wu, Gilberthorpe,
948 Poole, & Park, 2004), biochemical (Lu et al., 2007; Shepherd et al., 2010) and
949 expression analyses (Elvers et al., 2004; Monk, Pearson, Mulholland, Smith, & Poole,
950 2008). The structure of cyanide-bound Cgb was solved by using X-ray crystallography
951 with a resolution of 1.35 Å (Shepherd et al., 2010). Cgb has structural homology with
952 Vgb from *V. stercoraria* (Tarricone, Galizzi, Coda, Ascenzi, & Bolognesi, 1997), the N-
953 terminal globin domain of Hmp from *E. coli* (Ilari et al., 2002) and the sperm whale
954 myoglobin (Mb) (Arcovito et al., 2007) (Fig. 8C). Although evidence that sdHbs
955 function generally as NOD enzymes is emerging, the mechanism of action is not
956 known, since the redox system that recycles ferrous haem has not been identified.

957 TrHbs are between 20-40 residues shorter than the sdHbs (Tinajero-Trejo,
958 Vreugdenhil, Sedelnikova, Davidge, & Poole, 2013). Like sdHbs, trHbs lack the
959 flavoreductase domain and they only have the globin domain (Fig. 8D), but they differ
960 from sdHbs since, once translated, they are mannosylated and bound to the cell wall
961 (Arya et al., 2013). By using X-ray crystallography, the structure of dimeric cyanide-
962 bound trHb from *C. jejuni* showed the presence of four α-helices organized in '2-on-2'
963 fold formed by the B-E and G-H helical pairs (Fig. 8D). In this structural class, the A,
964 C, D, and F helices are reduced or missing (Nardini et al., 2006). Although some trHbs
965 supply O_2 in pathogens, others are involved in NO stress tolerance. In *Mycobacterium*
966 *tuberculosis*, a truncated haemoglobin HbN is involved in NO detoxification being this
967 process critical for its virulence (Pathania, Navani, Gardner, Gardner, & Dikshit, 2002).
968 In *Mycobacterium bovis*, HbN protects aerobic respiration from NO, and this protein
969 oxidizes *in vitro* NO to NO_3^- (Ouellet et al., 2002).

970 In addition to *Mycobacterium* and *Campylobacter* species, a role in nitrosative
971 stress response has also been demonstrated genetically for trHbs from *Synechococcus*
972 (Scott et al., 2010), *Chlamydomonas reinhardtii* (Hemschemeier et al., 2013; Johnson et
973 al., 2014), and the antarcticmarine bacterium *Pseudoalteromonas haloplanktis* (Parrilli
974 et al., 2010). *In vitro* and *in vivo* studies suggest that the trHb from *C. reinhardtii*
975 converts the NO generated by nitrate reductase (NIT1) into NO_3^- (Rice et al., 2015). The
976 structure of trHb from *C. reinhardtii* resembles other trHbs, but it also exhibits distinct
977 features associated with the coordination of the haem by a proximal histidine (H77) and
978 a distal lysine (K53) (Rice et al., 2015) (Fig. 8D). Site-directed mutagenesis analyses
979 have recently demonstrated that K53 coordination is related to the ability of *C.*
980 *reinhardtii* trHb to detoxify NO efficiently (Johnson, Russo, Nye, Schlessman, &
981 Lecomte, 2018).

982

983 ***3.2.1 Rhizobial haemoglobins***

984 As mentioned above, bacterial Hbs have been extensively studied in pathogenic bacteria
985 due to their contribution to scape the hypersensitive response by removing the NO
986 produced by their hosts. In the recent years, reports about the involvement of rhizobial
987 Hbs in the symbiotic interaction with plants are emerging. Nowadays, it is well-known
988 the capacity of root nodules to produce NO (see Section 4). How rhizobia cope with the
989 presence of NO either in the plant rhizosphere or inside the nodules is essential to
990 protect symbiotic nitrogen-fixation from nitrosative stress. Beside the respiratory Nor
991 which catalyses the reduction of NO to N_2O, Hbs have been proposed to be also
992 involved in NO degradation in rhizobia. In *E. meliloti*, transcriptomic analyses in
993 response to NO enabled the identification of *hmp* encoding a putative flavohaemoglobin
994 (fHb) (Meilhoc et al., 2010) (for details see Section 3.2). This gene is located close to

995 *nosRZDFYLX* in *E. meliloti* plasmid pSymA (Fig. 3). An *hmp* mutant showed higher
996 sensitivity to NO whereas overexpression of Hmp improved NO resistance in free-
997 living cultures (Meilhoc et al., 2010). Inoculation of *M. truncatula* plants with the *E.*
998 *meliloti hmp* mutant induced the formation of nodules with higher levels of NO, lower
999 N_2 fixation activity and earlier nodule senescence than those inoculated with the wild-
1000 type strain (Cam et al., 2012), suggesting a role of this fHb in symbiosis.

1001 In *B. diazoefficiens*, a search for Hb-like sequences enabled the identification of a
1002 putative single domain haemoglobin (sdHb, designated Bjgb) very similar to those from
1003 *V. stercoraria* or *C. jejuni* as well as to the haem domain from the fHbs present in *E.*
1004 *coli*, *S.* Typhimurium or *E. meliloti* 1021 (Sánchez, Cabrera, et al., 2011) (further details
1005 about sdHbs in Section 3.2). In *B. diazoefficiens*, Bjgb belongs to a coordinated NO_3^-
1006 assimilation and NO detoxification system encoded by the *narK-bjgb-flp-nasC* operon
1007 (for details see Section 2.2 and Figs. 6A and B). The involvement of Bjgb in NO
1008 detoxification was demonstrated by testing the sensitivity of a *bjgb* mutant to the
1009 presence of the NO donor sodium nitroprusside (SNP) (Cabrera et al., 2011, 2016).
1010 However, the capacity of Bjgb to bind NO *in vitro* is at the moment unknown.
1011 Furthermore, the addition of SNP also provoked a decrease in the viability of a *flp*
1012 mutant. These results revealed the importance of Bjgb and Flp for protection against
1013 nitrosative stress in *B. diazoefficiens* under free-living conditions. It might be posible
1014 that Flp, in addition to donating electrons to NasC, is also the redox partner responsible
1015 for flavin-mediated NADH reduction of the Bjgb haem cofactor (Fig. 6B). This would
1016 draw parallels with the classical fHb system. However, this hypothesis is currently
1017 under investigation. Cabrera and colleagues have proposed that Bjgb-Flp would
1018 mitigate the NO produced by NasC as by-product of NO_3^-/NO_2^- reduction (Cabrera et
1019 al., 2016) (Fig. 6B).

4. NO metabolism in the rhizobia-legume symbiosis

The establishment of an effective rhizobia-legume symbiosis is a complex process in which the exchange of specific signals between the symbionts is essential. The communication between the micro- and the macrosymbiont lets rhizobia attachment to root hairs and entrapment by root hair curling, which results in the formation of an infection thread. Then, rhizobia are liberated into the cytoplasm of the infected cortical cells, and they are surrounded by a plant membrane, termed the symbiosome membrane. In this way, bacteria are contained inside a vesicle, called the symbiosome. Eventually, bacteria are differentiated into a N_2-fixing form, known as bacteroids (for recent reviews see Poole, Ramachandran, & Terpolilli, 2018; Udvardi & Poole, 2013).

Inside the bacteroids, nitrogenase biosynthesis requires the expression of structural, and regulatory genes as well genes needed for nitrogenase activity. The structural *nifH* and *nifDK* genes encode the nitrogenase complex of all known diazotrophs. This complex catalyzes the reduction of N_2 to NH_3 with the following stoichiometry;

$$N_2 + 8e^- + 8H^+ + 16\text{MgATP} \rightarrow 2NH_3 + H_2 + 16\text{MgADP} + 16P_i$$

This equation shows that nitrogenase activity requires high levels of energy in ATP to overcome the stability of the N_2 triple bond. Nitrogenases comprise two proteins, a reductase component, known as the Fe-protein (NifH), that contains a single 4Fe-4S cluster and two ATP binding sites. A second catalytic protein (NifDK), known as the MoFe-protein, VFe-protein or FeFe-protein, houses an electron transfer P cluster, as well as the active-site metal cofactor (FeMo-co, FeV-co, and FeFe-co) (for key

reviews about nitrogenase see Rubio & Ludden, 2008; Seefeldt et al., 2018; Sickerman, Rettberg, Lee, Hu, & Ribbe, 2017).

Microoxia is a requirement not only for nitrogenase activity, but for the whole induction of N_2 fixation and the expression of genes related to symbiosis (*nif* and *fix*) (Fischer, 1994; Rutten & Poole, 2019). The detection and transduction of the "low-O_2 signal" is mediated by proteins conserved and integrated into regulatory networks of different rhizobial species. The regulation of nitrogen fixation is beyond the scope of this review, however information on this topic can be found in a variety of reviews (Dixon & Kahn, 2004; Fischer, 1994; Poole et al., 2018; Rutten & Poole, 2019; Terpolilli, Hood, & Poole, 2012).

The low-O_2 concentration in the nodule is subjected to a delicate balance, since on one hand O_2 is needed to synthesize the ATP that nitrogenase activity demands, but it must not be allowed to reach levels that inactivate this central enzyme. These conflicting needs are solved thanks to the function of three systems, mainly: i) the variable oxygen diffusion barrier in the nodule endodermis; ii) the presence of leghaemoglobin (Lb); iii) the expression of the *cbb*$_3$ high-affinity terminal oxidase in the bacteroids. The oxygen diffusion barrier maintains the microoxic environment within the infected zone of the nodules and is composed by a complex structure that occludes the intercellular space in the middle part of the cortex, limiting O_2 permeability. It involves several morphological and metabolic mechanisms in different areas of the nodule, such as the presence of occlusions in the intercellular spaces of the middle cortex, an osmotic mechanism in the internal cortex, and a precision oxygen diffusion control mechanism that occurs in the infection zone (Minchin, 1997; Minchin, James, & Becana, 2008). Lb is a high-affinity O_2 plant carrier, which buffers free O_2 around 7-11 nM. It is the most abundant protein in nodules (~300 µM) and its

concentration can reach up to 2-3 mM in very active nodules (Davies, Mathieu, & Puppo, 1999). Lb is present at a concentration of several orders of magnitude higher than free O_2 (Ott et al., 2005). Most O_2 within nodules is therefore bound by Lb, which transports O_2 through the cytosol of infected cells to the membrane of the symbiosome at steady-state concentrations to ensure bacteroid respiration, while protecting nitrogenase activity (Downie, 2005). A strong correlation between the Lb content and N_2 fixation activity of nodules has been demonstrated (Dakora, 1995). The cbb_3 oxidase encoded by the *fixNOQP* genes, has high affinity by O_2 (K_M = 7 nM) which allows microoxic respiration of the bacteroid during N_2 fixation (Delgado, Bedmar, & Downie, 1998; Preisig, Zufferey, Thony-Meyer, Appleby, & Hennecke, 1996).

NO is produced in plant root nodules of legumes such as *M. truncatula* (Baudouin, Pieuchot, Engler, Pauly, & Puppo, 2006), *M. sativa* (Pii, Crimi, Cremonese, Spena, & Pandolfini, 2007), *G. max* (Sánchez et al., 2010), or *P. vulgaris* (Gómez-Hernández et al., 2011). The function of NO in the rhizobia-legume simbiosis is different from that known in pathogenic interactions. During *E. meliloti-M. truncatula* symbiosis, NO has a positive role during the infection steps (del Giudice et al., 2011). On the contrary, in madure nodules NO provokes a strong inhibition of nitrogenase expression and activity (Kato, Kanahama, & Kanayama, 2010; Sánchez et al., 2010). In nodules, NO can also bind deoxy-Lb, contributing to the formation of nitrosyl-leghaemoglobin (LbNO) complexes. It has been proposed that Lb, through the formation of LbNO, has a major role in detoxifying either NO_2^- or NO in root nodules from soybean plants (Sánchez et al., 2010) (Fig. 9). This role has also been attributed to the non-symbiotic haemoglobins (ns-Hbs) which are also present in legumes (for a recent review see Rubio et al., 2019). Another role of NO in nodules is the tyrosine nitration of glutamine synthetase causing the inhibition of its activity, and subsequently,

of NH_4^+ assimilation (Blanquet et al., 2015; Melo, Silva, Ribeiro, Seabra, & Carvalho, 2011). NO has also been shown to be a signal for nodule senescence in *M. truncatula* nodules (Cam et al., 2012). A similar effect has been reported in determinate nodules of *Lotus japonicus* where overexpression of a phytoglobin (LjGlb1-1) resulted in the reduction of NO levels in nodules and a delay of nodule senescence (Fukudome et al., 2019).

Given the dual effect of NO in legume root nodules, in order to maintain an efficient infection and N_2 fixation, the level of NO inside them must be finely tuned. NO levels in the nodules result from a balance between NO synthesis and consumption, two processes which rely on both partners of the symbiosis (Hichri et al., 2016). In the root nodules, both the bacterial and plant partners are responsible for NO production (Berger et al., 2018; Hichri et al., 2015). From the plant perspective, NO is synthesized by the plant NR, the ETC (electron transport chain)-dependent enzymatic NO_2^- reduction using NADPH as donor to produce NO (MIT-ETC), and the NO synthase (NOS)-like activity (Figure 9) (for a review see Astier, Gross, & Durner, 2018; Chamizo-Ampudia, Sanz-Luque, Llamas, Galván, & Fernández, 2017). From the bacterial perspective, denitrification is the main process involved in NO production. However, new rhizobial systems involved in NO metabolism in the nodules are emerging, that will be discussed in the next Section.

4.1 Contribution of the bacteroids to NO metabolism in the nodules

The first evidence of the implication of rhizobial denitrification in NO metabolism in root nodules was the reported *in situ* expression of *B. diazoefficiens* denitrification genes in soybean nodules (Mesa, Alché, Bedmar, & Delgado, 2004). Levels of β-galactosidase activity from a *nirK-lacZ*, *norC-lacZ*, or *nosZ-lacZ* fusions

were similar in both bacteroids and nodule sections from plants that were solely N_2-dependent or grown in the presence of 4 mM KNO_3. These findings suggest that low O_2, and not NO_3^-, is the dominant main factor controlling expression of the denitrification genes in soybean nodules (Mesa et al., 2004). In fact, some environmental stresses, such as flooding that triggers a reduction in the O_2 concentration in the nodule, significantly increased the expression and activity of denitrification enzymes in soybean nodules, including a significant increase in Nap activity (Meakin et al., 2007; Sánchez et al., 2010).

Inoculation of soybean plants with *B. diazoefficiens* denitrification mutants did not affect symbiotic N_2 fixation when plant growth was exclusively dependent on atmospheric N_2 (Mesa et al., 2004). However, when KNO_3 was added to the medium, NirK and NorCB denitrification enzymes play a role in nodule formation rather than in nodule function (Mesa et al., 2004). In response to flooding conditions, inoculation with a *B. diazoefficiens nirK* mutant had a slight advantage for N_2 fixation over inoculation with the wild-type (WT) or a *norC* mutant (Sánchez, Tortosa, et al., 2011). These findings allowed Sánchez and colleagues to suggest that NO formed by NirK in soybean nodules, in response to flooding, has a negative effect on nitrogenase activity. Recently, it was shown that NO_3^- and flooding can also increase the production of the potent greenhouse gas N_2O by soybean nodules (Tortosa et al., 2015). As mentioned aboved, denitrification in *B. diazoefficiens* bacteroids has been proposed as the main process responsible for NO and N_2O production in soybean nodules, since levels of these gases are significantly reduced in nodules produced by a *B. diazoefficiens napA* mutant strain, where denitrification is blocked (Sánchez et al., 2010; Tortosa et al., 2015). However, basal levels of NO and N_2O were still detected in nodules from the *napA* mutant (Sánchez et al., 2010; Tortosa et al., 2015). Similarly as it was proposed in free-living *B.*

diazoefficiens cells (see Section 2.2), it may be possible that the assimilatory NR (NasC) is an additional source of NO in soybean nodules. However, the expression and physiological role of *B. diazoefficiens* NO_3^- assimilation in the nodules has not been demonstrated yet.

With respect to NO detoxifying mechanisms acting in the bacteroids, in addition to the denitrifying cNor enzyme, the *B. diazoefficiens* sdHb (Bjgb) has been proposed to be also involved in NO removal in soybean nodules contributing to the protection of nitrogenase against NO. To assess whether the Bjgb plays a role during soybean-B. *diazoefficiens* simbiosis, the effect of inoculation of soybean plants with a *bjgb* mutant on biological nitrogen fixation has been recently investigated by using the ^{15}N isotope dilution technique (Salas et al., 2020). These studies have shown that soybean plants inoculated with the *bjgb* mutant had higher tolerance to flooding than those inoculated with the parental strain. Furthermore, the negative effect of flooding on nitrogenase activity and *nifH* expression was less severe in *bjgb* than in WT nodules. Nodules induced by the *bjgb* mutant expressed higher Nor activity and consequently accumulated less NO than WT nodules. Given this body of experimental evidence, it has been proposed that *B. diazoefficiens* Bjgb, instead of functioning as a direct NO-detoxifying protein in the nodules, it would contribute indirectly by modulating cytoplasmic NO levels, the signaling molecule required for induction of the denitrifying Nor which is the major protein involved in NO removal in soybean nodules (Salas et al., 2020) (Fig. 9).

Similarly, as described in soybean nodules, *E. meliloti napA* and *nirK* denitrification genes also appear to be involved in NO formation in *M. trucantula* nodules, at least at the mature stage (Horchani et al., 2011). Although *E. meliloti* denitrification remains the main enzymatic way to produce NO, recent studies suggested

that the NO_3^- assimilatory pathway involving the nitrate reductase (NarB) and nitrite reductase (NirBD) participates indirectly to NO synthesis in partnership with denitrification (Ruiz et al., 2019). Even though these genes are clearly expressed in the fixation zone of *M. trucantula* root nodules, they do not play a crucial role in simbiosis.

NO-detoxification systems in *M. trucantula* nodules are also essential for keeping a low steady-state intracellular NO concentration to maintain an efficient symbiosis. The main enzyme involved in NO consumption in *M. truncatula* nodules is the *E. meliloti* Nor (Blanquet et al., 2015). In addition to Nor, a combined role for *E. meliloti* flavohaemoglobin (Hmp), and $NnrS_1$ and $NnrS_2$ proteins in NO degradation has also been reported in *M. truncatula* nodules (Fig. 9). In fact, *in situ* detection of NO by confocal microscopy using fluorescent dyes revealed that nodules induced by *hmp*, *nnrS1* or *nnrS2* mutants showed increased NO levels which affected the maintenance of an efficient symbiosis with *M. truncatula* (Blanquet et al., 2015). The gene *nnrS* is usually located close to that encoding the transcriptional regulator NnrR (Fig. 3), and it encodes a haem- and cooper-containing transmembrane protein (Bartnikas et al., 2002). However, the regulatory function of NnrS is at the moment unknown. It has been reported in *Vibrio cholerae* that NnrS contributes to nitrosative-stress tolerance by protecting Fe-S cluster enzymes from NO-Fe complexes formation (Stern, Liu, Bakken, Shapleigh, & Zhu, 2013).

In nodules of common bean, *R. etli* NirK contributes to NO formation and Nor to NO removal in response to NO_3^-, as levels of LbNO complexes raised in nodules produced by a *R. etli norC* mutant and decreased in those from a *nirK* mutant, compared to LbNO levels from WT nodules (Gómez-Hernández et al., 2011). Interestingly, the presence of NO_3^- in the plant nutrient solution reduced nitrogenase-specific activity in WT and *norC* nodules. Nevertheless, the inhibition of nitrogenase activity by NO_3^- was

not detected in *nirK* nodules, probably due to the low levels of cytotoxic NO produced in those nodules (Gómez-Hernández et al., 2011). As mentioned in Section 2.1.1, *R. etli* lacks genes encoding the respiratory nitrate reductases (Nap or Nar). In common bean nodules, NO_3^- included in the nutrient solution can be reduced to NO_2^- by the plant NR that has been proposed in plants to be the most important enzymatic source of NO from NO_2^- reduction (Chamizo-Ampudia et al., 2017). In the bacteroids, the *R. etli* assimilatory NR (NarB) might be a candidate to reduce NO_3^- to NO_2^- (see Section 2.2), acting as a source of NO_3^--dependent NO production in common bean nodules. The contribution of *R. etli* NarB to NO production in *P. vulgaris* nodules is currently under investigation.

[Insert Figure 9 here]

5. Concluding remarks and future perspectives

The cultivation of legumes, through their symbiotic association with N_2-fixing bacteria, globally named as rhizobia, constitutes an agricultural practice that can safe huge amounts of environmental polluting synthetic nitrogen fertilizers, decrease greenhouse gas (GHG) emissions as well as protect ground water from toxicity, while improving soil fertility. Nowadays, it is well-known the production of NO during the symbiotic interaction. This molecule is produced at different steps of the infection process, during nodule organogenesis and development, as well as in mature nodules where nitrogenase reduces atmospheric N_2 into biologically useful forms in a process called symbiotic nitrogen fixation (SNF).

The amount of data regarding the NO sources and sinks by the plant partner of the rhizobia-legume symbiosis, as well as the role of this molecule during the early steps of the symbiotic interaction have significantly increased in recent years and numerous reviews have been published (Berger, Boscari, Frendo, & Brouquisse, 2019; Berger et al., 2018; Hichri et al., 2015, 2016, among others). However, only limited information is available concerning the contribution of the microsymbiont to the NO homeostasis in mature and functional root nodules. Thus, this review highlights the importance of rhizobia in controlling NO accumulation in the nodules. Several reports have suggested the multiple functions of NO in rhizobia where it acts through signaling cascades as a regulator of gene expression especially those genes encoding the nitric oxide reductase (Nor) which is considered today the main rhizobial enzyme that removes NO in root nodules. When legumes are sujected to extreme weather events connected to climate change such as soil flooding, NO accumulation occurs and this molecule becomes a potent inhibitor of nitrogenase. A fine balance of NO homeostasis in the nodules is essential to protect SNF from nitrosative stress. In addition to denitrification, new processes involved in NO production are emerging in rhizobia. This is the case of NO_3^- assimilation where the NR (NasC or NarB) has been reported to produce NO either directly or indirectly by cooperating with the denitrification pathway. Future investigations are needed in order to establish the role of these new NO-producing pathways in the nodules. To cope with the presence of NO inside the nodules, beside the respiratory Nor which catalyzes the reduction of NO to N_2O, rhizobial Hbs have been reported to be also involved in NO detoxification in the nodules. However, the function of these Hbs is different depending on the rhizobial species. In *E. meliloti*, the fHb Hmp, is directly involved in NO removal in *M. truncatula* nodules, and protect them from early senescense. On the contrary, *B.*

diazoefficiens sdHb, Bjgb, does not have a direct role on NO detoxification. Instead it modulates the concentration of NO in the cytoplasm of the bacteroids where it acts as a signaling molecule that induces the expression of Nor which, as mention above, is the major protein involved in NO removal in soybean nodules. Induction of Nor reduces NO levels in the nodules and protect nitrogen fixation from abiotic stresses such as NO_3^- and flooding.

In conclusion, this review highlights the significance of rhizobia to protect SNF from the toxic effect of NO by controlling its accumulation in the nodules. We propose the use of rhizobial strains that modulate NO levels inside the nodules as a strategy to enhance legume production. Selecting strains that overexpress Nor or Hbs, as well as unravelling the mechanism involved in the inhibition of nitrogenase by NO is a challenging issue for future investigations.

Acknowledgments

The authors gratefully acknowledge the group members and collaborators who contributed to these studies over many years. Work in the laboratory of MJD is supported by FEDER-co-financed grants AGL2011-23383 and AGL2015-63651-P to SM, and AGL2013-45087-R and AGL2017-85676-R to MJD (MINECO, Spain). Grant P12-AGR-1968 to EJB, P18-RT-1401 to MJD and SM, and support from the Junta de Andalucía to Group BIO-275 is also acknowledged. Work in DJR and AJG laboratory was supported by grants BB/M00256X/1 and BB/S008942/1 (BBSRC, UK) to AJG. AS acknowledged the FPU Program (MINECO, Spain; grant FPU14/04527) for the

financial support. JJC acknowledged to Alfonso Martín Escudero Foundation the support by their abroad postdoc grant program.

6. References

Al-Attar, S., & de Vries, S. (2015). An electrogenic nitric oxide reductase. *FEBS Letters, 589*, 2050-2057.

Andrews, S. C., Shipley, D., Keen, J. N., Findlay, J. B., Harrison, P. M., & Guest, J. R. (1992). The haemoglobin-like protein (HMP) of *Escherichia coli* has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin $NADP^+$ reductases. *FEBS Letters, 302*, 247-252.

Anjum, M. F., Stevanin, T. M., Read, R. C., & Moir, J. W. (2002). Nitric oxide metabolism in *Neisseria meningitidis*. *Journal of Bacteriology, 184*, 2987-2993.

Arcovito, A., Benfatto, M., Cianci, M., Hasnain, S. S., Nienhaus, K., Nienhaus, G. U., et al. (2007). X-ray structure analysis of a metalloprotein with enhanced active-site resolution using *in situ* x-ray absorption near edge structure spectroscopy. *Proceedings of the National Academy of Sciences of the United States of America, 104*, 6211-6216.

Arya, S., Sethi, D., Singh, S., Hade, M. D., Singh, V., Raju, P., et al. (2013). Truncated hemoglobin, HbN, is post-translationally modified in *Mycobacterium tuberculosis* and modulates host-pathogen interactions during intracellular infection. *Journal of Biological Chemistry, 288*, 29987-29999.

Astier, J., Gross, I., & Durner, J. (2018). Nitric oxide production in plants: an update. *Journal of Experimental Botany, 69*, 3401-3411.

Bali, S., Palmer, D. J., Schroeder, S., Ferguson, S. J., & Warren, M. J. (2014). Recent advances in the biosynthesis of modified tetrapyrroles: the discovery of an alternative pathway for the formation of heme and heme d_1. *Cellular and Molecular Life Sciences, 71*, 2837-2863.

Bang, I. S., Liu, L., Vázquez-Torres, A., Crouch, M. L., Stamler, J. S., & Fang, F. C. (2006). Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin Hmp. *Journal of Biological Chemistry, 281*, 28039-28047.

Barnett, M. J., Fisher, R. F., Jones, T., Komp, C., Abola, A. P., Barloy-Hubler, F., et al. (2001). Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* pSymA megaplasmid. *Proceedings of the National Academy of Sciences of the United States of America, 98*, 9883-9888.

Barrios, H., Fischer, H. M., Hennecke, H., & Morett, E. (1995). Overlapping promoters for two different RNA polymerase holoenzymes control *Bradyrhizobium japonicum nifA* expression. *Journal of Bacteriology, 177*, 1760-1765.

Bartberger, M. D., Liu, W., Ford, E., Miranda, K. M., Switzer, C., Fukuto, J. M., et al. (2002). The reduction potential of nitric oxide (NO) and its importance to NO biochemistry. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 10958-10963.

Bartesaghi, S., & Radi, R. (2018). Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration. *Redox Biology, 14*, 618-625.

Bartnikas, T. B., Wang, Y., Bobo, T., Veselov, A., Scholes, C. P., & Shapleigh, J. P. (2002). Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a haem-copper protein. *Microbiology, 148*, 825-833.

Baudouin, E., Pieuchot, L., Engler, G., Pauly, N., & Puppo, A. (2006). Nitric oxide is formed in *Medicago truncatula-Sinorhizobium meliloti* functional nodules. *Molecular Plant-Microbe Interactions, 19*, 970-975.

Bauer, E., Kaspar, T., Fischer, H. M., & Hennecke, H. (1998). Expression of the *fixR-nifA* operon in *Bradyrhizobium japonicum* depends on a new response regulator, RegR. *Journal of Bacteriology, 180*, 3853-3863.

Becker, A., Berges, H., Krol, E., Bruand, C., Ruberg, S., Capela, D., et al. (2004). Global changes in gene expression in *Sinorhizobium meliloti* 1021 under microoxic and symbiotic conditions. *Molecular Plant-Microbe Interactions, 17*, 292-303.

Bedmar, E. J., Bueno, E., Correa, D., Torres, M. J., Delgado, M. J., & Mesa, S. (2013). Ecology of denitrification in soils and plant-associated bacteria. In B. Rodelas, & J. González-López (Eds.), *Beneficial plant-microbial interactions: Ecology and applications* (pp. 164-182). Boca Raton, FL: CRC Press.

Bedmar, E. J., Robles, E. F., & Delgado, M. J. (2005). The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. *Biochemical Society Transactions, 33*, 141-144.

Bennett, S. P., Soriano-Laguna, M. J., Bradley, J. M., Svistunenko, D. A., Richardson, D. J., Gates, A. J., et al. (2019). NosL is a dedicated copper chaperone for assembly of the Cu_Z center of nitrous oxide reductase. *Chemical Science, 10*, 4985-4993.

Bergaust, L. L., Hartsock, A., Liu, B., Bakken, L. R., & Shapleigh, J. P. (2014). Role of *norEF* in denitrification, elucidated by physiological experiments with *Rhodobacter sphaeroides*. *Journal of Bacteriology, 196*, 2190-2200.

Berger, A., Boscari, A., Frendo, P., & Brouquisse, R. (2019). Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis. *Journal of Experimental Botany, 70*, 4505-4520.

Berger, A., Brouquisse, R., Pathak, P. K., Hichri, I., Singh, I., Bhatia, S., et al. (2018). Pathways of nitric oxide metabolism and operation of phytoglobins in legume nodules: missing links and future directions. *Plant, Cell and Environment, 41*, 2057-2068.

Blanquet, P., Silva, L., Catrice, O., Bruand, C., Carvalho, H., & Meilhoc, E. (2015). *Sinorhizobium meliloti* controls nitric oxide-mediated post-translational modification of a *Medicago truncatula* nodule protein. *Molecular Plant-Microbe Interactions, 28*, 1353-1363.

Bobik, C., Meilhoc, E., & Batut, J. (2006). FixJ: a major regulator of the oxygen limitation response and late symbiotic functions of *Sinorhizobium meliloti*. *Journal of Bacteriology, 188*, 4890-4902.

Boccara, M., Mills, C. E., Zeier, J., Anzi, C., Lamb, C., Poole, R. K., et al. (2005). Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host. *The Plant Journal, 43*, 226-237.

Bonamore, A., & Boffi, A. (2008). Flavohemoglobin: structure and reactivity. *IUBMB Life, 60*, 19-28.

Bonnet, M., Stegmann, M., Maglica, Ž., Stiegeler, E., Weber-Ban, E., Hennecke, H., et al. (2013). $FixK_2$, a key regulator in *Bradyrhizobium japonicum*, is a substrate for the protease ClpAP *in vitro*. *FEBS Letters, 587*, 88-93.

Borrero-de Acuña, J. M., Timmis, K. N., Jahn, M., & Jahn, D. (2017). Protein complex formation during denitrification by *Pseudomonas aeruginosa*. *Microbial Biotechnology, 10*, 1523-1534.

Brandes, N., Rinck, A., Leichert, L. I., & Jakob, U. (2007). Nitrosative stress treatment of *E. coli* targets distinct set of thiol-containing proteins. *Molecular Microbiology, 66*, 901-914.

Bricio, C., Álvarez, L., San Martin, M., Schurig-Briccio, L. A., Gennis, R. B., & Berenguer, J. (2014). A third subunit in ancestral cytochrome *c*-dependent nitric oxide reductases. *Applied and Environmental Microbiology, 80*, 4871-4878.

Buddha, M. R., Tao, T., Parry, R. J., & Crane, B. R. (2004). Regioselective nitration of tryptophan by a complex between bacterial nitric-oxide synthase and tryptophanyl-tRNA synthetase. *Journal of Biological Chemistry, 279*, 49567-49570.

Bueno, E., Bedmar, E. J., Richardson, D. J., & Delgado, M. J. (2008). Role of *Bradyrhizobium japonicum* cytochrome c_{550} in nitrite and nitrate respiration. *FEMS Microbiology Letters, 279*, 188-194.

Bueno, E., Gómez-Hernández, N., Girard, L., Bedmar, E. J., & Delgado, M. J. (2005). Function of the *Rhizobium etli* CFN42 *nirK* gene in nitrite metabolism. *Biochemical Society Transactions, 33*, 162-163.

Bueno, E., Mania, D., Frostegård, Å., Bedmar, E. J., Bakken, L. R., & Delgado, M. J. (2015). Anoxic growth of *Ensifer meliloti* 1021 by N_2O-reduction, a potential mitigation strategy. *Frontiers in Microbiology, 6*, 537.

Bueno, E., Mesa, S., Sánchez, C., Bedmar, E. J., & Delgado, M. J. (2010). NifA is required for maximal expression of denitrification genes in *Bradyrhizobium japonicum*. *Environmental Microbiology, 12*, 393-400.

Bueno, E., Robles, E. F., Torres, M. J., Krell, T., Bedmar, E. J., Delgado, M. J., et al. (2017). Disparate response to microoxia and nitrogen oxides of the *Bradyrhizobium japonicum napEDABC*, *nirK* and *norCBQD* denitrification genes. *Nitric Oxide, 68*, 137-149.

Burney, S., Niles, J. C., Dedon, P. C., & Tannenbaum, S. R. (1999). DNA damage in deoxynucleosides and oligonucleotides treated with peroxynitrite. *Chemical Research in Toxicology, 12*, 513-520.

Butland, G., Spiro, S., Watmough, N. J., & Richardson, D. J. (2001). Two conserved glutamates in the bacterial nitric oxide reductase are essential for activity but not assembly of the enzyme. *Journal of Bacteriology, 183*, 189-199.

Cabrera, J. J., Salas, A., Torres, M. J., Bedmar, E. J., Richardson, D. J., Gates, A. J., et al. (2016). An integrated biochemical system for nitrate assimilation and nitric oxide detoxification in *Bradyrhizobium japonicum*. *Biochemical Journal, 473*, 297-309.

Cabrera, J. J., Sánchez, C., Gates, A. J., Bedmar, E. J., Mesa, S., Richardson, D. J., et al. (2011). The nitric oxide response in plant-associated endosymbiotic bacteria. *Biochemical Society Transactions, 39*, 1880-1885.

Cam, Y., Pierre, O., Boncompagni, E., Herouart, D., Meilhoc, E., & Bruand, C. (2012). Nitric oxide (NO): a key player in the senescence of *Medicago truncatula* root nodules. *New Phytologist, 196*, 548-560.

Caranto, J. D., & Lancaster, K. M. (2017). Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. *Proceedings of the National Academy of Sciences of the United States of America, 114*, 8217-8222.

Carreira, C., Pauleta, S. R., & Moura, I. (2017). The catalytic cycle of nitrous oxide reductase - The enzyme that catalyzes the last step of denitrification. *Journal of Inorganic Biochemistry, 177*, 423-434.

Cébron, A., & Garnier, J. (2005). *Nitrobacter* and *Nitrospira* genera as representatives of nitrite-oxidizing bacteria: detection, quantification and growth along the lower Seine River (France). *Water Research, 39*, 4979-4992.

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galván, A., & Fernández, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trends in Plant Science, 22*, 163-174.

Cole, J. (1996). Nitrate reduction to ammonia by enteric bacteria: redundancy, or a strategy for survival during oxygen starvation? *FEMS Microbiology Letters, 136*, 1-11.

Crane, B. R., Sudhamsu, J., & Patel, B. A. (2010). Bacterial nitric oxide synthases. *Annual Review of Biochemistry, 79*, 445-470.

Crow, A., Matsuda, Y., Arata, H., & Oubrie, A. (2016). Structure of the membrane-intrinsic nitric oxide reductase from *Roseobacter denitrificans*. *Biochemistry, 55*, 3198-3203.

Dakora, F. D. (1995). A functional relationship between leghaemoglobin and nitrogenase based on novel measurements of the two proteins in legume root nodules. *Annals of Botany, 75*, 49-54.

Daskalakis, V., Ohta, T., Kitagawa, T., & Varotsis, C. (2015). Structure and properties of the catalytic site of nitric oxide reductase at ambient temperature. *Biochimica et Biophysica Acta, 1847*, 1240-1244.

Davies, M. J., Mathieu, C., & Puppo, A. (1999). Leghemoglobin: properties and reactions. *Advances in Inorganic Chemistry, 46*, 495-542.

de Boer, A. P., van der Oost, J., Reijnders, W. N., Westerhoff, H. V., Stouthamer, A. H., & van Spanning, R. J. (1996). Mutational analysis of the *nor* gene cluster which encodes nitric-oxide reductase from *Paracoccus denitrificans*. *European Journal of Biochemistry, 242*, 592-600.

de Lajudie, P. M., Andrews, M., Ardley, J., Eardly, B., Jumas-Bilak, E., Kuzmanovic, N., et al. (2019). Minimal standards for the description of new genera and species of rhizobia and agrobacteria. *International Journal of Systematic and Evolutionary Microbiology, 69*, 1852-1863.

de Vries, S., Suharti, S., & Pouvreau, L. A. M. (2007). Nitric oxide reductase: Structural variations and catalytic mechanism. In H. Bothe, S. J. Ferguson, & W. E. Newton (Eds.), *Biology of the nitrogen cycle* (pp. 57-67). Amsterdam: Elsevier.

del Giudice, J., Cam, Y., Damiani, I., Fung-Chat, F., Meilhoc, E., Bruand, C., et al. (2011). Nitric oxide is required for an optimal establishment of the *Medicago truncatula-Sinorhizobium meliloti* symbiosis. *New Phytologist, 191*, 405-417.

Delamuta, J. R., Ribeiro, R. A., Ormeno-Orrillo, E., Melo, I. S., Martínez-Romero, E., & Hungria, M. (2013). Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 63*, 3342-3351.

Delgado, M. J., Bedmar, E. J., & Downie, J. A. (1998). Genes involved in the formation and assembly of rhizobial cytochromes and their role in symbiotic nitrogen fixation. *Advances in Microbial Physiology, 40*, 191-231.

Delgado, M. J., Bonnard, N., Tresierra-Ayala, A., Bedmar, E. J., & Muller, P. (2003). The *Bradyrhizobium japonicum napEDABC* genes encoding the periplasmic

nitrate reductase are essential for nitrate respiration. *Microbiology, 149*, 3395-3403.

Dixon, R., & Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nature Reviews Microbiology, 2*, 621-631.

Downie, J. A. (2005). Legume haemoglobins: symbiotic nitrogen fixation needs bloody nodules. *Current Biology, 15*, 196-198.

Eady, R. R., Antonyuk, S. V., & Hasnain, S. S. (2016). Fresh insight to functioning of selected enzymes of the nitrogen cycle. *Current Opinion in Chemical Biology*, *31*, 103-112.

Ellington, M. J., Bhakoo, K. K., Sawers, G., Richardson, D. J., & Ferguson, S. J. (2002). Hierarchy of carbon source selection in *Paracoccus pantotrophus*: strict correlation between reduction state of the carbon substrate and aerobic expression of the *nap* operon. *Journal of Bacteriology, 184*, 4767-4774.

Elvers, K. T., Wu, G., Gilberthorpe, N. J., Poole, R. K., & Park, S. F. (2004). Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in *Campylobacter jejuni* and *Campylobacter coli*. *Journal of Bacteriology, 186*, 5332-5341.

Ermler, U., Siddiqui, R. A., Cramm, R., & Friedrich, B. (1995). Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 Å resolution. *The EMBO Journal, 14*, 6067-6077.

Fernández, N., Cabrera, J. J., Salazar, S., Parejo, S., Rodríguez, M. C., Lindemann, A., et al. (2016). Molecular determinants of negative regulation of the *Bradyrhizobium diazoefficiens* transcription factor $FixK_2$. In F. González-Andrés, & E. James (Eds.), *Biological Nitrogen Fixation and Beneficial Plant-Microbe Interactions* (pp. 57-72). Cham, Switzerland: Springer International Publishing.

Fernández, N., Cabrera, J. J., Varadarajan, A. R., Lutz, S., Ledermann, R., Roschitzki, B., et al. (2019). An integrated systems approach unveils new aspects of microoxia-mediated regulation in *Bradyrhizobium diazoefficiens*. *Frontiers in Microbiology, 10*, 924.

Fischer, H. M. (1994). Genetic regulation of nitrogen fixation in rhizobia. *Microbiological Reviews, 58*, 352-386.

Franck, W. L., Qiu, J., Lee, H. I., Chang, W. S., & Stacey, G. (2015). DNA microarray-based identification of genes regulated by NtrC in *Bradyrhizobium japonicum*. *Applied and Environmental Microbiology, 81*, 5299-5308.

Freitas, T. A., Hou, S., Dioum, E. M., Saito, J. A., Newhouse, J., González, G., et al. (2004). Ancestral hemoglobins in archaea. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 6675-6680.

Fukudome, M., Watanabe, E., Osuki, K. I., Imaizumi, R., Aoki, T., Becana, M., et al. (2019). Stably transformed *Lotus japonicus* plants overexpressing phytoglobin LjGlb1-1 show decreased nitric oxide levels in roots and nodules as well as delayed nodule senescence. *Plant & Cell Physiology, 60*, 816-825.

Galibert, F., Finan, T. M., Long, S. R., Puhler, A., Abola, P., Ampe, F., et al. (2001). The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science, 293*, 668-672.

Gardner, P. R. (2005). Nitric oxide dioxygenase function and mechanism of flavohemoglobin, hemoglobin, myoglobin and their associated reductases. *Journal of Inorganic Biochemistry, 99*, 247-266.

Gardner, P. R. (2012). Hemoglobin: a nitric-oxide dioxygenase. *Scientifica, 2012*, 683729.

Gardner, P. R., Gardner, A. M., Martin, L. A., & Salzman, A. L. (1998). Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proceedings of the National Academy of Sciences of the United States of America, 95*, 10378-10383.

Gates, A. J., Luque-Almagro, V. M., Goddard, A. D., Ferguson, S. J., Roldán, M. D., & Richardson, D. J. (2011). A composite biochemical system for bacterial nitrate and nitrite assimilation as exemplified by *Paracoccus denitrificans*. *Biochemical Journal, 435*, 743-753.

Gell, D. A. (2018). Structure and function of haemoglobins. *Blood Cells, Molecules and Diseases, 70*, 13-42.

Gibson, Q. H., & Roughton, F. J. (1957). The kinetics and equilibria of the reactions of nitric oxide with sheep haemoglobin. *The Journal of Physiology, 136*, 507-524.

Gibson, Q. H., & Roughton, F. J. (1965). Further studies on the kinetics and equilibria of the reaction of nitric oxide with haemoproteins. *Proceedings of the Royal Society of London. Series B, Biological Sciences, 163*, 197-205.

Gilberthorpe, N. J., & Poole, R. K. (2008). Nitric oxide homeostasis in *Salmonella typhimurium*: roles of respiratory nitrate reductase and flavohemoglobin. *Journal of Biological Chemistry, 283*, 11146-11154.

Gilles-González, M. A., González, G., Sousa, E. H., & Tuckerman, J. (2008). Oxygen-sensing histidine-protein kinases: assays of ligand binding and turnover of response-regulator substrates. *Methods in Enzymology, 437*, 173-189.

Girard, L., Brom, S., Dávalos, A., López, O., Soberón, M., & Romero, D. (2000). Differential regulation of *fixN*-reiterated genes in *Rhizobium etli* by a novel *fixL-fixK* cascade. *Molecular Plant-Microbe Interactions, 13*, 1283-1292.

Goddard, A. D., Bali, S., Mavridou, D. A., Luque-Almagro, V. M., Gates, A. J., Roldán, M. D., et al. (2017). The *Paracoccus denitrificans* NarK-like nitrate and nitrite transporters-probing nitrate uptake and nitrate/nitrite exchange mechanisms. *Molecular Microbiology, 103*, 117-133.

Goddard, A. D., Moir, J. W., Richardson, D. J., & Ferguson, S. J. (2008). Interdependence of two NarK domains in a fused nitrate/nitrite transporter. *Molecular Microbiology, 70*, 667-681.

Gómez-Hernández, N., Reyes-González, A., Sánchez, C., Mora, Y., Delgado, M. J., & Girard, L. (2011). Regulation and symbiotic role of *nirK* and *norC* expression in *Rhizobium etli*. *Molecular Plant-Microbe Interactions, 24*, 233-245.

González, V., Santamaría, R. I., Bustos, P., Hernández-González, I., Medrano-Soto, A., Moreno-Hagelsieb, G., Chandra Janga, S., Ramírez, M. A., Jiménez-Jacinto, V., Collado-Vides, J., and Dávila, G. 2006. The partitioned *Rhizobium etli* genome: Genetic and metabolic redundancy in seven interacting replicons. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 3834-3839

González, P. J., Rivas, M., & Moura, J. J. (2017). Structure, function and mechanisms of respiratory nitrate reductases. In I. Moura, J. J. Moura, S. R. Pauleta, & L. B. Maia (Eds.), *Metalloenzymes in denitrification: Applications and environmental impacts* (pp. 39-58). Cambridge: The Royal Society of Chemistry.

Göttfert, M., Grob, P., & Hennecke, H. (1990). Proposed regulatory pathway encoded by the *nodV* and *nodW* genes, determinants of host specificity in *Bradyrhizobium japonicum*. *Proceedings of the National Academy of Sciences of the United States of America, 87*, 2680-2684.

Granados-Baeza, M. J., Gómez-Hernández, N., Mora, Y., Delgado, M. J., Romero, D., & Girard, L. (2007). Novel reiterated Fnr-type proteins control the production of

the symbiotic terminal oxidase *cbb*$_3$ in *Rhizobium etli* CFN42. *Molecular Plant-Microbe Interactions, 20*, 1241-1249.

Gusarov, I., Shatalin, K., Starodubtseva, M., & Nudler, E. (2009). Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. *Science, 325*, 1380-1384.

Hallin, S., Philippot, L., Löffler, F. E., Sanford, R. A., & Jones, C. M. (2018). Genomics and ecology of novel N_2O-reducing microorganisms. *Trends in Microbiology, 26*, 43-55.

Hartsock, A., & Shapleigh, J. P. (2010). Identification, functional studies, and genomic comparisons of new members of the NnrR regulon in *Rhodobacter sphaeroides*. *Journal of Bacteriology, 192*, 903-911.

Hausladen, A., Gow, A. J., & Stamler, J. S. (1998). Nitrosative stress: metabolic pathway involving the flavohemoglobin. *Proceedings of the National Academy of Sciences of the United States of America, 95*, 14100-14105.

Hein, S., & Simon, J. (2019). Bacterial nitrous oxide respiration: electron transport chains and copper transfer reactions. *Advances in Microbial Physiology, 75*, 137-175.

Hein, S., Witt, S., & Simon, J. (2017). Clade II nitrous oxide respiration of *Wolinella succinogenes* depends on the NosG, -C1, -C2, -H electron transport module, NosB and a Rieske/cytochrome *bc* complex. *Environmental Microbiology, 19*, 4913-4925.

Hemschemeier, A., Duner, M., Casero, D., Merchant, S. S., Winkler, M., & Happe, T. (2013). Hypoxic survival requires a 2-on-2 hemoglobin in a process involving nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 10854-10859.

Hendriks, J., Oubrie, A., Castresana, J., Urbani, A., Gemeinhardt, S., & Saraste, M. (2000). Nitric oxide reductases in bacteria. *Biochimica et Biophysica Acta, 1459*, 266-273.

Hichri, I., Boscari, A., Castella, C., Rovere, M., Puppo, A., & Brouquisse, R. (2015). Nitric oxide: a multifaceted regulator of the nitrogen-fixing symbiosis. *Journal of Experimental Botany, 66*, 2877-2887.

Hichri, I., Meilhoc, E., Boscari, A., Bruand, C., Frendo, P., & Brouquisse, R. (2016). Nitric oxide: Jack- of- all- trades of the nitrogen- fixing symbiosis? In D. Wendehenne (Ed.), *Advances in Botanical Research* (pp. 193-218). London & New York: Academic Press.

Hidalgo-García, A., Torres, M. J., Salas, A., Bedmar, E. J., Girard, L., & Delgado, M. J. (2019). *Rhizobium etli* produces nitrous oxide by coupling the assimilatory and denitrification pathways. *Frontiers in Microbiology, 10*, 980.

Hino, T., Matsumoto, Y., Nagano, S., Sugimoto, H., Fukumori, Y., Murata, T., et al. (2010). Structural basis of biological N_2O generation by bacterial nitric oxide reductase. *Science, 330*, 1666-1670.

Honisch, U., & Zumft, W. G. (2003). Operon structure and regulation of the *nos* gene region of *Pseudomonas stutzeri*, encoding an ABC-Type ATPase for maturation of nitrous oxide reductase. *Journal of Bacteriology, 185*, 1895-1902.

Horchani, F., Prevot, M., Boscari, A., Evangelisti, E., Meilhoc, E., Bruand, C., et al. (2011). Both plant and bacterial nitrate reductases contribute to nitric oxide production in *Medicago truncatula* nitrogen-fixing nodules. *Plant Physiology, 155*, 1023-1036.

Horrell, S., Kekilli, D., Strange, R. W., & Hough, M. A. (2017). Recent structural insights into the function of copper nitrite reductases. *Metallomics, 9*, 1470-1482.

Hu, Z., Wessels, H., van Alen, T., Jetten, M. S. M., & Kartal, B. (2019). Nitric oxide-dependent anaerobic ammonium oxidation. *Nature Communications, 10*, 1244.

Hughes, M. N. (2008). Chemistry of nitric oxide and related species. *Methods in Enzymology, 436*, 3-19.

Hutfless, E. H., Chaudhari, S. S., & Thomas, V. C. (2018). Emerging roles of nitric oxide synthase in bacterial physiology. *Advances in Microbial Physiology, 72*, 147-191.

Ilari, A., Bonamore, A., Farina, A., Johnson, K. A., & Boffi, A. (2002). The X-ray structure of ferric *Escherichia coli* flavohemoglobin reveals an unexpected geometry of the distal heme pocket. *Journal of Biological Chemistry, 277*, 23725-23732.

Jang, J., Ashida, N., Kai, A., Isobe, K., Nishizawa, T., Otsuka, S., et al. (2018). Presence of Cu-type (NirK) and cd_1-type (NirS) nitrite reductase genes in the denitrifying bacterium *Bradyrhizobium nitroreducens* sp. nov. *Microbes and Environments, 33*, 326-331.

Jepson, B. J., Marietou, A., Mohan, S., Cole, J. A., Butler, C. S., & Richardson, D. J. (2006). Evolution of the soluble nitrate reductase: defining the monomeric periplasmic nitrate reductase subgroup. *Biochemical Society Transactions, 34*, 122-126.

Jepson, B. J., Mohan, S., Clarke, T. A., Gates, A. J., Cole, J. A., Butler, C. S., et al. (2007). Spectropotentiometric and structural analysis of the periplasmic nitrate reductase from *Escherichia coli*. *Journal of Biological Chemistry, 282*, 6425-6437.

Jiménez-Leiva, A., Cabrera, J. J., Bueno, E., Torres, M. J., Salazar, S., Bedmar, E. J., et al. (2019). Expanding the regulon of the *Bradyrhizobium diazoefficiens* NnrR transcription factor: new insights into the denitrification pathway. *Frontiers in Microbiology, 10*, 1926.

Johnson, E. A., Rice, S. L., Preimesberger, M. R., Nye, D. B., Gilevicius, L., Wenke, B. B., et al. (2014). Characterization of THB1, a *Chlamydomonas reinhardtii* truncated hemoglobin: linkage to nitrogen metabolism and identification of lysine as the distal heme ligand. *Biochemistry, 53*, 4573-4589.

Johnson, E. A., Russo, M. M., Nye, D. B., Schlessman, J. L., & Lecomte, J. T. J. (2018). Lysine as a heme iron ligand: A property common to three truncated hemoglobins from *Chlamydomonas reinhardtii*. *Biochimica et Biophysica Acta - General Subjects, 1862*, 2660-2673.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., & Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology, 5*, 619-633.

Kahle, M., Ter Beek, J., Hosler, J. P., & Ädelroth, P. (2018). The insertion of the non-heme Fe_B cofactor into nitric oxide reductase from *P. denitrificans* depends on NorQ and NorD accessory proteins. *Biochimica et Biophysica Acta - Bioenergetics, 1859*, 1051-1058.

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., et al. (2002). Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Research, 9*, 189-197.

Kapp, O. H., Moens, L., Vanfleteren, J., Trotman, C. N., Suzuki, T., & Vinogradov, S. N. (1995). Alignment of 700 globin sequences: extent of amino acid substitution and its correlation with variation in volume. *Protein Science, 4*, 2179-2190.

Kartal, B., & Keltjens, J. T. (2016). Anammox biochemistry: a tale of heme *c* proteins. *Trends in Biochemical Sciences, 41*, 998-1011.

Kastrau, D. H., Heiss, B., Kroneck, P. M., & Zumft, W. G. (1994). Nitric oxide reductase from *Pseudomonas stutzeri*, a novel cytochrome *bc* complex. Phospholipid requirement, electron paramagnetic resonance and redox properties. *European Journal of Biochemistry, 222*, 293-303.

Kato, K., Kanahama, K., & Kanayama, Y. (2010). Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in *Lotus* root nodules. *Journal of Plant Physiology, 167*, 238-241.

Kaur, R., Pathania, R., Sharma, V., Mande, S. C., & Dikshit, K. L. (2002). Chimeric *Vitreoscilla* hemoglobin (VHb) carrying a flavoreductase domain relieves nitrosative stress in *Escherichia coli*: new insight into the functional role of VHb. *Applied and Environmental Microbiology, 68*, 152-160.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., et al. (1960). Structure of myoglobin: a three-dimensional Fourier synthesis at 2 Å. resolution. *Nature, 185*, 422-427.

Kim, S. O., Orii, Y., Lloyd, D., Hughes, M. N., & Poole, R. K. (1999). Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. *FEBS Letters, 445*, 389-394.

Körner, H., Sofia, H. J., & Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiology Reviews, 27*, 559-592.

Kraft, B., Strous, M., & Tegetmeyer, H. E. (2011). Microbial nitrate respiration – Genes, enzymes and environmental distribution. *Journal of Biotechnology, 155*, 104-117.

Kuenen, J. G. (2020). Anammox and beyond. *Environmental Microbiology*, *22*, 525-536.

Kullik, I., Fritsche, S., Knobel, H., Sanjuán, J., Hennecke, H., & Fischer, H. M. (1991). *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the σ^{54} gene (*rpoN*). *Journal of Bacteriology, 173*, 1125-1138.

Lewinska, A., & Bartosz, G. (2006). Yeast flavohemoglobin protects against nitrosative stress and controls ferric reductase activity. *Redox Report, 11*, 231-239.

Lin, J. T., & Stewart, V. (1998). Nitrate assimilation by bacteria. *Advances in Microbial Physiology, 39*, 1-30.

Liu, L., Zeng, M., Hausladen, A., Heitman, J., & Stamler, J. S. (2000). Protection from nitrosative stress by yeast flavohemoglobin. *Proceedings of the National Academy of Sciences of the United States of America, 97*, 4672-4676.

López, M. F., Cabrera, J. J., Salas, A., Delgado, M. J., & López-García, S. L. (2017). Dissecting the role of NtrC and RpoN in the expression of assimilatory nitrate and nitrite reductases in *Bradyrhizobium diazoefficiens*. *Antonie Van Leeuwenhoek, 110*, 531-542.

Lu, C., Mukai, M., Lin, Y., Wu, G., Poole, R. K., & Yeh, S. R. (2007). Structural and functional properties of a single domain hemoglobin from the food-borne pathogen *Campylobactor jejuni*. *Journal of Biological Chemistry, 282*, 25917-25928.

Luque-Almagro, V. M., Lyall, V. J., Ferguson, S. J., Roldán, M. D., Richardson, D. J., & Gates, A. J. (2013). Nitrogen oxyanion-dependent dissociation of a two-component complex that regulates bacterial nitrate assimilation. *Journal of Biological Chemistry, 288*, 29692-29702.

Mahinthichaichan, P., Gennis, R. B., & Tajkhorshid, E. (2018). Bacterial denitrifying nitric oxide reductases and aerobic respiratory terminal oxidases use similar delivery pathways for their molecular substrates. *Biochimica et Biophysica Acta - Bioenergetics, 1859*, 712-724.

Maia, L. B., Moura, I., & Moura, J. J. (2017). Molybdenum and tungsten-containing enzymes: an overview. In R. Hille, C. Schulzke, & M. L. Kirk (Eds.), *Molybdenum and tungsten enzymes: Biochemistry* (pp. 1-80). Cambridge: The Royal Society of Chemistry.

Maia, L. B., & Moura, J. J. (2015). Nitrite reduction by molybdoenzymes: a new class of nitric oxide-forming nitrite reductases. *Journal of Biological Inorganic Chemistry, 20*, 403-433.

Marietou, A., Richardson, D., Cole, J., & Mohan, S. (2005). Nitrate reduction by *Desulfovibrio desulfuricans*: a periplasmic nitrate reductase system that lacks NapB, but includes a unique tetraheme *c*-type cytochrome, NapM. *FEMS Microbiology Letters, 248*, 217-225.

Martínez-Espinosa, R. M., Richardson, D. J., Butt, J. N., & Bonete, M. J. (2006). Respiratory nitrate and nitrite pathway in the denitrifier haloarchaeon *Haloferax mediterranei*. *Biochemical Society Transactions, 34*, 115-117.

Matsumoto, Y., Tosha, T., Pisliakov, A. V., Hino, T., Sugimoto, H., Nagano, S., et al. (2012). Crystal structure of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*. *Nature Structural & Molecular Biology, 19*, 238-245.

McLean, S., Bowman, L. A., & Poole, R. K. (2010). Peroxynitrite stress is exacerbated by flavohaemoglobin-derived oxidative stress in *Salmonella typhimurium* and is relieved by nitric oxide. *Microbiology, 156*, 3556-3565.

Meakin, G. E., Bueno, E., Jepson, B., Bedmar, E. J., Richardson, D. J., & Delgado, M. J. (2007). The contribution of bacteroidal nitrate and nitrite reduction to the formation of nitrosylleghaemoglobin complexes in soybean root nodules. *Microbiology, 153*, 411-419.

Meilhoc, E., Cam, Y., Skapski, A., & Bruand, C. (2010). The response to nitric oxide of the nitrogen-fixing symbiont *Sinorhizobium meliloti*. *Molecular Plant-Microbe Interactions, 23*, 748-759.

Melo, P. M., Silva, L. S., Ribeiro, I., Seabra, A. R., & Carvalho, H. G. (2011). Glutamine synthetase is a molecular target of nitric oxide in root nodules of *Medicago truncatula* and is regulated by tyrosine nitration. *Plant Physiology, 157*, 1505-1517.

Mesa, S., Alché , J. D., Bedmar, E., & Delgado, M. J. (2004). Expression of *nir*, *nor* and *nos* denitrification genes from *Bradyrhizobium japonicum* in soybean root nodules. *Physiologia Plantarum, 120*, 205-211.

Mesa, S., Bedmar, E. J., Chanfon, A., Hennecke, H., & Fischer, H. M. (2003). *Bradyrhizobium japonicum* NnrR, a denitrification regulator, expands the FixLJ-$FixK_2$ regulatory cascade. *Journal of Bacteriology, 185*, 3978-3982.

Mesa, S., Hauser, F., Friberg, M., Malaguti, E., Fischer, H. M., & Hennecke, H. (2008). Comprehensive assessment of the regulons controlled by the FixLJ-$FixK_2$-$FixK_1$ cascade in *Bradyrhizobium japonicum*. *Journal of Bacteriology, 190*, 6568-6579.

1749 Mesa, S., Reutimann, L., Fischer, H. M., & Hennecke, H. (2009). Posttranslational
1750 control of transcription factor $FixK_2$, a key regulator for the *Bradyrhizobium*
1751 *japonicum*-soybean symbiosis. *Proceedings of the National Academy of*
1752 *Sciences of the United States of America, 106*, 21860-21865.
1753 Mesa, S., Ucurum, Z., Hennecke, H., & Fischer, H. M. (2005). Transcription activation
1754 *in vitro* by the *Bradyrhizobium japonicum* regulatory protein $FixK_2$. *Journal of*
1755 *Bacteriology, 187*, 3329-3338.
1756 Mesa, S., Velasco, L., Manzanera, M. E., Delgado, M. J., & Bedmar, E. J. (2002).
1757 Characterization of the *norCBQD* genes, encoding nitric oxide reductase, in the
1758 nitrogen fixing bacterium *Bradyrhizobium japonicum*. *Microbiology, 148*, 3553-
1759 3560.
1760 Minchin, F. R. (1997). Regulation of oxygen diffusion in legume nodules. *Soil Biology*
1761 *and Biochemistry, 29*, 88-89.
1762 Minchin, F. R., James, E. K., & Becana, M. (2008). Oxygen diffusion, production of
1763 reactive oxygen and nitrogen species, and antioxidants in legume nodules. In M.
1764 J. Dilworth, E. K. James, J. I. Sprent, & W. E. Newton (Eds.), *Nitrogen-fixing*
1765 *leguminous symbioses* (pp. 321-362). Dordrecht: Springer.
1766 Möller, M. N., Ríos, N., Trujillo, M., Radi, R., Denicola, A., & Álvarez, B. (2019).
1767 Detection and quantification of nitric oxide-derived oxidants in biological
1768 systems. *Journal of Biological Chemistry, 294*, 14776-14802.
1769 Monk, C. E., Pearson, B. M., Mulholland, F., Smith, H. K., & Poole, R. K. (2008).
1770 Oxygen- and NssR-dependent globin expression and enhanced iron acquisition
1771 in the response of campylobacter to nitrosative stress. *Journal of Biological*
1772 *Chemistry, 283*, 28413-28425.
1773 Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R., & Castillo, F. (1999).
1774 Prokaryotic nitrate reduction: molecular properties and functional distinction
1775 among bacterial nitrate reductases. *Journal of Bacteriology, 181*, 6573-6584.
1776 Moreno-Vivián, C., Luque-Almagro, V. M., Cabello, P., Roldán, M. D., & Castillo, F.
1777 (2011). Transport and assimilation of inorganic nitrogen in bacteria. In J. W. B.
1778 Moir (Ed.), *Nitrogen Cycling in Bacteria* (pp. 101-122). Norfolk: Caister
1779 Academic Press.
1780 Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., & Storz, G. (2004).
1781 Prominent roles of the NorR and Fur regulators in the *Escherichia coli*
1782 transcriptional response to reactive nitrogen species. *Proceedings of the*
1783 *National Academy of Sciences of the United States of America, 101*, 745-750.
1784 Nakano, M. M. (2002). Induction of ResDE-dependent gene expression in *Bacillus*
1785 *subtilis* in response to nitric oxide and nitrosative stress. *Journal of*
1786 *Bacteriology, 184*, 1783-1787.
1787 Nakano, M. M. (2006). Essential role of flavohemoglobin in long-term anaerobic
1788 survival of *Bacillus subtilis*. *Journal of Bacteriology, 188*, 6415-6418.
1789 Nardini, M., Pesce, A., Labarre, M., Richard, C., Bolli, A., Ascenzi, P., et al. (2006).
1790 Structural determinants in the group III truncated hemoglobin from
1791 *Campylobacter jejuni*. *Journal of Biological Chemistry, 281*, 37803-37812.
1792 Nellen-Anthamatten, D., Rossi, P., Preisig, O., Kullik, I., Babst, M., Fischer, H. M., et
1793 al. (1998). *Bradyrhizobium japonicum* $FixK_2$, a crucial distributor in the FixLJ-
1794 dependent regulatory cascade for control of genes inducible by low oxygen
1795 levels. *Journal of Bacteriology, 180*, 5251-5255.
1796 Nojiri, M. (2017). Structure and function of copper nitrite reductase. In I. Moura, J. J.
1797 Moura, S. R. Pauleta, & L. B. Maia (Eds.), *Metalloenzymes in denitrification:*

Applications and environmental impacts (pp. 91-113). Cambridge: The Royal Society of Chemistry.

Opperman, D. J., Murgida, D. H., Dalosto, S. D., Brondino, C. D., & Ferroni, F. M. (2019). A three-domain copper-nitrite reductase with a unique sensing loop. *International Union of Crystallography Journal, 6*, 248-258.

Ott, T., van Dongen, J. T., Günther, C., Krusell, L., Desbrosses, G., Vigeolas, H., et al. (2005). Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology, 15*, 531-535.

Ouellet, H., Ouellet, Y., Richard, C., Labarre, M., Wittenberg, B., Wittenberg, J., et al. (2002). Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 5902-5907.

Parrilli, E., Giuliani, M., Giordano, D., Russo, R., Marino, G., Verde, C., et al. (2010). The role of a 2-on-2 haemoglobin in oxidative and nitrosative stress resistance of Antarctic *Pseudoalteromonas haloplanktis* TAC125. *Biochimie, 92*, 1003-1009.

Patel, B. A., Moreau, M., Widom, J., Chen, H., Yin, L., Hua, Y., et al. (2009). Endogenous nitric oxide regulates the recovery of the radiation-resistant bacterium *Deinococcus radiodurans* from exposure to UV light. *Proceedings of the National Academy of Sciences of the United States of America, 106*, 18183-18188.

Pathania, R., Navani, N. K., Gardner, A. M., Gardner, P. R., & Dikshit, K. L. (2002). Nitric oxide scavenging and detoxification by the *Mycobacterium tuberculosis* haemoglobin, HbN in *Escherichia coli*. *Molecular Microbiology, 45*, 1303-1314.

Pauleta, S. R., Carepo, M. S. P., & Moura, I. (2019). Source and reduction of nitrous oxide. *Coordination Chemistry Reviews, 387*, 436-449.

Pauleta, S. R., Carreira, C., & Moura, I. (2017). Insights into nitrous oxide reductase. In I. Moura, J. J. Moura, S. R. Pauleta, & L. B. Maia (Eds.), *Metalloenzymes in denitrification: Applications and environmental impacts* (pp. 141-169). Cambridge: The Royal Society of Chemistry.

Perutz, M. F., Rossmann, M. G., Cullis, A. F., Muirhead, H., Will, G., & North, A. C. (1960). Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5-Å. resolution, obtained by X-ray analysis. *Nature, 185*, 416-422.

Phillips, S. E. (1980). Structure and refinement of oxymyoglobin at 1.6 Å resolution. *Journal of Molecular Biology, 142*, 531-554.

Pii, Y., Crimi, M., Cremonese, G., Spena, A., & Pandolfini, T. (2007). Auxin and nitric oxide control indeterminate nodule formation. *BMC Plant Biology, 7*, 21.

Pilegaard, K. (2013). Processes regulating nitric oxide emissions from soils. *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*, 20130126.

Pinchbeck, B. J., Soriano-Laguna, M. J., Sullivan, M. J., Luque-Almagro, V. M., Rowley, G., Ferguson, S. J., et al. (2019). A dual functional redox enzyme maturation protein for respiratory and assimilatory nitrate reductases in bacteria. *Molecular Microbiology, 111*, 1592-1603.

Pisliakov, A. V., Hino, T., Shiro, Y., & Sugita, Y. (2012). Molecular dynamics simulations reveal proton transfer pathways in cytochrome *c*-dependent nitric oxide reductase. *PLoS Computational Biology, 8*, e1002674.

Poole, P., Ramachandran, V., & Terpolilli, J. (2018). Rhizobia: from saprophytes to endosymbionts. *Nature Reviews Microbiology, 16*, 291-303.

Poole, R. K. (2005). Nitric oxide and nitrosative stress tolerance in bacteria. *Biochemical Society Transactions, 33*, 176-180.

Poole, R. K. (2020). Flavohaemoglobin: the pre-eminent nitric oxide-detoxifying machine of microorganisms. *F1000Research, 9*.

Potter, L., Angove, H., Richardson, D., & Cole, J. (2001). Nitrate reduction in the periplasm of gram-negative bacteria. *Advances in Microbial Physiology, 45*, 51-112.

Preisig, O., Zufferey, R., Thony-Meyer, L., Appleby, C. A., & Hennecke, H. (1996). A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. *Journal of Bacteriology, 178*, 1532-1538.

Prendergast-Miller, M. T., Baggs, E. M., & Johnson, D. (2011). Nitrous oxide production by the ectomycorrhizal fungi *Paxillus involutus* and *Tylospora fibrillosa*. *FEMS Microbiology Letters, 316*, 31-35.

Regensburger, B., & Hennecke, H. (1983). RNA polymerase from *Rhizobium japonicum*. *Archives of Microbiology, 135*, 103-109.

Reutimann, L., Mesa, S., & Hennecke, H. (2010). Autoregulation of $fixK_2$ gene expression in *Bradyrhizobium japonicum*. *Molecular Genetics and Genomics, 284*, 25-32.

Rhee, K. Y., Erdjument-Bromage, H., Tempst, P., & Nathan, C. F. (2005). S-nitroso proteome of *Mycobacterium tuberculosis*: Enzymes of intermediary metabolism and antioxidant defense. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 467-472.

Rice, S. L., Boucher, L. E., Schlessman, J. L., Preimesberger, M. R., Bosch, J., & Lecomte, J. T. (2015). Structure of *Chlamydomonas reinhardtii* THB1, a group 1 truncated hemoglobin with a rare histidine-lysine heme ligation. *Acta Crystallographica Section F: Structural Biology Communications, 71*, 718-725.

Richardson, A. R., Dunman, P. M., & Fang, F. C. (2006). The nitrosative stress response of *Staphylococcus aureus* is required for resistance to innate immunity. *Molecular Microbiology, 61*, 927-939.

Richardson, D. J. (2011). Redox complexes of the nitrogen cycle. In J. W. B. Moir (Ed.), *Nitrogen cycling in bacteria: Molecular analysis* (pp. 23-39). Norfolk, U.K.: Caister Academic Press.

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S., & Taylor, C. J. (2001). Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cellular and Molecular Life Sciences, 58*, 165-178.

Richardson, D. J., van Spanning, R. J. M., & Ferguson, S. J. (2007). The Prokaryotic nitrate reductases. In S. J. Ferguson, & W. E. Newton (Eds.), *Biology of the nitrogen cycle* (pp. 21-35). Amsterdam: Elsevier.

Rinaldo, S., Arcovito, A., Giardina, G., Castiglione, N., Brunori, M., & Cutruzzolà, F. (2008). New insights into the activity of *Pseudomonas aeruginosa* cd_1 nitrite reductase. *Biochemical Society Transactions, 36*, 1155-1159.

Rinaldo, S., Giardina, G., & Cutruzzolà, F. (2017). Nitrite reductase-cytochrome cd_1. In I. Moura, J. J. Moura, S. R. Pauleta, & L. B. Maia (Eds.), *Metalloenzymes in denitrification: Applications and environmental impacts* (pp. 59-90). Cambridge: The Royal Society of Chemistry.

Robles, E. F., Sánchez, C., Bonnard, N., Delgado, M. J., & Bedmar, E. J. (2006). The *Bradyrhizobium japonicum napEDABC* genes are controlled by the FixLJ-$FixK_2$-NnrR regulatory cascade. *Biochemical Society Transactions, 34*, 108-110.

Rowley, G., Hensen, D., Felgate, H., Arkenberg, A., Appia-Ayme, C., Prior, K., et al. (2012). Resolving the contributions of the membrane-bound and periplasmic nitrate reductase systems to nitric oxide and nitrous oxide production in *Salmonella enterica* serovar Typhimurium. *Biochemical Journal, 441*, 755-762.

Rubio, L. M., & Ludden, P. W. (2008). Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Annual Review of Microbiology, 62*, 93-111.

Rubio, M. C., Calvo-Begueria, L., Díaz-Mendoza, M., Elhiti, M., Moore, M., Matamoros, M. A., et al. (2019). Phytoglobins in the nuclei, cytoplasm and chloroplasts modulate nitric oxide signaling and interact with abscisic acid. *The Plant Journal, 100*, 38-54.

Ruiz, B., Le Scornet, A., Sauviac, L., Rémy, A., Bruand, C., & Meilhoc, E. (2019). The nitrate assimilatory pathway in *Sinorhizobium meliloti*: contribution to NO production. *Frontiers in Microbiology, 10*, 1526.

Rutten, P. J., & Poole, P. S. (2019). Oxygen regulatory mechanisms of nitrogen fixation in rhizobia. *Advances in Microbial Physiology, 75*, 325-389.

Salas, A., Tortosa, G., Hidalgo-García, A., Delgado, A., Bedmar, E. J., Richardson, D. J., et al. (2020). The hemoglobin Bjgb from *Bradyrhizobium diazoefficiens* controls NO homeostasis in soybean nodules to protect symbiotic nitrogen fixation. *Frontiers in Microbiology, 10*, 2915.

Salgo, M. G., Stone, K., Squadrito, G. L., Battista, J. R., & Pryor, W. A. (1995). Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochemical and Biophysical Research Communications, 210*, 1025-1030.

Sánchez, C., Cabrera, J. J., Gates, A. J., Bedmar, E. J., Richardson, D. J., & Delgado, M. J. (2011). Nitric oxide detoxification in the rhizobia-legume symbiosis. *Biochemical Society Transactions, 39*, 184-188.

Sánchez, C., Gates, A. J., Meakin, G. E., Uchiumi, T., Girard, L., Richardson, D. J., et al. (2010). Production of nitric oxide and nitrosylleghemoglobin complexes in soybean nodules in response to flooding. *Molecular Plant-Microbe Interactions, 23*, 702-711.

Sánchez, C., Itakura, M., Okubo, T., Matsumoto, T., Yoshikawa, H., Gotoh, A., et al. (2014). The nitrate-sensing NasST system regulates nitrous oxide reductase and periplasmic nitrate reductase in *Bradyrhizobium japonicum*. *Environmental Microbiology, 16*, 3263-3274.

Sánchez, C., & Minamisawa, K. (2018). Redundant roles of *Bradyrhizobium oligotrophicum* Cu-type (NirK) and cd_1-type (NirS) nitrite reductase genes under denitrifying conditions. *FEMS Microbiology Letters, 365*.

Sánchez, C., Mitsui, H., & Minamisawa, K. (2017). Regulation of nitrous oxide reductase genes by NasT-mediated transcription antitermination in *Bradyrhizobium diazoefficiens*. *Environmental Microbiology Reports, 9*, 389-396.

Sánchez, C., Tortosa, G., Granados, A., Delgado, A., Bedmar, E. J., & Delgado, M. J. (2011). Involvement of *Bradyrhizobium japonicum* denitrification in symbiotic nitrogen fixation by soybean plants subjected to flooding. *Soil Biology and Biochemistry, 43*, 212-217.

Santolini, J. (2019). What does "NO-Synthase" stand for? *Frontiers in Bioscience (Landmark Ed), 24*, 133-171.

Scott, N. L., Xu, Y., Shen, G., Vuletich, D. A., Falzone, C. J., Li, Z., et al. (2010). Functional and structural characterization of the 2/2 hemoglobin from *Synechococcus* sp. PCC 7002. *Biochemistry, 49*, 7000-7011.

Sebbane, F., Lemaître, N., Sturdevant, D. E., Rebeil, R., Virtaneva, K., Porcella, S. F., et al. (2006). Adaptive response of *Yersinia pestis* to extracellular effectors of innate immunity during bubonic plague. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 11766-11771.

Seefeldt, L. C., Peters, J. W., Beratan, D. N., Bothner, B., Minteer, S. D., Raugei, S., et al. (2018). Control of electron transfer in nitrogenase. *Current Opinion in Chemical Biology, 47*, 54-59.

Seth, D., Hausladen, A., Wang, Y. J., & Stamler, J. S. (2012). Endogenous protein S-Nitrosylation in *E. coli*: regulation by OxyR. *Science, 336*, 470-473.

Seth, D., Hess, D. T., Hausladen, A., Wang, L., Wang, Y. J., & Stamler, J. S. (2018). A multiplex enzymatic machinery for cellular protein S-nitrosylation. *Molecular Cell, 69*, 451-464.

Shapleigh, J. P. (2006). The denitrifying prokaryotes. In M. Dworkin, S. Falkow, E. Rosenberg, & K. H. Schleifer (Eds.), *The Prokaryotes* (pp. 769-792). New York: Springer Science +Business Media.

Shatalin, K., Gusarov, I., Avetissova, E., Shatalina, Y., McQuade, L. E., Lippard, S. J., et al. (2008). *Bacillus anthracis*-derived nitric oxide is essential for pathogen virulence and survival in macrophages. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 1009-1013.

Shepherd, M., Barynin, V., Lu, C., Bernhardt, P. V., Wu, G., Yeh, S. R., et al. (2010). The single-domain globin from the pathogenic bacterium *Campylobacter jejuni*: novel D-helix conformation, proximal hydrogen bonding that influences ligand binding, and peroxidase-like redox properties. *Journal of Biological Chemistry, 285*, 12747-12754.

Shiro, Y., Sugimoto, H., Tosha, T., Nagano, S., & Hino, T. (2012). Structural basis for nitrous oxide generation by bacterial nitric oxide reductases. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 367*, 1195-1203.

Shoun, H., & Fushinobu, S. (2017). Denitrification in fungi. In I. Moura, J. J. Moura, S. R. Pauleta, & L. B. Maia (Eds.), *Metalloenzymes in denitrification: Applications and environmental impacts* (pp. 331-348). Cambridge: The Royal Society of Chemistry.

Sickerman, N. S., Rettberg, L. A., Lee, C. C., Hu, Y., & Ribbe, M. W. (2017). Cluster assembly in nitrogenase. *Essays in Biochemistry, 61*, 271-279.

Simon, J., & Klotz, M. G. (2013). Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochimica et Biophysica Acta, 1827*, 114-135.

Souza, J. M., Peluffo, G., & Radi, R. (2008). Protein tyrosine nitration – functional alteration or just a biomarker? *Free Radical Biology and Medicine, 45*, 357-366.

Stern, A. M., Liu, B., Bakken, L. R., Shapleigh, J. P., & Zhu, J. (2013). A novel protein protects bacterial iron-dependent metabolism from nitric oxide. *Journal of Bacteriology, 195*, 4702-4708.

Stern, A. M., & Zhu, J. (2014). An introduction to nitric oxide sensing and response in bacteria. *Advances in Applied Microbiology, 87*, 187-220.

Sullivan, M. J., Gates, A. J., Appia-Ayme, C., Rowley, G., & Richardson, D. J. (2013). Copper control of bacterial nitrous oxide emission and its impact on vitamin B_{12}-dependent metabolism. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 19926-19931.

Svensson, L., Marklund, B. I., Poljakovic, M., & Persson, K. (2006). Uropathogenic *Escherichia coli* and tolerance to nitric oxide: the role of flavohemoglobin. *Journal of Urology, 175*, 749-753.

Svensson, L., Poljakovic, M., Säve, S., Gilberthorpe, N., Schön, T., Strid, S., et al. (2010). Role of flavohemoglobin in combating nitrosative stress in uropathogenic *Escherichia coli*-implications for urinary tract infection. *Microbial Pathogenesis, 49*, 59-66.

Takaya, N. (2002). Dissimilatory nitrate reduction metabolisms and their control in fungi. *Journal of Bioscience and Bioengineering, 94*, 506-510.

Tarricone, C., Galizzi, A., Coda, A., Ascenzi, P., & Bolognesi, M. (1997). Unusual structure of the oxygen-binding site in the dimeric bacterial hemoglobin from *Vitreoscilla* sp. *Structure, 5*, 497-507.

Terasaka, E., Okada, N., Sato, N., Sako, Y., Shiro, Y., & Tosha, T. (2014). Characterization of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*: enzymatic activity and active site structure. *Biochimica et Biophysica Acta, 1837*, 1019-1026.

Terpolilli, J. J., Hood, G. A., & Poole, P. S. (2012). What determines the efficiency of N_2-fixing *Rhizobium*-legume symbioses? *Advances in Microbial Physiology, 60*, 325-389.

Tinajero-Trejo, M., & Shepherd, M. (2013). The globins of *Campylobacter jejuni*. *Advances in Microbial Physiology, 63*, 97-145.

Tinajero-Trejo, M., Vreugdenhil, A., Sedelnikova, S. E., Davidge, K. S., & Poole, R. K. (2013). Nitric oxide reactivities of the two globins of the foodborne pathogen *Campylobacter jejuni*: roles in protection from nitrosative stress and analysis of potential reductants. *Nitric Oxide, 34*, 65-75.

Toledo, J. C. Jr., & Augusto, O. (2012). Connecting the chemical and biological properties of nitric oxide. *Chemical Research in Toxicology, 25*, 975-989.

Torregrosa-Crespo, J., González-Torres, P., Bautista, V., Esclapez, J. M., Pire, C., Camacho, M., et al. (2017). Analysis of multiple haloarchaeal genomes suggests that the quinone-dependent respiratory nitric oxide reductase is an important source of nitrous oxide in hypersaline environments. *Environmental Microbiology Reports, 9*, 788-796.

Torres, M. J., Argandoña, M., Vargas, C., Bedmar, E. J., Fischer, H. M., Mesa, S., et al. (2014). The global response regulator RegR controls expression of denitrification genes in *Bradyrhizobium japonicum*. *PLoS One, 9*, e99011.

Torres, M. J., Ávila, S., Bedmar, E. J., & Delgado, M. J. (2018). Overexpression of the periplasmic nitrate reductase supports anaerobic growth by *Ensifer meliloti*. *FEMS Microbiology Letters, 365*.

Torres, M. J., Bueno, E., Jiménez-Leiva, A., Cabrera, J. J., Bedmar, E. J., Mesa, S., et al. (2017). $FixK_2$ is the main transcriptional activator of *Bradyrhizobium diazoefficiens nosRZDYFLX* genes in response to low oxygen. *Frontiers in Microbiology, 8*, 1621.

Torres, M. J., Rubia, M. I., Bedmar, E. J., & Delgado, M. J. (2011). Denitrification in *Sinorhizobium meliloti*. *Biochemical Society Transactions, 39*, 1886-1889.

Torres, M. J., Rubia, M. I., de la Peña, T. C., Pueyo, J. J., Bedmar, E. J., & Delgado, M. J. (2014). Genetic basis for denitrification in *Ensifer meliloti*. *BMC Microbiology, 14*, 142.

Torres, M. J., Simon, J., Rowley, G., Bedmar, E. J., Richardson, D. J., Gates, A. J., et al. (2016). Nitrous oxide metabolism in nitrate-reducing bacteria: physiology and regulatory mechanisms. *Advances in Microbial Physiology, 68*, 353-432.

Tortosa, G., Hidalgo, A., Salas, A., Bedmar, E. J., Mesa, S., & Delgado, M. J. (2015). Nitrate and flooding induce N_2O emissions from soybean nodules. *Symbiosis, 67*, 125-133.

Tosha, T., & Shiro, Y. (2017). Structure and function of nitric oxide reductases. In I. Moura, J. J. Moura, S. R. Pauleta, & L. B. Maia (Eds.), *Metalloenzymes in denitrification: Applications and environmental impacts* (pp. 114-140). Cambridge: The Royal Society of Chemistry.

Treusch, A. H., Leininger, S., Kletzin, A., Schuster, S. C., Klenk, H. P., & Schleper, C. (2005). Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environmental Microbiology, 7*, 1985-1995.

Udvardi, M., & Poole, P. S. (2013). Transport and metabolism in legume-rhizobia symbioses. *Annual Review of Plant Biology, 64*, 781-805.

van Spanning, R. J. (2011). Structure, function, regulation and evolution of the nitrite and nitrous oxide reductases: Denitrification enzymes with a beta-propeller fold. In J. W. B. Moir (Ed.), *Nitrogen cycling in bacteria* (pp. 135-161). Norfolk, U.K.: Caister Academic Press.

van Spanning, R. J., Richardson, D. J., & Ferguson, S. J. (2007). Introduction to the biochemistry and molecular biology of denitrification. In H. Bothe, S. J. Ferguson, & W. E. Newton (Eds.), *Biology of the nitrogen cycle* (pp. 3-20). Amsterdam: Elsevier.

Velasco, L., Mesa, S., Delgado, M. J., & Bedmar, E. J. (2001). Characterization of the *nirK* gene encoding the respiratory, Cu-containing nitrite reductase of *Bradyrhizobium japonicum*. *Biochimica et Biophysica Acta, 1521*, 130-134.

Velasco, L., Mesa, S., Xu, C. A., Delgado, M. J., & Bedmar, E. J. (2004). Molecular characterization of *nosRZDFYLX* genes coding for denitrifying nitrous oxide reductase of *Bradyrhizobium japonicum*. *Antonie Van Leeuwenhoek, 85*, 229-235.

Vine, C. E., & Cole, J. A. (2011). Nitrosative stress in *Escherichia coli*: reduction of nitric oxide. *Biochemical Society Transactions, 39*, 213-215.

Vine, C. E., Purewal, S. K., & Cole, J. A. (2011). NsrR-dependent method for detecting nitric oxide accumulation in the *Escherichia coli* cytoplasm and enzymes involved in NO production. *FEMS Microbiology Letters, 325*, 108-114.

Wang, W., Kinkel, T., Martens-Habbena, W., Stahl, D. A., Fang, F. C., & Hansen, E. J. (2011). The *Moraxella catarrhalis* nitric oxide reductase is essential for nitric oxide detoxification. *Journal of Bacteriology, 193*, 2804-2813.

Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., et al. (1991). DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science, 254*, 1001-1003.

Wu, G., Wainwright, L. M., & Poole, R. K. (2003). Microbial globins. *Advances in Microbial Physiology, 47*, 255-310.

Wunsch, P., & Zumft, W. G. (2005). Functional domains of NosR, a novel transmembrane iron-sulfur flavoprotein necessary for nitrous oxide respiration. *Journal of Bacteriology, 187*, 1992-2001.

Young, N. D., Debelle, F., Oldroyd, G. E., Geurts, R., Cannon, S. B., Udvardi, M. K., et. al. (2011). The *Medicago* genome provides insight into the evolution of rhizobial symbioses. *Nature, 480*, 520-524.

Yukl, E. T., de Vries, S., & Moënne-Loccoz, P. (2009). The millisecond intermediate in the reaction of nitric oxide with oxymyoglobin is an iron(III)-nitrato complex, not a peroxynitrite. *Journal of the American Chemical Society, 131*, 7234-7235.

Zamorano-Sánchez, D., Reyes-González, A., Gómez-Hernández, N., Rivera, P., Georgellis, D., & Girard, L. (2012). FxkR provides the missing link in the *fixL-fixK* signal transduction cascade in *Rhizobium etli* CFN42. *Molecular Plant-Microbe Interactions, 25*, 1506-1517.

Zhang, L., Trncik, C., Andrade, S. L. A., & Einsle, O. (2017). The flavinyl transferase ApbE of *Pseudomonas stutzeri* matures the NosR protein required for nitrous oxide reduction. *Biochimica et Biophysica Acta, 1858*, 95-102.

Zhang, L., Wüst, A., Prasser, B., Müller, C., & Einsle, O. (2019). Functional assembly of nitrous oxide reductase provides insights into copper site maturation. *Proceeding of the National Academy of Sciences of the United States of America*, *116*, 12822-12827.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews, 61*, 533-616.

Figure legends

Figure 1 Nitric oxide (NO•) reactions and biological targets. Reaction with superoxide (O_2•$^-$) generates peroxynitrite ($ONOO^-$); with oxygen (O_2), nitrogen dioxide (NO_2•); with NO_2•, dinitrogen trioxide (N_2O_3). $ONOO^-$ at neutral pH will protonate and generate NO_2• and hydroxyl radicals (HO•) and in the presence of carbon dioxide (CO_2) will generate NO_2• and carbonate anion radical (CO_3•$^-$). In the presence of reductants, $ONOO^-$ will be reduced to nitrite (NO_2^-). NO_2• can react with tyrosyl radicals to generate 3-nitrotyrosine (NO_2-Tyr) or with a reductant to form NO_2^-. N_2O_3 can be rapidly hydrolyzed to NO_2^-, it can also be formed from NO_2^- in acidic pH, and it can react with thiols (RSH) to generate S-nitrosothiols (SNOs). NO also reacts readily with dinitrosyl iron complexes (DNICs); and transition metals, such as iron (Fe-NO). DNICs, SNOs, and NO_2-Tyr alter protein structure and function. $ONOO^-$ causes double-stranded DNA breaks. A large number of transcription factors are also affected by NO.

Figure 2 Properties and location of redox proteins involved in denitrification. The membrane-bound (NarGHI), and periplasmic (NapABC) nitrate reductases, nitrite reductases (NirK and NirS), nitric oxide reductases (cNor, qNor and Cu_ANor) and nitrous oxide reductase (NosZ) are shown. Dashed lines indicate electron transfer. Q/QH_2 means UQ/UQH_2 or MK/MKH_2. NDH, NADH dehydrogenase. SDH, Succinate dehydrogenase. See the text for further details.

Figure 3. The organization of denitrification genes in *B. diazoefficiens*, *R. etli* and *E. meliloti*. Colours indicate the following functions of the respective gene products: red, periplasmic nitrate reductase; dark blue, nitrite reductase; green, nitric oxide reductase;

purple, nitrous oxide reductase; brown, regulatory genes, light blue, pseudoazurins; pink, heme biosynthesis; yellow, cytochrome *c* oxidase cbb_3; orange, flavohemoglobin; grey, other genes with unknown function.

Figure 4 Regulatory network of *B. diazoefficiens* denitrification. Positive regulation is denoted by arrows and positive symbols, unknown control mechanisms are indicated by dashed lines. Question marks denote that the NO sensing mechanism by NnrR, as well as the redox signal involved in RegSR control are still unknown. See text for further details.

Figure 5 NO signalling regulatory cascades in *S. meliloti*, *R. etli* and *B. diazoefficiens*. Positive regulation is denoted by arrows, negative regulation is indicated by perpendicular lines. Question marks denote that the NO sensing mechanism by NnrR and FixL as well as the redox signal involved in RegSR control are still unknown. See text for further details.

Figure 6 Schematic representation of genes (A) and proteins (B, C) involved in nitrate assimilation and their contribution to NO production in *B. diazoefficiens* (B), *R. etli* and *E. meliloti* (C). Colours indicate the following functions of the respective gene products: red, nitrate reductase (NasC, NarB); blue, nitrite reductases (NirA, NirBD); light green, NO_3^- ABC transporter (NrtABC); dark green, NO_3^-/NO_2^- transporter (NarK), pink, single domain haemoglobin (Bjgb); yellow, regulatory genes (NasST); purple, uroporphyrin-III C-methyltransferase (CysG). Electron transfer is indicated by dashed lines. Question marks denote mechanisms still unknown.

Figure 7 Bacterial nitric oxide reductases. (A) cNor estructure from *Roseobacter denitrificans* containing the structural subunits NorB with a haem *b* and a binuclear active centre (haem b_3 and Fe_B) and NorC with haem *c* (PDB ID: 4XYD; Crow, Matsuda, Arata, & Oubrie, 2016). (B) The quinol dependent qNor structure of *Geobacillus stearothermophilus* is similar to NorB from cNor differing in an N-terminal extension with homology to NorC without a haem *c* and the replacement of non-haem metal Fe_B by Zn_B (PDB ID: 3AYG, Matsumoto et al., 2012). (C) Predicted model of Cu_ANor from *Bacillus azotoformans* shows a NorB subunit and another subunit containing Cu_A which uses cytochrome *c* as electron donor. Electron transfer is indicated by dashed lines.

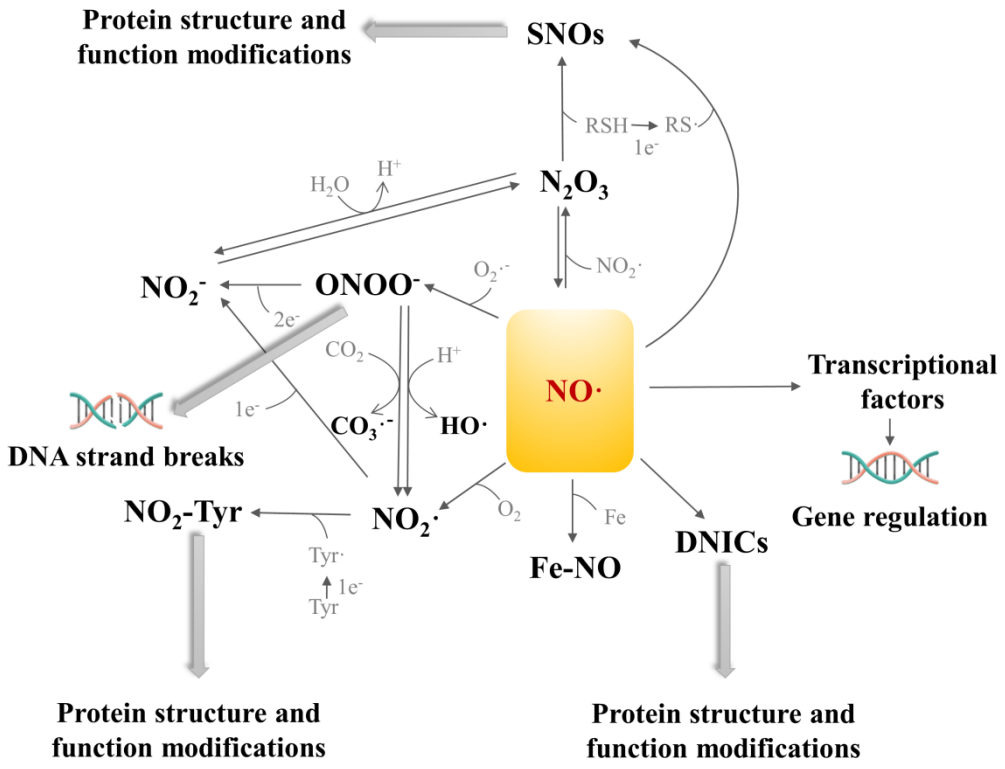
Figure 8 Haemoglobin tertiary structures**.** (A) Sperm whale Mb (PDB ID:1MBO: Phillips, 1980). (B) Flavohaemoglobin from *E. coli* (PDB ID: 1GVH, Ilari, Bonamore, Farina, Johnson, & Boffi, 2002). (C) Single domain haemoglobin from *C. jejuni* (PDB ID: 2WY4, Shepherd et al., 2010). (D) Truncated hahemoglobin from *C. reinhardtii* (PDB ID: 4XDI, Rice et al., 2015). α-helices (labelled A-H), haem, and the HisF8, PheCD1 and Lys53 residues are shown. In B, the presence of FAD in the ferredoxin reductase domain from the fHb crystal structure is also shown. (E) Haem group, where N highlighted in red denotes a coordinate bond.

Figure 9 Illustration of NO metabolism in *G. max, M. truncatula, and P. vulgaris* root nodules. The large light grey square represents the plant cell, and the small squares represent the bacteroid where the periplasm is shown in dark grey and the cytoplasm is shown in white. In addition to the reported plant sources of NO in legume nodules, denitrification pathways in the *B. diazoefficiens*, *R. etli* and *E. meliloti* bacteroids also

2181 contribute to the formation of this molecule. In addition to denitrification, NasC might
2182 be another source of NO in *B. diazoefficiens* bacteroids where it acts as a signal
2183 molecule to activate *nor* genes. Bjgb would remove NO produced by NasC. In *E.*
2184 *meliloti* bacterois, Nor, Hmp, NnrS1 and NnrS2 have been proposed to be involved in
2185 NO detoxification. Unknown mechanisms is indicated with a question mark.

2186

2187

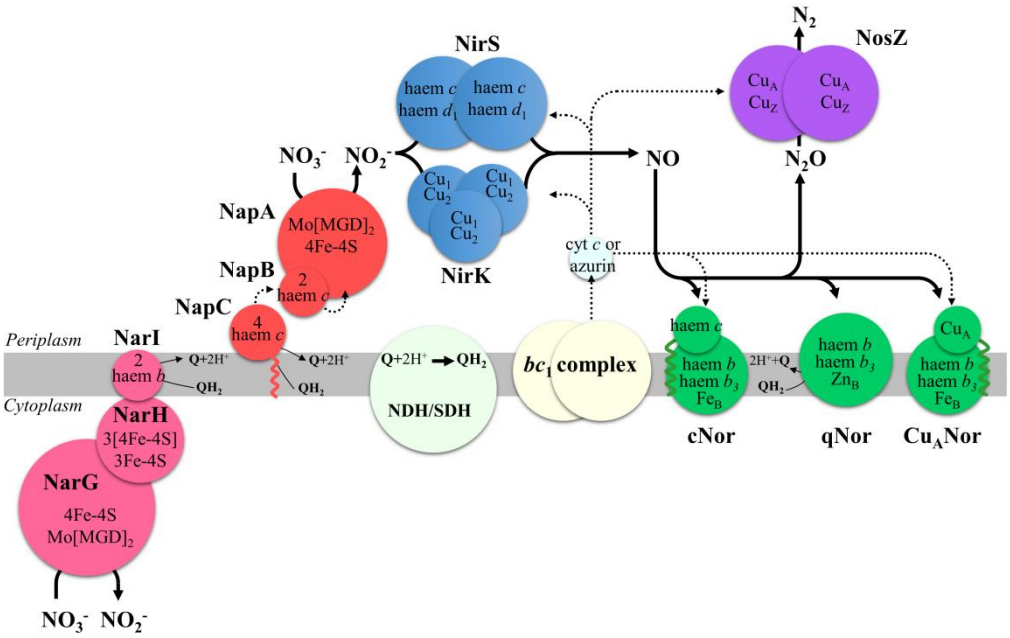


2188

2189

2190 **Figure 1**

2191

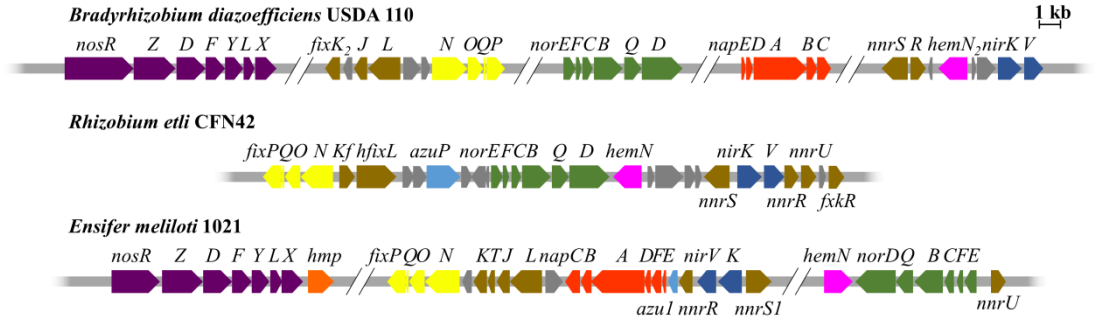


2192

2193

2194 **Figure 2**

2195

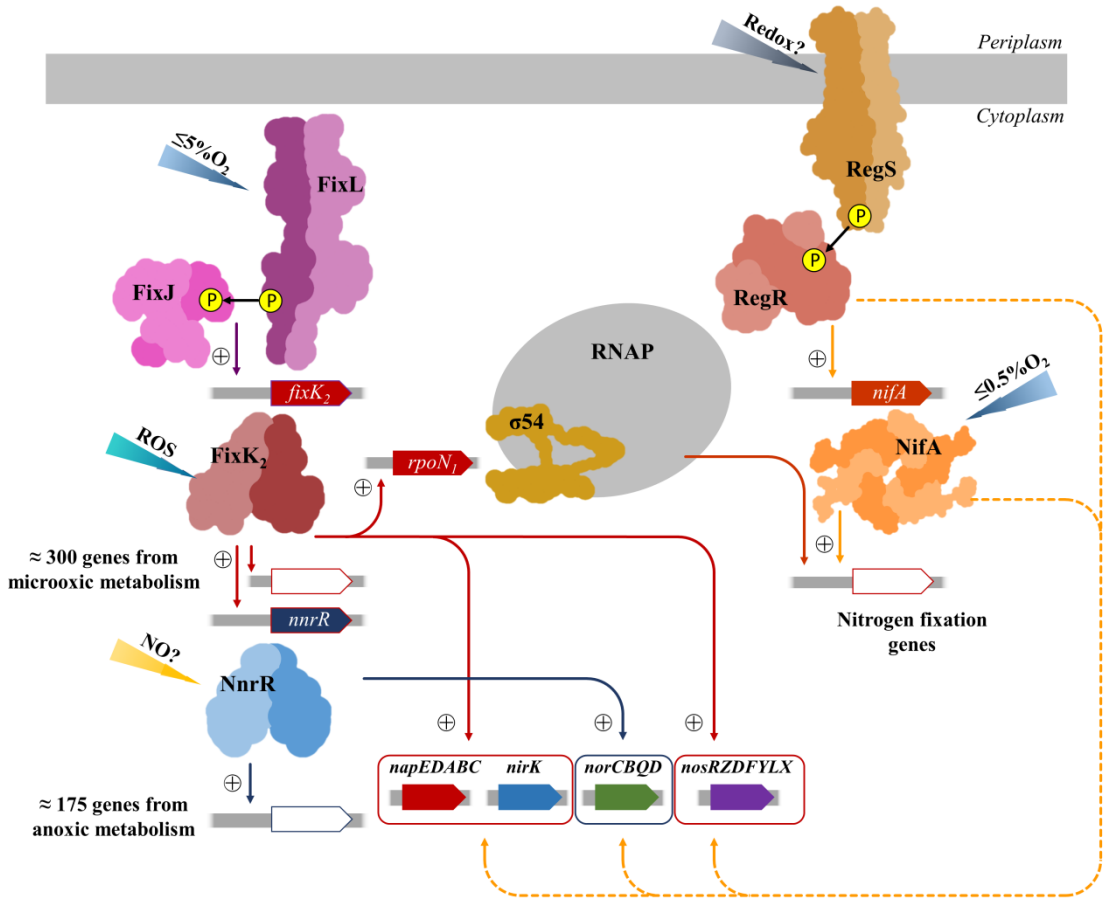


2196

2197

2198 **Figure 3**

2199

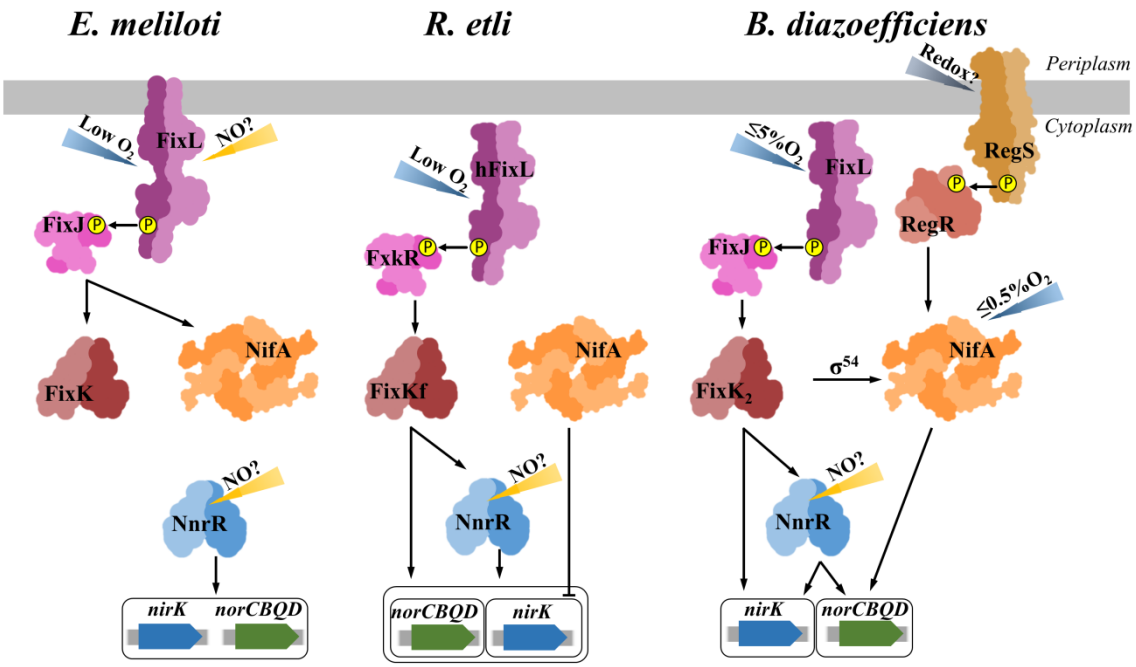


2200

2201

2202 **Figure 4**

2203

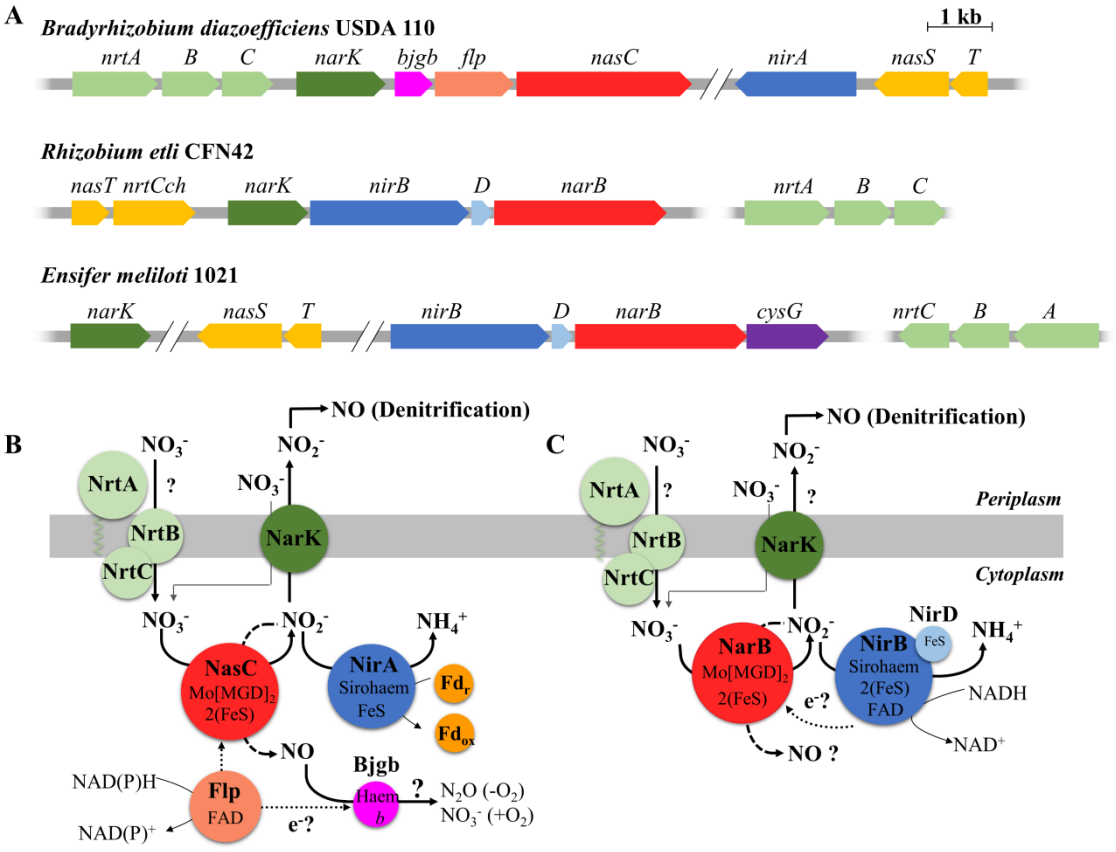


2204

2205

2206 **Figure 5**

2207

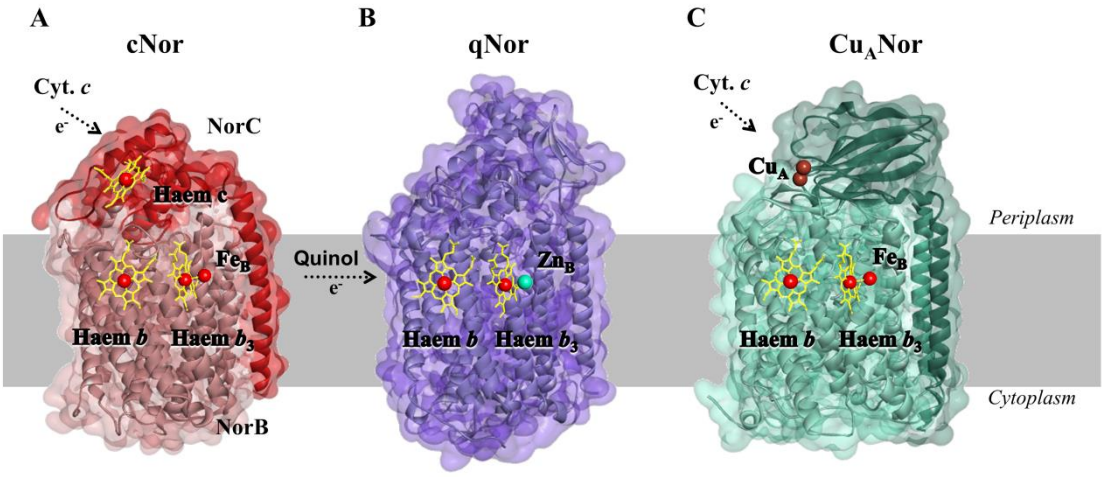


2208

2209

2210 **Figure 6**

2211



2212

2213 **Figure 7**

2214

2215

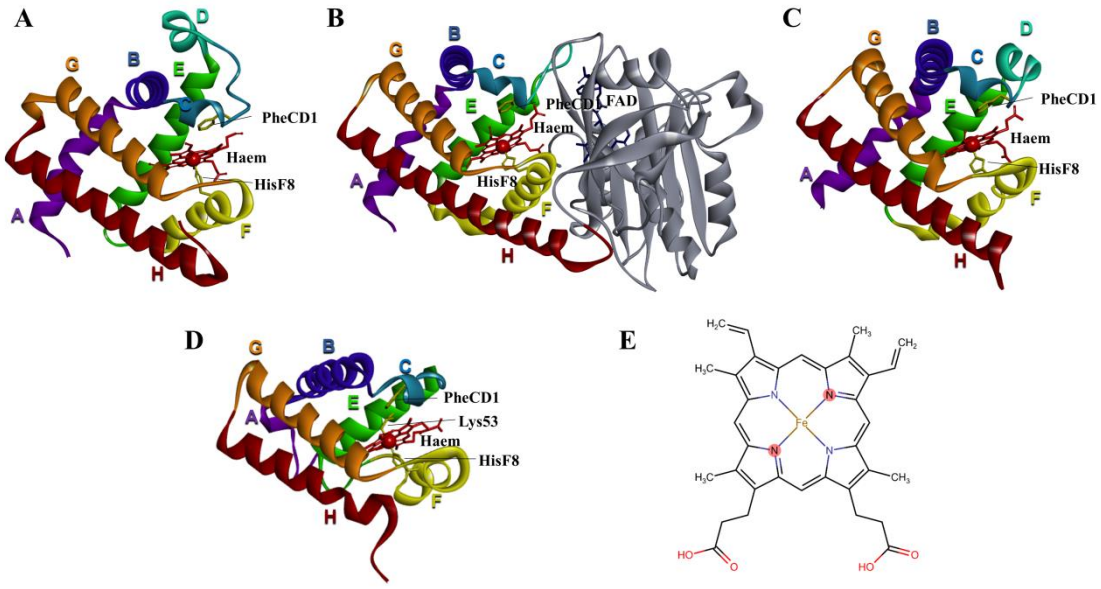


Figure 8

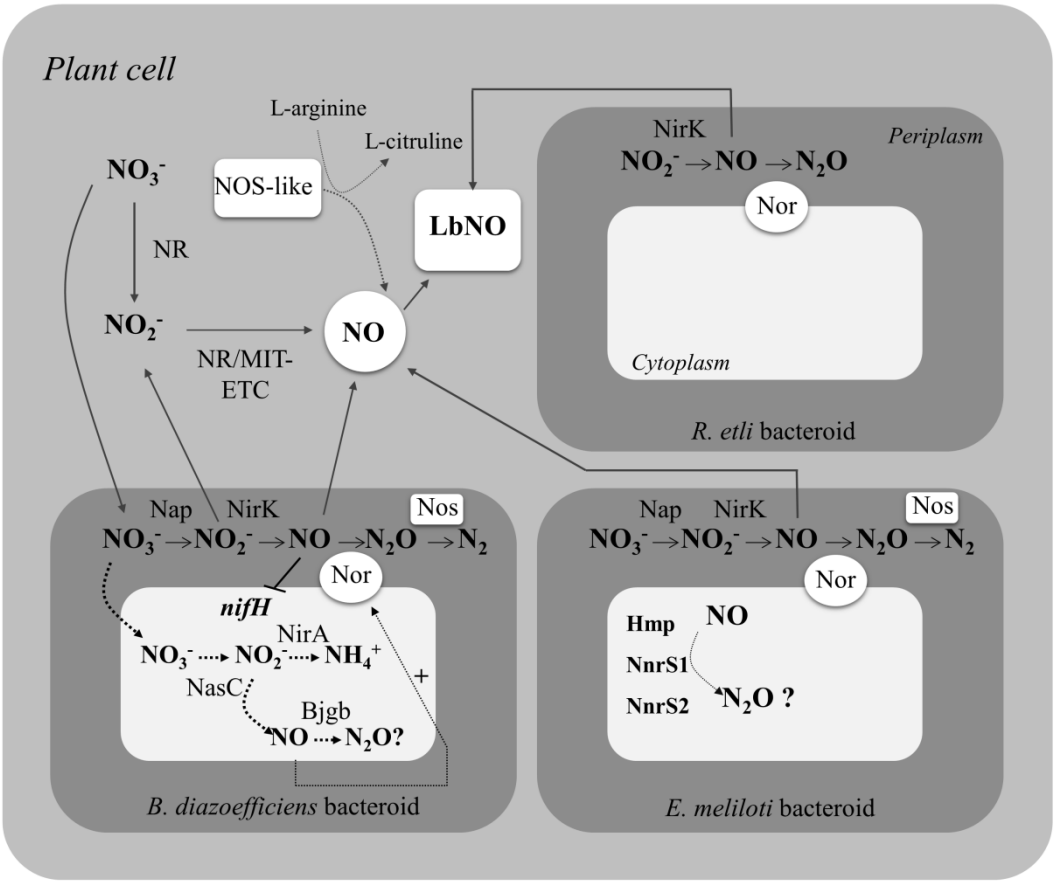

Plant cell
L-arginine
L-citruline
NOS-like
LbNO
NR
NR/MIT-ETC
NO
NirK
Periplasm
Nor
Cytoplasm
R. etli bacteroid
Nap
NirK
Nos
Nor
nifH
NirA
NasC
Bjgb
+
B. diazoefficiens bacteroid
Hmp
NnrS1
NnrS2
E. meliloti bacteroid

Figure 9