Nitrous Oxide Metabolism in Nitrate-Reducing Bacteria: Physiology and Regulatory Mechanisms

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ABBREVIATIONS: AOB, ammonia oxidizing bacteria; bis-MGD, bismolybdopterin guanine dinucleotide; Crp, cAMP receptor protein; Cys, cysteine; Cyt, cytochrome; cd_1Nir, cd_1-type nitrite reductase; CuNir, Cu-type nitrite reductase; DNRA, dissimilatory nitrate reduction to ammonium; ETC, electron transport chain; FMN, flavin mononucleotide; NorVW, flavorubredoxin; FNR, fumarate and nitrate reductase regulatory protein; HCO, heme-copper oxidase; His, Histidine; Hmp, flavohaemoglobin; H-T-H, helix-turn-helix motif; LbNO, nitrosyl-leghaemoglobin MK, menaquinone; MKH_2, menahydroquinone; NDH, NADH dehydrogenase; Nap, periplasmic nitrate reductase; Nar, membrane-bound nitrate reductase; Nir, nitrite reductase; NnrR, nitrite and nitric oxide reductase regulator; Nor, nitric oxide reductase;

NOS, nitric oxide synthase; N_2OR, nitrous oxide reductase; Nrf, nitrite reduction with formate; NrfA, cytochrome *c* nitrite reductase; NssR, nitrosative stress sensing regulator; PMF, proton motive force; RNAP, RNA polymerase; RNS, reactive nitrogen species; Tat, twin arginine translocation; UQ, ubiquinone; UQH_2, ubihydroquinone.

Contents

Abstract

Nitrous oxide (N_2O) is an important greenhouse gas with large global warming potential and also leads to ozone depletion through photo-chemical nitric oxide (NO) production in the stratosphere. The negative effects of N_2O on climate and stratospheric ozone make N_2O mitigation an international challenge. More than 60% of N_2O emissions globally are emitted from agricultural soils mainly due to the application of synthetic N in the form of fertilisers to soils. Thus, mitigation strategies must be developed which increase (or at least do not negatively impact) on agricultural efficiency whilst decrease levels of N_2O emissions. This aim is particularly important in the context of the ever expanding population and sunsequent increased burden on the food chain. More than two-thirds of N_2O emissions from soils arise from bacterial and fungal denitrification and nitrification processes. In ammonia oxidizing bacteria (AOB) or nitrifiers, N_2O is formed through the oxidation of hydroxylamine (NH_2OH) to nitrite (NO_2^-). In denitrifiers, nitrate (NO_3^-) is reduced to N_2 via NO_2^-, NO and N_2O production. In addition to denitrification, respiratory NO_3^-/NO_2^- ammonification also named dissimilatory nitrate reduction to ammonium (DNRA) is another important nitrate reducing mechanism in soil, responsible for the loss of NO_3^- and production of N_2O from reduction of NO that is formed as a by-product of NO_3^-/NO_2^- reduction. This review will synthesize our current understanding of the environmental, regulatory and biochemical control of N_2O emissions by nitrate-reducing bacteria and point to new solutions for agricultural greenhouse gas mitigation.

1. INTRODUCTION

Nitrous oxide (N_2O) is a powerful greenhouse gas (GHG) and a major cause of ozone layer depletion with an atmospheric lifetime of 114 years. Although N_2O only accounts for around 0.03 % of total GHG emissions, it has an almost 300-fold greater potential for global warming effects, based on its radiative capacity, compared with that of carbon dioxide (CO_2). Hence, when the impact of individual GHGs on global warming is expressed in terms of the Intergovernmental Panel on Climate Change (IPCC) approved unit of CO_2 equivalents, N_2O accounts for approximately 10 % of total emissions (IPCC, 2014). Human activities are currently considered to emit 6.7 Tg N-N_2O per year mainly from agriculture, which accounts for about 60 % of N_2O emissions (IPCC, 2014; Smith et al., 2008; 2012). This contribution has been exacerbated through the intensification of agriculture, the so-called 'green revolution', which has increased the presence of nitrogen (N) in soil through the application, since the early 1900s, of synthetic nitrogen-based fertilizers whose production steadily increased after the invention of the Haber-Bosch process. Since 1997, many of the non-biological emissions of N_2O, for example, those associated with the transport industry, have been systematically lowered, whereas emissions from agriculture are essentially unchanged (IPCC, 2014). Given the clear evidence about the damaging effects on climate of atmospheric N_2O, strategies to ameliorate N_2O emission arising from intensive agricultural practices have to be developed in order to increase agricultural efficiency and decrease current levels of N_2O emissions in particular in the context of the continuing population growth (Richardson et al., 2009; Thomson et al. 2012). Strategies that might be adopted include: (i) management of soil chemistry and microbiology to ensure that bacterial denitrification runs to completion, thus generating N_2 instead of N_2O; (ii) reducing the dependence on fertilizers through engineering crop

plants to fix nitrogen themselves or through the application of nitrogen-fixing bacteria, (iii) promotion of sustainable agriculture, that is producing more output from the same area of land while reducing the negative environmental impacts, and (iv) an increased understanding of the environmental and molecular factors which contribute to the biological generation and consumption of N_2O. Pathways for biological N_2O production include dissimilatory nitrate/nitrite reduction to N_2 (denitrification) (Zumft, 1997), dissimilatory nitrate reduction to ammonia (DNRA) (Bleakley and Tiedje, 1982), nitrifier denitrification, hydroxylamine oxidation by ammonia oxidizing bacteria (AOB), and NO detoxification (also known as nitrosative stress defense). N_2O is also produced by methane-oxidizing bacteria (Campbell et al., 2011) and ammonia oxidizing archaea (AOA; Liu et al., 2010; Stieglmeier et al., 2014). N_2O production by nitrite-oxidizing bacteria (NOB), anaerobic methane (N-AOM) and anaerobic AOB (anammox bacteria) has also been reported (for reviews see Stein, 2011; Schreiber et al., 2012). Among them, denitrification and DNRA are the major microbial processes in soil that are capable of removing NO_3^- since they are two competing, energy-conserving NO_3^- /NO_2^- reduction pathways (Fig. 7.1). During denitrification, NO_3^- is reduced to the gaseous products, N_2O and dinitrogen gas (N_2), in a step-wise manner via NO_2^- and nitric oxide (NO) as intermediates (Zumft, 1997). N_2O and N_2 release to the atmosphere causes N loss from terrestrial and aquatic environments, and N_2O is an ozone-depleting greenhouse gas. DNRA shares the NO_3^- to NO_2^- reaction step with denitrification but reduces NO_2^- to NH_4 (Bleakley and Tiedje, 1982; Simon and Klotz, 2013). In contrast to NO_3^- and NO_2^-, NH_4 is retained in soils and sediments and has a higher tendency for incorporation into microbial or plant biomass. Hence, the relative contributions of denitrification versus respiratory ammonification activities have important consequences for N retention, plant growth and climate. In addition to denitrification

that produces N_2O when abiotic conditions or the lack of an N_2O reductase encoding gene prevent its reduction to N_2, DNRA seemingly releases N_2O as a by-product of the NO_3^-/NO_2^- reduction process (Fig. 7.1). In denitrifiers, it has been well established the role of the Cu-containing (NirK) and *cd*$_1$-type (NirS) nitrite reductases as well as the membrane-bound respiratory NO reductases (*c*Nor and qNor enzymes) in NO and N_2O formation (Fig. 7.1). In DNRA, the ammonium-generating respiratory cytochrome *c* nitrite reductase (NrfA), the assimilatory siroheme-containing nitrite reductase (NirB) and the NO-detoxifying flavorubredoxin (NorVW) are the main candidates to be involved in NO and N_2O production (Fig. 7.1). Recent findings have proposed the involvement of the membrane-bound respiratory nitrate reductase (NarG, Gilberthorpe and Poole, 2008; Rowley et al., 2012) and the assimilatory nitrate reductase (NasC, Cabrera et al., 2015) in NO and N_2O metabolism (Fig. 7.1).

While there are several enzymatic and microbial routes to N_2O production, the bacterial N_2O reductase (N_2OR), is the only known enzyme capable of reducing N_2O to N_2 (Fig. 7.1). The typical N_2OR enzyme, NosZ, from denitrifiers has been considered for long time the only enzyme involved in N_2O mitigation. Recently, however, a closely related enzyme variant named atypical NosZ has been identified in diverse microbial taxa forming a distinct clade of N_2OR (Sanford et al., 2012; Jones et al., 2013). Organisms containing atypical NosZ enzymes also possess divergent *nos* clusters with genes that are evolutionarily distinct from the typical *nos* genes of denitrifiers (Table 7.1). Interestingly, DNRA bacteria such as *Wolinella succinogenes* as well as some other non-denitrifiers contain this atypical N_2OR that probably acts on the N_2O produced by detoxifying activities that remove the NO formed as a by-product of nitrite accumulation during the DNRA process (Simon et al., 2004; Sanford et al., 2012; Jones et al., 2013). However, another group of DNRA including enterobacteria such as

Escherichia coli or *Salmonella enterica* that also can produce N_2O do not have an enzyme that can consume it. Thus, these bacteria might contribute significantly to global N_2O emissions. A greater understanding of the key enzymes and environmental and regulatory factors involved in N_2O metabolism in denitrifiers and DNRA may allow the development of more effective N_2O mitigation strategies in soil nitrate reducing communities. The goal of this review is to present an overview of the enzymatic mechanisms of N_2O production and consumption by nitrate reducing bacteria, as well as the environmental signals and the regulatory pathways or networks involved.

2. NITROUS OXIDE METABOLISM IN NITRATE-AMMONIFYING BACTERIA

The metabolism of N_2O in organisms that grow by respiratory nitrate or nitrite ammonification is poorly understood. The respective organisms reduce NO_3^- to NO_2^- using a membrane-bound nitrate reductase (Nar) and/or a periplasmic nitrate reductase (Nap) (Richardson *et al*., 2001; Kern & Simon, 2009; Simon & Klotz, 2013). Subsequently, NO_2^- is reduced to NH_4 by a cytochrome *c* nitrite reductase (NrfA), which obtains electrons from the quinone/quinol pool through one of several different electron transport enzyme systems, depending on the organism (Simon, 2002; Kern & Simon, 2009; Simon & Klotz, 2013). Prominent examples of respiratory ammonifiers of NO_3^-/NO_2^- are Gamma-, Delta- and Epsilonproteobacteria such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Shewanella oneidensis*, *Shewanella loihica*, *Anaeromyxobacter dehalogenans*, *Campylobacter jejuni* and *Wolinella succinogenes* but also some less well-known members of the genus *Bacillus* (phylum Firmicutes), for example *Bacillus vireti, Bacillus azotoformans* or *Bacillus bataviensis* (Simon, 2002; Heylen & Keltjens, 2012; Simon & Klotz, 2013; Mania *et al*., 2014). With the exception of *S. loihica*, nitrate-ammonifying bacteria usually lack both the Cu-containing (NirK)

and cd_1-type (NirS) nitrite reductases as well as typical membrane-bound respiratory NO reductases (*c*Nor and qNor enzymes) found in denitrifiers. Apparently, however, the catalysis of respiratory ammonification of NO_3^-/NO_2^- is also a source of N_2O. In a first step leading to N_2O production, NO is generated either chemically and/or enzymatically from nitrite. The detailed mechanisms of these conversions, however, are yet to be elucidated. Since NO is a highly toxic compound that exerts nitrosative stress on cells and organisms, it needs to be detoxified (Poole, 2005). It is therefore not surprising that N_2O generation from NO has been described for numerous non-respiratory enzymes, including flavodiiron proteins (Fdp), flavorubredoxin (NorVW), cytochrome c_{554} (CycA; present in nitrifiers), cytochrome *c´*-beta (CytS) and cytochrome *c´*-alpha (CytP) (Simon & Klotz, 2013 and references therein). In these cases, NO reduction to N_2O is thought to serve predominantly in NO detoxification. In the light of such an N_2O-producing capacity, it is not surprising that some NO_3^-/NO_2^--ammonifiers such as *W. succinogenes*, *A. dehalogenans* and *B. vireti* have been reported to grow by anaerobic N_2O respiration using N_2O as sole electron acceptor (Yoshinari, 1980, Sanford *et al*., 2012; Kern & Simon, 2016; Mania *et al*., 2016). Moreover, the cells of some other species have been reported to reduce N_2O and many genomes of ammonifiers indeed contain a *nos* gene cluster (see section 2.2.2). These *nos* clusters comprise a *nosZ* gene encoding the "atypical" nitrous oxide reductase and some of them even a cytochrome *c* nitrous oxide reductase (*c*NosZ) (Table 7.1) (Simon *et al*., 2004; Zumft & Kroneck, 2007; Kern and Simon, 2009; Sanford *et al*., 2012; Jones *et al*., 2013; Simon and Klotz, 2013). The *c*NosZ enzyme is a variant of the canonical NosZ found in denitrifiers that contains a C-terminal monoheme cytochrome *c* domain, which is thought to donate electrons to the active copper site (Simon *et al*., 2004). Export of *c*NosZ to the

periplasm is accomplished by the Sec secretion pathway rather than by the Tat pathway used by the canonical NosZ.

2.1. Gammaproteobacteria

N_2O metabolism by Gammaproteobacteria that perform dissimilatory nitrate/nitrite reduction to ammonia (DNRA) has been mainly investigated in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. These bacteria belong to the Enterobacteriaceae family of Gammaproteobacteria which have their natural habitats in soil, water (fresh and marine) environments or the intestines of both warm and cold blooded animals. In humans, while *Salmonella* species are pathogenic and can result in an inflamed intestine and gastroenteritits, *E. coli* strains can form part of the normal flora having beneficial traits for humans.

In many species of Enterobacteriaceae, there are two biochemically distinct nitrate reductases: one membrane-bound with the active site located in the cytoplasm (Nar) and a periplasmic nitrate reductase (Nap). Nar enzymes have been most studied in *E. coli* and *Paracoccus* (reviewed by Potter et al., 2001, Richardson et al., 2001; González et al., 2006; Richardson et al., 2007; Richardson, 2011). Nar is common to both ammonification and denitrification and has been crystallographically resolved from *E. coli* (Bertero et al., 2003; Jormakka et al., 2004). It is a 3-subunit enzyme composed of NarGHI, where NarG is the catalytic subunit of about 140 kDa that contains a bismolybdopterin guanine dinucleotide (bis-MGD) cofactor and a [4Fe-4S] cluster. NarH, of about 60 kDa, contains one [3Fe-4S] and three [4Fe-4S] clusters. NarG and NarH are located in the cytoplasm and associate with NarI, an integral membrane protein of about 25 kDa with five transmembrane helices and the N-terminus facing the

periplasm (Fig. 7.2A). Nar proteins are encoded by genes of a *narGHJI* operon. Whereas *narGHI* encode the structural subunits, *narJ* codes for a cognate chaperone required for the proper maturation and membrane insertion of Nar. The organization of this operon is conserved in most species that express Nar. *E. coli* and *S.* Typhimurium have a functional duplicate of the *narGHJI* operon named *narZYWV*, which has a central role in the physiology of starved and stressed cells, rather than anaerobic respiration *per se* (Blasco et al., 1990, Spector et al., 1999). In the cytoplasm, a NADH-dependent assimilatory nitrite reductase (Nir) reduces nitrite to ammonia as rapidly as it is formed from nitrate by Nar (Fig. 7.2A). The *nir* operon includes *nirB* and *nirD* as structural genes for the two enzyme sub-units; a third gene, *nirC*, probably encodes a nitrite transport protein; and finally *cysG*, the product of which is required for the synthesis of the novel haem group, sirohaem (Peakman et al., 1990).

Enteric bacteria such as *E.coli* and *S.* Typhimurium have evolved a second respiratory pathway to survive in electron acceptor-limited anaerobic conditions. Under anoxic and microoxic conditions in the presence of low levels of nitrate, the periplasmic nitrate reductase (Nap) system and the periplasmic nitrite reductase (Nrf) system are expressed (Figs. 7.2 and 7.3A). NapA is the catalytic subunit responsible for the two electron reduction of NO_3^- to NO_2^-, while NrfA reduces NO_2^- to NH_4 through a six-electron reduction proposed to involve bound intermediates of nitric oxide (NO) and hydroxylamine (NH_2OH) (Einsle et al., 2002). In *E. coli*, the reduction of NO_3^- to NH_4 can be coupled to energy-conserving electron transport pathways with formate as an electron donor (Potter et al., 2001).The Nap system is found in many different Gram-negative bacteria (reviewed by Potter et al., 2001; González et al., 2006; Richardson et al., 2007; Richardson, 2011; Simon and Klotz, 2013). The best studied Nap enzymes were isolated from *Paracoccus pantotrophus*, *E. coli*, *Rhodobacter sphaeroides*, and

Desulfovibrio desulfuricans. The crystal structure of *E. coli* NapA has been solved (Jepson et al., 2007). Similar to NarG, NapA binds bis-MGD and a [4Fe-4S] cluster. In the majority of known cases, NapA forms a complex with the dihaem cytochrome *c* NapB. Generally, mature NapA is transported across the membrane by the Tat apparatus and this process requires the cytoplasmic chaperone NapD, which is encoded in all known *nap* gene clusters (Grahl et al., 2012). In the mayority of Nap systems, electron transfer from quinol to NapAB complex requires a tetrahaem cytochrome *c* NapC, a member of the NapC/NrfH family (Fig. 7.2A). However, in *E. coli* a second quinol-oxidizing system has been identified, the NapGH complex which consists of two proposed Fe/S proteins. NapH is a membrane-bound quinol dehydrogenase containing four transmembrane domains while NapG is a periplasmic electron transfer adapter protein (Fig. 7.2A). The structure and detailed function of the NapGH proteins, however, remain unclear as these have not been purified. In addition to *napDAGHBC* genes directly involved in nitrate reduction, *E. coli napFDAGHBC* operon also contains *napF* encoding an accessory protein. NapF is a cytoplasmic Fe/S protein that is thought to have a role in the post-translational modification of NapA prior to the export of folded NapA into the periplasm (Nilavongse et al., 2006).

The best-known periplasmic ammonium-generating nitrite reductase is the decahaem homodimeric cytochrome *c* nitrite reductase NrfA (Figs. 7.2 and 7.3A) (reviewed by Clarke et al., 2008; Einsle, 2011; Simon and Klotz, 2013). This enzyme reduces NO_2^- produced by Nap to NH_4 by using six electrons that are commonly obtained through the oxidation of formate (nitrite reduction with formate, Nrf). This allows NO_2^- to be used as a terminal electron acceptor, facilitating anaerobic respiration while allowing nitrogen to remain in a biologically available form. NrfA, first described in *E. coli* is expressed within the periplasm of a wide range of Gamma-, Delta- and

Epsilonproteobacteria. In *E. coli*, *nrfABCDEFG* genes are involved in the synthesis and activity of NrfA with *nrfA* coding for the actual enzyme, *nrfB* coding for a small, pentahaem electron transfer protein, *nrfC* and *nrfD* for a membrane-integral quinol dehydrogenase (Fig. 7.3A), and *nrfE*, *nrfF*, and *nrfG* for components of a dedicated assembly machinery required for attachment of the active site haem group. The electron transfer between NrfCD and NrfA in *E. coli* is mediated by the pentahaem cytochrome *c* NrfB (Clarke et al., 2007). Crystal structures of NrfA from *E. coli* are currently available (Bamford et al., 2002; Clarke et al., 2008). NrfA contains four His/His ligated *c*-haems for electron transfer and a structurally differentiated haem that provides the catalytic center for nitrite reduction. The catalytic haem has proximal ligation from lysine, or histidine, and an exchangeable distal ligand bound within a pocket that includes a conserved His. Recent experiments where electrochemical, structural and spectroscopic analyses were combined revealed that the distal His is proposed to play a key role in orienting the nitrite for N−O bond cleavage (Lockwood et al., 2015).

2.1.1 Enzymes involved in NO and N_2O metabolism

The cytotoxin nitric oxide (NO) is the major precursor of N_2O in many biological pathways, and the accumulation of N_2O in bacteria which lack NosZ, can be used as a direct reporter of intracelular NO production (Rowley et al., 2012). In prokaryotes, NO formation was considered to occur only in denitrification, anaerobic ammonium oxidation and other related respiratory pathways (Zumft, 1997; Bothe et al., 2007; Jetten, 2008; Schreiber et al., 2013; Maia and Moura, 2014). NO formation from NO_2^- constitutes the first committed step in denitrification and is an essential step in anaerobic ammonium oxidation and other respiratory pathways, where nitrogen

compounds are used to derive energy. For those respiratory functions, prokaryotes developed NirS-type (cytochrome cd_1) or NirK-type (copper containing) nitrite reductases to reduce NO_2^- to NO. Several studies have suggested that NO is also generated in prokaryotes by non-respiratory pathways via NO synthase (NOS) enzymes, homologous to the oxygenase domain of the mammalian NOS. NOS catalyses aerobic NO formation from arginine, using cellular redox equivalents that are not normally committed to NO production (reviewed by Spiro, 2011; Maia and Moura, 2015). *Salmonella* species and *E. coli* lack the typical respiratory NirS or NirK enzymes, as well as NOS, however they do produce NO as a side-product of nitrate or nitrite metabolism. Studies with *E. coli* mutants suggested that nitrite-dependent NO formation was assumed to arise from the "side" activity of the assimilatory sirohaem-containing, NirB, as well as from NrfA that both catalyse NO_2^- reduction to NH_4 (Corker and Poole, 2003; Weiss, 2006) (Fig. 7.2A). However, NO formation from NO_2^- in *S.* Typhimurium does not involve NirB or NrfA. Recently, reduction of NO_2^- by the membrane-bound nitrate reductase NarG has been proposed as one major source of NO in *E. coli* and *S. enterica serovar* Typhimurium (Fig. 7.2A). By contrary, a small contribution (less than 3 %) from the periplasmatic Nap to NO formation has been reported in both bacteria (Gilberthorpe and Poole, 2008; Vine et al., 2011; Rowley et al., 2012).

In addition to the catalysis of the six-electron reduction of NO_2^- to NH_4, *E. coli* NrfA has also the ability to act as an NO reductase. Kinetic, spectroscopic, voltammetric, and crystallization studies with purified NrfA have demonstrated the capacity of this enzyme to reduce NO (Clarke et al., 2008; Einsle, 2011). This capacity has also been reported in whole cells studies using wild-type and *nrf* mutant strains of *E. coli and S.* Typhimurium where a contribution by NrfA to NO stress tolerance has been demonstrated (Poock et al., 2002; Poole, 2005; Mills et al., 2008). *E. coli* and *S.*

Typhimurium are known to possess other NO-consuming systems to overcome NO produced by the immune system as well as to defend themselves against their own toxic metabolites. They comprise the soluble flavohaemoglobin Hmp, and the di-iron-centred flavorubredoxin NorV with its NADH-dependent oxidoreductase NorW (NorVW). Hmp is phylogenetically widespread, being found in denitrifying bacteria and non-denitrifiers (Vinogradov et al., 2013). This enzyme has a globin like domain, and an FAD-containing domain that binds NAD(P)H. In the presence of oxygen, Hmp oxidizes NO to nitrate, an activity that has been described as an NO dioxygenase or NO denitrosylase. A detailed description of Hmp enzymatic and structural properties have been published in several reviews (Gardner, 2005; Poole, 2005; Spiro, 2011; Forrester and Foster, 2012). Aside from NO dioxygenation, Hmp has also been shown to execute NO reduction to N_2O under anoxic conditions (Kim et al., 1999), which operates at approximately 1% of the rate of the aerobic dioxygenation reaction (Mills et al., 2001). Although this Hmp-based NO reduction may operate under anaerobic conditions, it remains somewhat unclear whether it provides physiologically relevant protection from nitrosative stress. Consequently, Hmp may not be a significant source of N_2O. The main candidate to reduce NO to N_2O in non-denitrifying bacteria is NorVW (Fig. 7.2A). The physiological role of this enzyme seems to be NO detoxification under anaerobic or micro-oxic conditions. This reaction may be particularly important in organisms (such as *E. coli* or *S.* Typhimurium) which make low concentrations of NO as a by-product of the reduction of NO_2^- to NH_4 and they lack the respiratory Nor enzymes typical from denitrifiers (reviewed by Poole, 2005; Spiro, 2011; Spiro, 2012). In NorVW, NO is reduced by a flavo-diiron protein, which receives electrons from a rubredoxin domain or protein. The rubredoxin is itself reduced by an NADH-dependent flavoenzyme. The flavo-diiron protein of *E. coli* and *S.* Typhimurium has a fused rubredoxin domain, and

so is called flavorubredoxin (also called NorV). In complex with the NADH-dependent oxidoreductase (NorW), this enzyme functions as an NO reductase *in vitro* (Gomes et al., 2002). Consistently, in *E. coli* and *Salmonella* it has been reported that protection against NO stress during anaerobic respiratory conditions was mainly attributed to the action of the flavorubredoxin NorV (Gardner et al., 2002; Mills et al., 2008; Mühlig et al., 2014). However, it should be noted that *S.* Typhimurium mutant strains lacking functional copies of *hmpA*, *norV* and *nrfA* are still able to resist anaerobic NO stress, albeit very poorly, indicating a role for other NO detoxification mechanisms in this bacterium (Mills et al., 2008). As observed in *S.* Typhimurium, *E. coli* single mutants defective in NirB, NrfA; NorV or Hmp and even the mutant defective in all four proteins reduced NO at the same rate as the parent. Clearly, therefore, there are mechanisms of NO reduction by enteric bacteria that remain to be characterized (Vine and Cole, 2011).

Although N_2O has been proposed to be a product of NO reduction in nitrate-ammonifiers, studies about the contribution of this bacterial group to N_2O emissions from agricultural soils as well as the mechanisms behind this are poorly understood. In this context, there have been a few reports of N_2O release by pure cultures of Enterobacteriaceae, including *E. coli*, *Klebsiella pneumoniae* and *S.* Typhimurium during NO_3^- metabolism that presumably reflects NO being converted into N_2O (Smith, 1983; Bleakley and Tiedje, 1982). In complex medium nutrient-sufficient batch culture experiments, the rate of N_2O production during nitrate ammonification was around 5% of NO_3^- (Bleakley and Tiedje, 1982). Thus, it has been suggested that enteric nitrate-ammonifying bacteria could be a significant source of N_2O in soil (Bleakley and Tiedje, 1982). In this context, it has been proposed that under high C-to-NO_3^- conditions, nitrate ammonification may be faster and produce greater quantities of N_2O than

denitrification, depending on enzyme regulation (Yin *et al*., 2002). Recently, it has been demonstrated the potential for N_2O production by soil-isolated nitrate-ammonifying bacteria under different C and N availabilities. By performing chemostat cultures, it has been shown that maximum N_2O production was correlated with high NO_2^- production under C-limitation/NO_3^-sufficiency conditions (Streminska et al., 2012).

As mentioned above, one major source of N_2O in *S.* Typhimurium is the reduction of NO produced by the membrane-bound nitrate reductase NarG (Gilberthorpe and Poole, 2008) (Fig. 7.2A). In this context, kinetics analyses of NO_3^- consumption, NO_2^- accumulation and N_2O production by chemostat cultures of *S.* Thyphimurium *nap* or *nar* mutants confirmed that Nar is the major enzymatic route for NO_3^- catabolism associated with N_2O production (Rowley et al., 2012). While in nitrate-sufficient cultures, a *narG* mutant produced ~30-fold more N_2O than the wild-type, under nitrate-limited conditions, *nap*, but not *nar*, was upregulated and very little N_2O production was observed. Thus, these authors conclude that a combination of NO_3^--sufficiency, NO_2^- accumulation and an active Nar-type nitrate reductase leads to NO and thence N_2O production, and this can account for up to 20% of the NO_3^- catabolized (Rowley et al., 2012).

2.1.2 Regulatory proteins

The main regulators that mediate NO detoxification and consequently N_2O formation in *Salmonella* and *E. coli* include NorR, NsrR, and FNR (reviewed by Spiro 2007, 2011, 2012; Tucker et al., 2011; Arkenberg et al., 2011; Mettert and Kiley, 2015) (Fig. 7.2B). NorR is a member of the σ54-dependent enhancer-binding protein (EBP) family of transcriptional activators that has a three-domain structure that is typical of EBPs, with

a C-terminal DNA-binding domain, a central domain from the AAA^+ family that has ATPase activity and interacts with RNA polymerase (Bush et al., 2010), and an N-terminal signalling domain. The N-terminal regulatory GAF domain of NorR contains a mononuclear non-haem iron centre, which reversibly binds NO. Binding of NO stimulates the ATPase activity of NorR, enabling the activation of transcription by RNA polymerase. The mechanism of NorR reveals an unprecedented biological role for a non-haem mononitrosyl–iron complex in NO sensing (D'Autreaux et al., 2005; Tucker et al., 2008). NorR is an transcriptional activator of *E. coli norVW* genes in response to NO (Hutchings et al., 2002; Gardner et al., 2003) (Fig. 7.2B).

NsrR is a NO-sensitive transcriptional repressor that contains an [Fe–S] cluster. The cluster is likely to be [4Fe–4S] and is the binding site for NO. Nitrosylation of this cluster leads to a loss of DNA binding activity and, hence, derepression of NsrR target genes (Bodenmiller and Spiro, 2006; Yukl et al., 2008; Tucker et al., 2008; Crack et al., 2015). The NsrR binding site is an 11-1-11 bp inverted repeat of the consensus motif AAGATGCYTTT (Bodenmiller & Spiro, 2006), although chromatin immunoprecipitation (ChIP-chip) analysis suggested that a single 11 bp motif (with the consensus sequence AANATGCATTT) can function as an NsrR-binding site *in vivo* (Partridge et al., 2009). Very recently, it has been demonstrated that although *nsrR* is expressed from a strong promoter, however its translation is extremely inefficient, leading to a low cellular NsrR concentration. Thus, promoters with low-affinity NsrR binding sites may partially escape NsrR-mediated repression (Chhabra and Spiro, 2015). Using comparative genomics approaches, the most conserved member of the predicted NsrR regulon was found to be *hmp* (Rodionov et al., 2005). However, microarray analysis revealed that NsrR represses nine operons encoding 20 genes in *E. coli*, including the *hmp*, and the well-studied *nrfA* promoter that directs the expression

of the periplasmic respiratory nitrite reductase (Filenko et al., 2007). Regulation of the *nrf* operon by NsrR is consistent with the ability of the periplasmic nitrite reductase to reduce nitric oxide and hence protect against reactive nitrogen species (Fig. 7.2B).

FNR (Fumarate-nitrate reduction regulator) belongs to the subgroup of the cyclic-AMP receptor protein family of bacterial transcription regulators. FNR is a O_2-sensitive protein involved in gene expression to coordinate the switch from aerobic to anaerobic metabolism when facultative anaerobes like *E. coli* are starved of O_2 (Constantinidou, et al., 2006; Partridge et al., 2007; Rolfe et al., 2012; Myers et al., 2013). The N-terminal region of FNR contains four essential cysteine residues that coordinate an O_2-sensitive [4Fe-4S] cluster (Crack et al., 2012; Zhang et al., 2012). In the absence of O_2, the [4Fe-4S] cluster is stable, and FNR exists as a homodimer that is capable of high affinity, site-specific DNA binding to an FNR box (TTGATNNNNATCAA). When bound to target DNA, FNR activates the expression of genes encoding proteins required for anaerobic metabolism and represses those utilized under aerobic conditions. In addition to its primary function in mediating an adaptive response to O_2-limitation, FNR plays a role in sensing and responding to NO. NO damages the *E. coli* FNR [4Fe–4S] cluster *in vitro*, resulting in decreased FNR DNA binding activity (Crack et al., 2013). In the absence of nitrogen oxides, *hmp is* repressed by FNR, but the addition of either nitrite or nitrate causes a derepression of *hmp* gene expression (Cruz-Ramos et al., 2002). Conversely, transcription from the *E. coli nrf* operon is activated by FNR in the absence of oxygen and induced further by NarL and NarP in response to low concentrations of nitrate or to nitrite (Tyson et al., 1994) (Fig. 7.2B). Consistent with the additional NO detoxifying function of Nrf, recent studies have suggested that *pnrf* is also regulated by the global transcription repressor NsrR (Filenko et al., 2007; Partridge et al., 2009). In this context, it has been demonstrated

that FNR-dependent activation of the *E. coli nrf* promoter is downregulated by NsrR together with the nucleoid-associated protein IHF, which bind to overlapping targets adjacent to the DNA site for FNR (Browning et al., 2010). Interestingly, alignment of the *pnrf* sequence from *Salmonella* Typhimurium with that of *E. coli* revealed a base difference in the DNA site for NsrR that would be expected to decrease NsrR binding. In fact, anaerobic expression from the *Salmonella nrf* promoter is unaffected by the disruption of *nsrR* (Browning et al., 2010), suggesting that in contrast to *E. coli*, *Salmonella nrf* promoter appears to have become "blind" to repression by NsrR, though it remains to be seen if this has any biological significance.

2.1.3 Nitrate-ammonification and denitrification pathways in Shewanella loihica

Until recently, the general understanding had been that denitrification and respiratory nitrate ammonification pathways do not coexist within a single organism. However, recent genome analyses found that at least three different bacterial species, *Opitutus terrae* strain PB90-1, *Marivirga tractuosa* strain DSM 4126, and the Gammaproteobacterium *Shewanella loihica* strain PV-4, possess the complete sets of genes encoding the pathways for denitrification and respiratory ammonification (Sanford et al., 2012). *S. loihica* strain PV-4 possesses two copies of *nrfA*, as well as the complete suite of genes encoding denitrification enzymes (*nirK*, *norB* and *nosZ*) (Sanford et al., 2012; Yoon et al., 2013). The functionality of both the denitrification and the respiratory ammonification pathways has been recently confirmed (Yoon et al., 2015a). Batch and continuous culture experiments using *S. loihica* strain PV-4 revealed that denitrification dominated at low carbon-to-nitrogen (C/N) ratios (that is, electron donor-limiting growth conditions), whereas ammonium was the predominant product at

high C/N ratios (that is, electron acceptor-limiting growth conditions) (Yoon et al., 2015a). In addtion to C/N ratio, pH and temperature also affected NO_3^-/NO_2^- fate being ammonium formation favored by incubation above pH 7.0 and temperatures of 30 ºC (Yoon et al., 2015a). Recent findings revealed that the NO_2^-/NO_3^- ratio also affected the distribution of reduced products, and respiratory ammonification dominated at high NO_2^-/NO_3^- ratios, whereas low NO_2^-/NO_3^- ratios favored denitrification (Yoon et al., 2015b). These findings implicate NO_2^- as a relevant modulator of NO_3^- fate in *S. loihica* strain PV-4, and, by extension, suggest that NO_2^- is a relevant determinant for N retention (i.e., ammonification) versus N loss and greenhouse gas emission (i.e., denitrification).

2.2. Epsilonproteobacteria

2.2.1. Respiratory reduction of nitrate and nitrite, detoxification of NO and the concomitant generation of N_2O

Epsilonproteobacteria comprise host-associated heterotrophic species (exemplary genera are *Campylobacter*, *Helicobacter* and *Wolinella*) as well as free-living species that have been isolated mostly from sulfidic terrestrial and marine habitats (*Sulfurospirillum*, *Sulfurimonas*, *Nautilia*) (Campbell *et al.*, 2006). Epsilonproteobacterial cells usually grow at the expense of microaerobic or anaerobic respiration and many species use hydrogen, formate or reduced sulfur compounds, such as sulfide or thiosulfate as electron donor substrates. Nitrate is a prominent electron acceptor in Epsilonproteobacteria and is initially reduced to nitrite by the Nap enzyme system of nitrate respiration. The non-fermentative rumen bacterium *W. succinogenes* has been used for a long time as an epsilonproteobacterial model organism to

investigate the multitude of electron transport chains that couple anaerobic respiration to ATP generation. *W. succinogenes* cells may use formate, hydrogen gas or sulfide as electron donors and either fumarate, nitrate, nitrite, N_2O, dimethyl sulfoxide (DMSO), polysulfide or sulfite as electron acceptors (Kröger *et al*., 2002; Simon, 2002; Klimmek *et al*., 2004; Kern & Simon, 2009; Kern *et al*., 2011a; Simon & Klotz, 2013; Simon & Kroneck, 2013; 2014; Hermann *et al.*, 2015; Kern & Simon, 2015 and references therein). The cells are also capable of microaerobic respiration and the complete genome sequence suggests the existence of further electron acceptors such as arsenate or tetrathionate (Baar *et al*., 2003). With respect to the physiology and enzymology of respiratory nitrate ammonification, *W. succinogenes* is arguably the best characterized member of the Epsilonproteobacteria (reviewed by Simon, 2002; Kern and Simon, 2009; Simon and Klotz, 2013). Like many other Epsilonproteobacteria, the cells employ a periplasmic nitrate reductase (NapA) for nitrate reduction to nitrite and the latter is subsequently reduced to ammonium by cytochrome *c* nitrite reductase (NrfA).

Epsilonproteobacterial *nap* gene clusters generally lack a *napC* gene but, instead, NapG and NapH proteins are encoded (Kern and Simon, 2008). The NapGH complex is thought to constitute a menaquinol-oxidizing complex, in which NapH presumably acts as a membrane-bound quinol dehydrogenase containing four transmembrane domains while NapG is a periplasmic Fe-S protein that is thought to deliver electrons to the diheme cytochrome *c* NapB (or a NapAB complex)(Fig. 7.2A). In *W. succinogenes*, the Nap system is encoded by the *napAGHBFLD* gene cluster. The role of individual *nap* genes in *W. succinogenes* has been assessed by characterizing non-polar gene inactivation mutants (Kern *et al*., 2007; Kern & Simon 2008; 2009). NapB and NapD were shown to be essential for growth by nitrate respiration, with

NapD being required for the production of mature NapA. The inactivation of either *napH* or *napG* almost abolished growth without affecting the formation and activity of NapA. The cytoplasmic Fe/S protein NapF was shown to interact with NapH. NapF could be involved in electron transfer to immature NapA. Inactivation of *napL* did only slightly affect the growth behaviour of mutant cells although the NapA-dependent nitrate reductase activity was clearly reduced. The function of NapL, however, is not known.

In contrast to *E. coli* and other Gammaproteobacteria, the epsilonbacterial NrfA cytochrome *c* nitrite reductase forms a subunit of a membrane-bound menaquinol-reactive complex that also contains a tetraheam cytochrome *c* of the NapC-type called NrfH (Simon *et al*., 2000; Rodrigues *et al*., 2006; Kern *et al*., 2008; Einsle, 2011; Simon & Kroneck, 2014) (Fig. 7.3B). Such NrfHA complexes form a membrane-associated respiratory complex on the extracellular side of the cytoplasmic membrane that catalyses electroneutral menaquinol oxidation by nitrite. In *W. succinogenes* the structural genes *nrfA* and *nrfH* are part of an *nrfHAIJ* gene cluster. The product of the *nrfI* gene is a membrane-bound cytochrome *c* synthase of the CcsBA-type, which is a crucial enzyme of the so-called system II of cytochrome *c* biogenesis (Simon & Hederstedt, 2011). *W. succinogenes* NrfI was shown to play a crucial role in NrfA biogenesis as it is required for the attachment of the CX_2CK-bound and thus lysine-ligated haem 1 in NrfA (Pisa *et al*., 2002; Kern *et al*., 2010). No function in nitrite respiration could be assigned to NrfJ as concluded from the characterization of a corresponding gene deletion mutant (Simon *et al*., 2000).

NrfA proteins have a remarkable substrate range since they catalyse the reduction of nitrite, NO and hydroxylamine to ammonium (Stach *et al*., 2000; Simon *et al*., 2011; Simon & Kroneck, 2014). NrfA was also reported to produce N_2O as a product of NO reduction under suitable conditions (Costa *et al*., 1990) and to react with N_2O to a so far unidentified product (Stach *et al*., 2000). Furthermore, NrfA catalyses the decomposition of hydrogen peroxide and the reduction of sulfite to hydrogen sulfide, which is an isoelectronic reaction to ammonium production from nitrite (Lukat *et al*., 2008; Kern *et al*., 2011b). The reactive promiscuity of NrfA has been shown to mediate the stress response to NO_2^-, NO, hydroxylamine and hydrogen peroxide in *W. succinogenes* cells indicating that NrfA has a detoxifying function in cell physiology (Kern *et al*., 2011b). Apart from NrfA, a cytoplasmic flavodiiron protein (Fdp) has been proposed to be involved in nitrosative stress defence in *W. succinogenes* (Kern *et al*., 2011b). As proposed previously for these type of Fdps (Saraiva *et al*., 2004), *W. succinogenes* Fdp is assumed to reduce NO to N_2O. However, this reaction has not been demonstrated for *W. succinogenes* Fdp since the protein has not been purified. Further possible NO reductases in *W. succinogenes* are the hybrid cluster protein (Hcp) and a homolog of *Helicobacter pylori* NorH (Ws1903) (Kern et al., 2011b; Justino *et al*., 2012; Luckmann et al., 2014). The contribution of these proteins to N_2O production, however, has to be clarified in the future.

The capacity of *W. succinogenes* to produce N_2O during growth by nitrate ammonification has been recently examined using nitrate-sufficient or nitrate-limited medium containing formate as electron donor (Luckmann *et al*., 2014). It was found that cells growing in nitrate-sufficient medium (80 mM formate and 50 mM nitrate) produced small amounts of N_2O (about 0.15% of nitrate-N), which derived from accumulated nitrite and, most likely, from the presence of NO. In contrast, nitrite is only

transiently formed during growth in nitrate-limited medium (80 mM formate and 10 mM nitrate) and both NO and N_2O could not be detected under these conditions (Luckmann *et al*., 2014). However, the question remains how NO is generated from nitrite by *W. succinogenes* since NapA and NrfA are unlikely to release NO as a by-product (as opposed to the membrane-bound Nar-type nitrate reductase complex; see section 2.1). In the experiments described by Luckmann *et al*. (2014), NO might have been generated by chemical reactions between components of the medium and nitrite. Taken together, there is clear evidence that *W. succinogenes* cells are able to produce N_2O as a result from NO production and subsequent detoxification. It is quite likely that these features do also hold true for other Epsilonproteobacteria that contain similar *nap*, *nrf* and *nos* gene clusters, for example free-living species of the genus *Sulfurospirillum* (Kern & Simon, 2009) as well as host-associated *Campylobacter* species (Payne *et al*., 1982; Schumacher & Kroneck, 1992). Interestingly, Kaspar & Tiedje (1981) reported that the nitrate-ammonifying rumen microbiota accumulated up to 0.3% of the added nitrate-N as N_2O.

2.2.2 Growth by N_2O respiration and reduction of N_2O by the atypical cytochrome c nitrous oxide reductase system

More than three decades ago, *W. succinogenes* and *Campylobacter fetus* cells have been reported to grow by N_2O respiration using formate as electron donor (Yoshinari, 1980; Payne et al., 1982). However, only recently a corresponding growth curve for *W. succinogenes* has been provided that allowed to determine a doubling time of 1.2 h and to estimate a growth yield of about 10 g dry cells per mole formate (Kern & Simon, 2015). Interestingly, this value is higher than the reported maximal cell yield of

fumarate respiration (8.5 g of dry cells per mole formate; Bronder *et al*., 1982) as well as of nitrate and nitrite respiration (5.6 g and 5.3 g of dry cells per mole formate, respectively; Bokranz *et al*., 1983). In the latter three mode of anaerobic respiration the proton motive force (*pmf*) is built up by the redox loop mechanism of formate dehydrogenase (Richardson & Sawers, 2002; Simon *et al*., 2008). Furthermore, it has been shown that menaquinol oxidation by fumarate or nitrite are electroneutral processes (Simon *et al*., 2000; Kröger *et al*., 2002; Lancaster *et al*., 2005) and, originally, the same was expected for menaquinol oxidation by nitrate or N_2O given the postulated architecture of the corresponding electron transport chains that are envisaged to comprise homologous menaquinol dehydrogenases (NapGH or NosGH; Figs 7.2A and 7.5; see also below) (Simon *et al*., 2004; Kern and Simon, 2008; Simon and Klotz, 2013 and references therein). In the light of the cell yield of N_2O respiration, it remains to be seen whether menaquinol oxidation by N_2O might involve a hitherto undiscovered *pmf*-generating process that is absent in nitrate respiration. Conceivable scenarios comprise involvement of the cytochrome bc_1 complex (electrogenic menaquinol oxidation through the Q cycle mechanism) and/or the as yet uncharacterised polytopic membrane protein, NosB, that might work as a menaquinol-reactive proton pump (Fig. 7.5). The presence of the corresponding gene is conserved in epsilonproteobacterial *nos* gene clusters with *nosB* being surrounded by the *nosZ* and *nosD* genes in most cases (Fig. 7.4) (van Spanning, 2011; Sanford *et al*., 2012).

The *W. succinogenes nos* gene cluster belongs to the atypical clusters and contains *nosZ, -B, -D, -G, -C1, -C2, -H, -F, -Y* and *-L* genes (Simon *et al*., 2004; Sanford *et al*., 2012) (Fig. 7.4). The NosG, -C1, -C2 and -H proteins were postulated to encode a putative electron transport pathway from menaquinol to *c*NosZ (Fig. 7.5). This

pathway comprises a NosGH menaquinol dehydrogenase complex and two cytochromes *c* (NosC1 and NosC2). NosG and NosH are highly similar to NapG and NapH and therefore expected to form a NosGH complex that is functionally equivalent to NapGH. NosC1 and NosC2 are monohaem cytochromes *c* located either in the periplasm or attached to the membrane via an N-terminal helix. Ultimately, electrons are thought to be transferred via the cytochrome *c* domain of *c*NosZ to the copper-containing catalytic site of N_2O reduction. The *nosF*, *-Y*, and *-D* genes are likely to encode a membrane-bound ABC transporter and the *nosL* gene is thought to predict a copper chaperon involved in metallocenter assembly (Zumft, 2005; Zumft & Kroneck, 2007). In analogy to what has been proposed for denitrifiers (see Fig. 7.8), the NosF, -Y, -D- and -L proteins might be involved in the maturation of atypical Nos systems. Many other Epsilonproteobacteria also possess atypical *nos* gene clusters resembling that of *W. succinogenes* and it is conspicuous that the presence and arrangement of the *nosB*, *-G*, *-H*, *-C1* and *-C2* genes seem to be strictly conserved (Fig. 7.4).

2.2.3 Transcriptional regulation of the W. succinogenes nos gene cluster

In *W. succinogenes*, the respiratory Nap, Nrf and *c*Nos enzymes involved in N_2O metabolism are up-regulated in response to the presence of either nitrate, the NO-releasing compounds sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO) or spermine NONOate or of N_2O, but not to nitrite or hydroxylamine (Kern *et al.*, 2011c; Kern & Simon, 2015). However, nitrate-responsive two-component systems homologous to NarXL/NarQP from *E. coli* and other enteric bacteria are not encoded in the *W. succinogenes* genome. Furthermore, well-characterized NO-responsive proteins such as NsrR and NorR as well as NO-reactive transcription regulators of the Crp-Fnr superfamily, for example the Fnr, NNR/NnrR, Dnr and NarR proteins, are also absent in

W. succinogenes. Instead, *W. succinogenes* cells employ three transcription regulators of the Crp-Fnr superfamily (homologs of *Campylobacter jejuni* NssR; Nitrosative stress sensing Regulator; Elvers *et al*., 2005), designated NssA, NssB and NssC, to mediate up-regulation of Nap, Nrf and *c*Nos via dedicated signal transduction routes (Fig. 7.6, Kern & Simon, 2015). Analysis of single *nss* mutants revealed that NssA controls production of the Nap and Nrf systems in fumarate-grown cells while NssB was required to induce the Nap, Nrf and *c*Nos systems specifically in response to NO-generators (Fig. 7.6). NssC was indispensable for *c*Nos production under any tested condition. Moreover, N_2O apparently induced the Nap and Nrf systems independently of any Nss protein. The data implied the presence of an N_2O sensing mechanism since up-regulation of Nap, Nrf and *c*Nos was found in N_2O-gassed formate/fumarate medium, i.e. in the absence of notable amounts of nitrate or NO.

Nss proteins contain an N-terminal effector domain and a C-terminal DNA binding domain. In *Campylobacter jejuni*, which lacks *nos* genes altogether, NssR was found to be involved in the expression of genes encoding a single domain haemoglobin (Cgb) and truncated haemoglobin (Ctb) in response to NO/nitrosative stress conditions (Elvers *et al*., 2005, Monk *et al*., 2008). An *nssR* disruption mutant was found to be hypersensitive to NO-related stress conditions (Elvers *et al*., 2005). The *C. jejuni* NssR protein was purified and shown to bind specifically to the *ctb* promoter by electrophoretic mobility shift assays (Smith *et al*., 2011). Most likely, this binding was accomplished via an FNR-like binding site with a TTAAC-N_4-GTTAA consensus sequence (Elvers *et al*., 2005) that, however, is absent upstream of the *C. jejuni nap* and *nrf* gene clusters. Interestingly, DNA regions upstream of the *W. succinogenes nap, nrf* and *nos* gene clusters contain potential Nss-binding sites (consensus sequence TTGA-N_6-TCAA) within reasonable distances to the respective transcriptional start sites. In the

future, it will be most interesting to characterize the different N-terminal effector domains of NssR, NssA, NssB and NssC and whether these are directly or indirectly involved in cytoplasmic signal sensing. To date, it cannot be excluded that such domains are reactive with nitrogen compounds such as nitrate, NO or even N_2O.

2.3. Nitrate-ammonifying *Bacillus* species

Stremińska *et al*. (2012) demonstrated that nitrate-ammonifying soil isolates of the genus *Bacillus* formed N_2O (up to 2.7 % of nitrate was found to be reduced to N_2O) under nitrate-sufficient conditions (low C-to-nitrate ratio). Furthermore, the genomes of several other *Bacillus* species including *Bacillus vireti*, *Bacillus azotoformans* and *Bacillus bataviensis* were reported to encode a cytochrome *c* nitrite reductase complex (NrfHA) in addition to the presence of one or more atypical *nos* gene clusters (Heylen & Keltjens, 2012; Mania *et al*., 2014). In fact, the *B. azotoformans* genome encodes three atypical N_2O reductases (lacking the monohaem cytochrome *c* domain found in Epsilonproteobacteria) in different genetic contexts (Heylen & Keltjens, 2012). Each of the gene clusters includes a copy of *nosB* but lacks *nosG*, *-H*, *-R* and *-X* genes. Cells of *B. vireti* have been described to grow as nitrate ammonifiers in the presence of 5 mM nitrate although their nitrous oxide reductase was also found to be active in generating N_2 under these conditions (Mania *et al*., 2014). More recently, evidence was provided that N_2O reduction is coupled to growth of *B. vireti* cells (Mania et al., 2015). On the other hand, the *B. vireti* genome does not encode any obvious gene for an NO-generating nitrite reductase (NirS or NirK) and thus the cells do not qualify to be termed a classical denitrifier (Mania *et al*., 2014; Liu *et al*., 2015). It seems reasonable to assume that the mentioned *Bacillus* species are respiratory nitrate ammonifiers that are also capable to reduce N_2O formed as a product of NO detoxification. Surely, it is

desirable to explore these environmentally important organisms using suitable gene deletion mutants but corresponding genetic systems remain to be established in most cases. Recently, mutants of *B. vireti* lacking either the *narG* or *nrfA* gene have been successfully constructed and their physiology will be investigated in the future (Michelle Nothofer, Tamara Heß and J. Simon, unpublished data).

3. NITROUS OXIDE METABOLISM IN DENITRIFYING BACTERIA

Despite various sources for nitrous oxide (N_2O) emission in soils (see the introduction from this chapter), it has been estimated that over 65% of atmospheric N_2O is derived from microbial nitrification and denitrification (Thomson *et al*., 2012). Of these processes, denitrification is currently considered to be the largest source of N_2O. Denitrification commonly proceeds with respiratory reduction of the water-soluble nitrogen (N)-oxyanion nitrate (NO_3^-), which is readily bioavailable and abundant in many terrestrial and aquatic ecosystems. The nitrite (NO_2^-) formed from dissimilatory NO_3^- reduction is subsequently converted to gaseous N-oxide intermediates, including the highly reactive cytotoxic free-radical and ozone-depleting agent nitric oxide (NO), and the potent and long-lived greenhouse gas N_2O, which can be further reduced to dinitrogen (N_2) gas. Here, each of the N-oxyanions and N-oxides described may act as an individual terminal electron acceptor. Therefore, the reactions of denitrification underpin alternative and elaborate respiratory chains that function in the absence of the terminal oxidant, oxygen (O_2) to enable facultative aerobic microorganisms to survive and multiply under anaerobic conditions.

When faced with a shortage of O_2, although many bacterial species may have the potenital to tailor their respiratory pathways, the identity (i.e. complement of active

denitrification enzymes) and environmental conditions largely determine whether a denitrifier serves as a source or sink for N_2O (Thomson *et al*., 2012). Denitrification is widespread within the domain of *Bacteria* and appears to be dominant within *Proteobacteria* (Shapleigh, 2006). However, there is evidence that some fungi (Takaya et al., 2002, Prendergast-Miller et al 2011) and archaea (Treush et al., 2005) may also denitrify. The reactions of denitrification are catalysed by periplasmic (Nap) or membrane-bound (Nar) nitrate reductase, nitrite reductases (CuNir/cd_1Nir), nitric oxide reductases (cNor, qNor, or qCuANor) and nitrous oxide reductase (Nos) encoded by *nap*/*nar*, *nirK*/*nirS*, *nor* and *nos* genes, respectively (Fig. 7.7). Reviews covering the physiology, biochemistry and molecular genetics of denitrification have been published elsewhere (Zumft et al., 1997; van Spanning et al., 2005, 2007; Kraft et al., 2011; Richardson, 2011; Bueno et al., 2012).

Most denitrifiers have Nap and Nar enzymes and depending on the species, Nap is employed for anaerobic nitrate respiration as a part of bacterial ammonification (see sections 2.1 and 2.2 from this review), to promote denitrification (see section 4 from this review) or as electron sink during aerobic (photo)organoheterotrophic growth on reduced carbon sources to ensure redox homeostasis to dissipate excess reductant. This is the case of *Paracoccus denitrificans* considered as a model denitrifier in Alphaproteobacteria. In this bacterium, nitrate is reduced to nitrite by the membrane-bound nitrate reductase (NarGHI). In addition to Nar, *Pa. denitrificans* synthesizes the periplasmic nitrate oxidoreductases (NapABC) where as in the mayority of Nap systems, electron transfer from quinol to NapAB complex requires a the tetraheme cytochrome *c* NapC (Fig. 7.7). While NarGHI reduces nitrate as the first step of growth-linked anaerobic denitrification, NapABC serves to dissipate excess reducing equivalents formed during aerobic growth. These enzymes have been studied at the

biochemical level and derive electrons from the ubiquinol pool (reviewed by Potter et al., 2001; Gonzalez et al., 2006; Richardson et al., 2007; Richardson, 2011; Simon and Klotz, 2013, for detailed information see chapter 2.1). With the excepcion of some archaeal and bacterial examples of Nar-type nitrate reductases with an active site on the outside of the cytoplasmic membrane (Martinez-Espinosa et al., 2007), most Nar enzymes are oriented such that the active site for nitrate reduction is exposed to the cytoplasm being dependent on a nitrate transport system. In *Pa. denitrificans*, NarK has been identified as a nitrate importer that moves nitrate into the cytoplasm and also exports nitrite, the product of nitrate reduction, to the periplasm to support respiratory denitrification. NarK, is a fusion protein of two transmembrane domains NarK1 and NarK2, NarK1 is a proposed proton-linked nitrate importer, and NarK2 is a putative nitrate/nitrite antiporter (Wood et al., 2002; Goddard et al., 2008).

As we mention above, two types of respiratory nitrite reductases (Nir) have been described in denitrifying bacteria, NirS and NirK (Rinaldo and Crutuzzolá, 2007; Rinaldo et al., 2008, van Spanning, 2011). They catalyze the one-electron reduction of nitrite to nitric oxide, however, neither of the enzymes is electrogenic. Both are located in the periplasmic space, and receive electrons from cytochrome *c* and/or a blue copper protein, pseudoazurin, via the cytochrome bc_1 complex (Fig. 7.7). NirS is a homodimeric enzyme with hemes *c* and d_1. Electrons are transferred via the haem *c* of NirS to haem d_1, where nitrite binds and is reduced to nitric oxide (Rinaldo et al., 2008). The best-characterized *nirS* gene clusters are those from *Pseudomonas aeruginosa* (*nirSMCFDLGHJEN*) and *Pa. denitrificans* (*nirXISECFDLGHJN*). In the model denitrifier *Pseudomonas stutzeri,* there are two *nir* clusters (*nirSTBMCFDLGH* and *nirJEN*) which are separated by one part of *nor* gene cluster encoding nitric oxide reductase. The *nirS* gene encodes the functional subunits of the dimeric NirS. All other

genes are required for proper synthesis and assemblage of the d_1 heme and related functions (reviewed by van Spanning, 2011). NirK enzymes are homotrimeric complexes harboring three type I, and three type II copper centers, which form the active site (Fig. 7.7). Nitrite binds to the type II site where it is reduced to nitric oxide by electrons transferred from the type I copper site. In contrast to the complex organization of the genes encoding the NirS proteins, the Cu-NirK enzyme is encoded by the *nirK* gene (Rinaldo and Crutuzzolá, 2007; van Spanning et al., 2011). Here it must be noted that expression of NirK requires only a single gene, sometimes accompanied with a second one expressing a protein called NirV. The latter enzyme is related to desulfurates and may well be required for proper insertion of the copper reaction centre. As yet, there has been no organism found to have both types of nitrite reductases, so apparently the presence of either type of reductase excludes the option of gaining the other type.

The major contributor to the biological production of N_2O in many environments is the respiratory NO reductase (Nor) found in denitrifying bacteria and in some ammonia-oxidizing organisms. Then, N_2O is consumed through respiratory reduction to N_2 catalysed by the N_2O reductase (N_2OR) which completes the final reduction step in the denitrification pathway (Zumft, 2007) and is generally considered the sole enzyme able to interact with N_2O. However, various authors have suggested the existence of an alternative N_2O consumption pathway in wich N_2O is reduced to ammonium (NH_4^+) by nitrogenase, the enzyme involved in N_2 fixation (Jensen & Burris, 1986; Yamazaki et al., 1987; Burgess and Lowe, 1996). In fact, both N_2OR and nitrogenase are found in many denitrifiers (Shapleigh, 2006). Recent isotope tracing experiments by using *Ps. stutzeri* showed that consumption of N_2O via assimilatory reduction to NH_4^+ did not ocurr (Desloover et al., 2014). However, the latter studies showed that respiratory N_2O

reduction can be coupled to N_2 fixation as N_2O is first reduced to N_2 before is further reduced to NH_4^+ and incorporated into cell protein. This mechanism plays a signiticant role as an additional sink for N_2O involved in climate change mitigation.

Given the importance of Nor and N_2OR enzymes for N_2O formation during denitrification, it appear to be essential to progress in the current knowledge about these enzymes considered natural targets in the search for options to mitigate N_2O emission from agricultural soils.

3.1 Nitric Oxide Reductases

Nitric oxide reductase (Nor) enzymes catalyze NO reduction at the outside of the cytoplasmic membrane and most of them have been characterized in denitrifying *Proteobacteria* (reviewed by Hendriks et al., 2000; Zumft, 2005; de Vries et al., 2007; Richardson, 2011; Spiro, 2012). The best known NO reductases are cNor and qNor that either use cytochrome *c*/cupredoxins or quinones as immediate redox partners and both belong to the superfamily of heme-copper oxygen reductases (HCOs) (Fig. 7.7). The catalytic site of NO reduction harbors a dinuclear haem b_3::FeB active site that is reduced by another haem *b* group bound by the same protein (NorB). In cNor enzymes, NorB receives electrons from the monohaem cytochrome *c* subunit NorC while qNor enzymes are quinol-reactive single-subunit enzymes that resemble NorB (Fig. 7.7). In *Pa. denitrificans*, pseudoazurin or cytochrome c_{550} were found to donate electron to the NorC subunits of a heterotetrameric $(NorBC)_2$ complex (Hendriks et al., 1998) (Fig. 7.7). The best-characterized cNors are those from *Pa.* denitrificans, *Ps. stutzer*i and *Ps. aeruginosa*. The structure of the NorBC complex from *Ps. aeruginosa* (Hino et al., 2010) confirmed the predicted presence of 12 membrane-spanning α-helices in NorB while NorC is anchored to the membrane by a single membrane-spanning segment.

Biochemical experiments indicated that the protons required for NO reduction are taken from the periplasmic side of the membrane, and that NorB does not function as a proton pump (Bell et al., 1992). The latter is confirmed in the structure by the absence of trans-membrane proton channels in NorB analogous to those found in the proton-translocating haem-copper oxidases (Hino et al., 2010). Based on the crystal structure of *c*Nor from *Ps. aeruginosa* and molecular dynamics simulations, three different proton transfer pathways were proposed, all leading from the periplasmic side of the membrane (Hino et al., 2010; Shiro et al., 2012; Pisliakov et al., 2012). It has been demonstrated by site-directed mutation that *Pa. denitrificans* cNor is sensitive to mutations along the previously suggested proton transfer pathway 1 but not the others. Thus, although no energy is conserved, proton transfer still occurs through a specific pathway in *P. denitrificans* cNor (ter Beek et al., 2013). Furthermore, the formation of the hyponitrite (HO–N=N–O−) species in the haem b_3 Fe–FeB dinuclear center of cNor from *Pa. denitrificans* has been recently demosntrated (Daskalakis et al., 2015).

In contrast to cNor, qNor enzymes are reactive with ubiquinol and/or menaquinol and contain an N-terminal extension that is absent from NorB in the cNor complex. While this N-terminal extension shows similarity to NorC, a haem *c*-binding motif is lacking. The crystal structure of *Geobacillus stearothermophilus* qNor revealed a water channel from the cytoplasm that might serve in proton delivery (Matsumoto et al., 2012). Thus, the possibility that qNor might catalyze electrogenic quinol oxidation coupled to nitric oxide reduction cannot be excluded. An unusual qNor subgroup (qCu_ANor), exemplified by the enzyme from *Bacillus azotoformans* contains NorB in a complex with a subunit harboring a Cu_A site (typically found in oxygen-reducing HCOs), which makes this enzyme competent in receiving electrons from membrane-bound cytochrome c_{551} in addition to the menaquinol pool (de Vries et al., 2007).

However, it has been recently reported that the *Bacillus* enzyme lacks menaquinol activity and have changed its name from qCu$_A$Nor to Cu$_A$Nor (Al-Attar & de Vries, 2015).

NorCB structural subunits of cNor are encoded by *norCB* genes, respectively, which are usually co-transcribed with accessory genes designed *norD*, *norE*, *norF* and *norQ*. The gene order *norEFCBQD* is not universal, *norQ* and *norD* are always linked to *norCB*, however *norE* and *norF* may be distantly located or absent in some genomes (Zumft, 2005). Intriguingly, in ancient thermophilic bacteria belonging to the Thermales and Aquificales phylogenetic groups, the *norC* and *norB* genes are always followed by a third gene (*norH*) encoding a small membrane protein that is required for efficient denitrification *in vivo*, likely allowing more efficient electron transport to cNor (Bricio et al., 2014). The functions of the accessory genes and their protein products are not well understood. It has been shown that NorD and NorE are intengral membrane proteins required for successful heterologous assembly of the NorCB complex (Butland et al., 2001). NorE is a member of the subunit III of the cytochrome *c* oxidases family. Inactivation of *norEF* genes has been shown to slow NO reduction in both *Pa. denitrificans* and *Rhodobacter sphaeroides* 2.4.3 (de Boer et al., 1996; Hartsock and Shapleigh, 2010). Recent physiological experiments have shown that *norEF* are not essential for Nor activity; however their absence does affect activity under conditions where endogenous Nir activity generates prolonged exposure to NO (Bergaust et al., 2014).

3.2. Nitrous oxide reductase

Nitrous oxide reductase (N_2OR) is the terminal enzyme of bacterial denitrification and reduces N_2O by two electrons, breaking the N−O bond to release N_2 and H_2O (reviewed

in Zumft and Kroneck 2007; van Spanning, 2011; Spiro, 2012; and Solomon et al., 2014) (Fig. 7.7). Since this discovery, N_2OR had been purified and biochemically characterized from 11 denitrifying bacteria including *Ps. stutzeri* (Coyle et al., 1985), and *Pa. denitrificans* (Snyder and Hollocher, 1987) among others. The crystal structure of the *Pa. denitrificans* N_2OR enzyme at 1.6 A resolution has been revealed (Haltia et al, 2003). N_2ORs are homodimers with molecular weights of 120−160 kDa, a copper content of ∼12 Cu atoms per dimer, and a sulfide content of ∼2 S^{2-} ions per dimer (Rasmussen et al., 2000). N_2OR contains two copper sites: Cu_A, a binuclear copper site with two Cys residues, two His residues, one Met residues, and the backbone carbonyl of a Trp residue as ligands, which acts as an electron transfer site (as in the haem−copper oxidases), and Cu_Z, a tetranuclear μ4-sulfide-bridged cluster liganded by seven His residues, which is thought to be the site of N_2O binding and reduction. The ligands of the Cu_A site were identified from mutagenesis studies, and its structure was determined by analogy to the structurally characterized Cu_A site in the haem−copper oxidases, which has close to identical properties to Cu_A in NosZ. By contrary, the structure of Cu_Z was determined by X-ray crystallography and is still a matter of active study (Zumft et al., 1992, Kroneck et al., 1989, Einsle et al., 2011). There is a high degree of similarity between NosZ isolated from different sources, with the exception of *Thiobacillus denitrificans* which is a membrane-bound protein (Hole et al., 1996), most are periplasmic. The sequence of NosZ is conserved, showing a distinct two-domain architecture with an N-terminal, seven-bladed β-propeller domain and a smaller, C-terminal domain that adopts a conserved cupredoxin fold typical for copper-binding proteins (for a detail description of NosZ structural properties see Wüst et al., 2012). Each domain harbors one of the copper-based metal centres of the enzyme, the

binuclear Cu_A site in the cupredoxin domain, and the tetranuclear copper-sulfide center Cu_Z in the centre of the β–propeller (Johnston et al., 2014, 2015).

A gene cluster has been identified that is required for N_2O reduction, which encodes the NosZ protein and several ancillary proteins required for its expression, maturation, and maintenance (Zumft, 2005). The core of this cluster, which is the minimum required for N_2O reduction, contains six genes (*nosRZDFYL*) and is sometimes associated with a further gene, *nosX* (Fig. 7.8). The cluster *nosZDFYL* is found in every N_2O-reducing prokaryote, whereas *nosR, nosX,* and other *nos* genes such as *nosC, nosG* and *nosH* are distributed mostly according to taxonomic patterns and are not ubiquitous (Table 7.1) (Zumft and Kroneck, 2007). In this context, it is worth to mention the case of the atypical N_2OR of *Wolinella succinogenes* that is encoded in a gene cluster that also contain *nosG, -C1, -C2* and *-H* genes which were postulated to encode a putative menaquinol dehydrogenase pathway to cNosZ alternative to the conventional cytochrome bc_1 complex (see section 2.2.2, Figs. 7.4 and 7.5). These gene clusters lack *nosR* or *nosX* that in α-, β- and γ-Proteobacteria encode two FMN-binding flavoproteins (NosR and NosX) that might constitute yet another electron transport pathway from the quinone pool to NosZ (Fig. 7.8, Table 7.1). In fact, NosR resembles NosH but contains an additional periplasmic FMN-binding domain (Wunsch and Zumft, 2005). Interestingly and in contrast to the mayority of *nosRZDFYLX* gene clusters present in denitrifiers, in *Pa. denitrificans* biosynthesis of N_2OR requires the expression of *nosCRZDFYLX* genes where a *nosC* gene iniciates the *nos* cluster. The gene's product, NosC, is a hypothetical protein with unknown function and close (>50% identical) homologs appear to be only distributed among other *Paracoccus* species. Notably, all known homologs of NosC contain a CXXCXXC motif that may bind a redox active cofactor, the significance of which is unknown.

The twin arginine translocation pathway (Tat system) is responsible for transporting the NosZ apoprotein into the periplasm, where its maturation is completed. In adition to NosZ, NosX is another component of N_2O respiration system exported by the Tat system (Wunsch et al., 2003; Zumft and Kroneck 2007). An increasing list of NosZ proteins (besides the NosZ of *W. succinogenes*) have Sec-type signal peptides and, in contrast to the usual Tat export pathway, seem to be exported by the Sec system (Table 7.1) (Simon et al., 2004). NosR and NosY are integral membrane proteins, and have Sec-specific signal peptides. Thus, it is clear that both the Tat and the Sec translocation system have to cooperate to assemble a functional N_2O respiratory system (Zumft and Kroneck, 2007). NosZ, despite to be targeted to the Tat system, makes an exception to the concept that cofactor acquisition occurs prior to translocation, since Cu_A and Cu_Z are assemblaged in the periplasm (Zumft, 2005) (Fig. 7.8).

Mutation analyses demonstrated that NosDFY or NosL are not involved in the biogenesis of the Cu_A site (reviewed by Zumft and Kroneck, 2007). Cu_A is thought to be loaded *in vivo* by the same route used for the loading of Cu_A in the haem−copper oxidases (Zumft and Kroneck, 2007). The sequence similarity between Cu_A centre and the subunit II of cytochrome c oxidase led to the issue of a putative evolutionary relationship of the two enzymes (Zumft, 2005). Thus, maturation of the NosZ Cu_A site may be well mediated via SenC like proteins, which are homologous of the family Sco proteins. By contrast to Cu_A site, the biogenesis of the Cu_Z site and its maintenance *in vivo* depends on the *nosDFY* or *nosL* ancillary genes. NosDFY encodes an ABC-type transporter where NosY is a membrane-spanning protein, NosF is a cytoplasmic ATPase, and NosD is a periplasmic protein from the carbohydrate-binding and sugar hydrolase protein family (Zumft and Kroneck, 2007) (Fig. 7.8). Mutant strains lacking NosDFY express Cu_Z deficient N_2OR, indicating that NosDFY is essential for Cu_Z

biogenesis. The exact role of this transporter system is not known, but it is proposed to be the sulfur transporter that supplies the sulfide required for Cu_Z biogenesis (Zumft and Kroneck, 2007) (Fig. 7.8). NosL encodes a outer membrane lipoprotein which preferentially binds a single Cu(I) and is thought to be the copper transporter associated with Cu_Z assembly. However, active N_2OR containing both copper sites can be obtained in the absence of NosL, so an alternative Cu chaperone must exist (Dreusch et al., 1997). It has been suggested that, in addition to transport a sulfur compound via the NosFY proteins, NosD gathers copper ions from the NosL protein. In fact, in most N_2O-respiring bacteria *nosL* location downstream of *nosDFY* is strongly conserved (see Fig. 7.4) (Zumft and Kroneck, 2007). It has been proposed that NosL is targeted to the outer membrane by an ABC type-transporter system, the Lol system, leading the periplasmic NosL anchored to the outer membrane (Zumft and Kroneck, 2007). Since NosFYD ABC transporter system has not similarity with the Lol system, is unlikely that NosFYD would act on NosL transport.

Once assembled the NosZ copper centers, it would be expected the existence of mechanisms that preserve and maintain catalytically active the protein and the proper state of the reaction centre even in the case of changes in the cellular environment. For example, if oxygen enters a denitrifying cell, it may react with the Cu_Z reaction center rendering as a redox inactive Cu_Z* state (Rasmussen et al., 2002, Wüst et al., 2012). This Cu_Z* also appears when there is insufficient supply of the natural electron donors. These differences in redox properties lead several studies to propose that Cu_Z and Cu_Z* are structurally different. In fact, the recent X-ray crystal structure of anaerobic NosZ indicates a significant structural difference, with Cu_Z containing two bridging sulfide ligands, while the previously described structure of Cu_Z* contains only one (Pomowski et al., 2011). In order to rescue an already assembled NosZ enzyme it would make sense

to mobilize an electron transfer machinery that is able to maintain Cu_Z or to reactivate the Cu_Z* reaction centre. In addition to low potential electron donors as cytochrome *c* or pseudoazurin, NosR and NosX proteins have also been proposed as candidates to make up such an electron donating mechanism (Wunsch et al., 2005) (Fig. 7.8). NosR encodes a transmembrane portion with six transmembrane helices, a flavin-binding site in the N-terminal (periplasmic) domain and two [4Fe−4S] ferrodoxin-type iron−sulfur clusters in the C-terminal (cytoplasmic) domain (Wunsch and Zumft, 1992) (Fig. 7.8). In the presence of modified forms of NosR where the flavin-binding domain is deleted or the ferrodoxin sites are modified, NosZ is obtained that contains both Cu_A and Cu_Z, but the spectroscopic and redox properties of Cu_Z are modified (Wunsch & Zumft, 2005). A similar phenotype is obtained in the absence of the NosX gene product for organisms that contain NosX, which codes for another periplasmic flavoprotein (Wunsch et al., 2005). This suggests that NosR and NosX are not involved in Cu_Z biogenesis but play a role in N_2O reduction *in vivo* altering the state of the Cu_Z site during turnover and sustaining the catalytic activity of NosZ. Taken together, these results propose the existence of an electron donation pathway via NosR as a quinol-NosX oxidoreductase. This route may be paralleled by one involving cytochrome bc_1, cytochrome c_{550} and pseudoazurin (Fig. 7.8).

In addition to its proposed role as electron donor to NosZ, it has also been suggested a regulatory role for NosR since it was showed to be required for the transcription of *nosZ* and *nosD* operons in *Ps. stutzeri* (Honisch and Zumft, 2003). However, the membrane location and domain organization of NosR, as well as the absence of a pedicted DNA-binding domain indicate an indirect control of NosR on its target genes. Moreover, deletion analyses of NosR showed that only the periplasmic flavin-containing domain is required for *nosZ* expression (Wunsch & Zumft, 2005).

3.3. Regulators

In general, the environmental requirements for expression of the denitrification pathway are: a) restricted O_2 availability; b) the presence of a nitrogen oxide (NOx) as terminal electron acceptor; and c) suitable electron donors such as organic carbon compounds. Thus, the key molecules that act as signals for the regulation of denitrification genes are oxygen, a NOx (nitrate, nitrite, or NO), and the redox state of the cell. These environmental signals are perceived by a diversed number of transcriptional regulators that integrate them into regulatory networks (Fig. 7.9) (for reviews see van Spanning et al., 2011; Spiro, 2011, 2012; Shapleigh, 2011; Spiro, 2012; Bueno et al., 2012).

Oxygen strongly influences the growth and physiology of bacteria, as well as the expression of denitrification genes. Generally, denitrification is regarded as an anoxic or microoxic process. Since denitrifiers are facultative aerobes, this means that they must choose between oxygen and nitrate if both are available. Due to the organization and structural features of the denitrification enzymes, the maximum efficiency of free energy transduction during denitrification is only 60% of that during aerobic respiration (Richardson, 2000; Simon et al., 2008). Thus, oxygen is preferred as terminal electron acceptor than nitrate, and hence the regulation of expression of either type of respiration occurs according to an energetic hierarchy. In all species, the onset of denitrification is triggered by oxygen depletion and nitrate availability. Expression of *nar*, *nir*, *nor* and *nos* genes in most denitrifiers is tightly controlled, only occurring under microoxic conditions and in the presence of a NOx. By contrary, *nap* expression is quite variable, with this enzyme being maximally expressed under oxic conditions in some bacteria, but under microoxic conditions in others, adjusting to fit the physiological role it plays (Shapleigh, 2011; Bueno et al., 2012). It has been reported that NosZ has a greater sensitivity to O_2 compared to other denitrification enzymes, with important implications

for N_2O emissions from habitats where O_2 fluctuates (Morley et al., 2008). However, it has been recently demonstrated the capacity of *Ps. stutzeri* species to consume N_2O under oxic conditions (Desloover et al., 2014), supporting previous observations showing that the *nosZ* gene can also be expressed at high O_2 concentrations (Miyahara et al., 2010). Supporting these findings, it has been recently reported in *Pa. denitrificans* the reduction of N_2O at high O_2 partial pressure (Qu et al., 2015).

In addition to O_2, nitrate/nitrite and NO have been proposed as signal molecules that are required for induction of denitrification. NO is a potent cytotoxin and consequently both NO-generating (Nir) and NO-consuming (Nor) enzymes of denitrification are very tightly controlled by this molecule in order to avoid NO accumulation. With respect to N_2O, there is an absence of regulation of denitrification genes by this molecule presumably because it is non-toxic gas, so the denitrifying populations do not apparently respond to N_2O accumulation by making more of the N_2O reductase.

3.3.1. Oxygen-response

The most important types of O_2 sensors involved in regulation of denitrification are FixL and FNR (Fig. 7.9). FixL is a membrane-bound O_2 sensor found in rhizobial species which together with its cognate response regulator FixJ, belong to the group of two-component regulatory systems. In *B. japonicum*, phosphorylated FixJ activates transcription of *fixK*$_2$. In turn, FixK$_2$ activates expression of genes involved in denitrification, among others (for detail information see section 5.1 from this chapter). FNR is an oxygen responsive regulator that belongs to the CRP/FNR superfamily of transcription factors that has been extensively described in section 2.1.2 from this review. Orthologous of FNR from other organisms (such as FnrP, ANR, and FnrN) are

presumed to work in a similar way. For example, the *nar* and *nap* operons in *E. coli* and *B. subtilis* are activated by FNR under anoxic conditions (Reents et al., 2006; Stewart and Bledsoe, 2005; Tolla and Savageau, 2011). *Pa. denitrificans* FnrP controls expression of the *nar* gene cluster and the *cco*-gene cluster encoding the cbb_3-type oxidase (Veldman et al., 2006; Bouchal et al., 2010). Oxygen tension is sensed in *Ps. aeruginosa* by the Anr regulator, which activates transcription of the *narK1K2GHJI* operon encoding nitrate reductase and two transporters in response to oxygen limitation (Schreiber et al., 2007).

3.3.2 Nitrate/nitrite-response regulators

Denitrifying bacteria as well as those that reduce anaerobically nitrate to ammonium (DNRA, see section 2.1.2) respond to nitrate/nitrite through three types of regulatory systems: NarXL, NarQP, and NarR (Fig. 7.9). NarXL and NarQP are members of two-component regulatory systems being the NarX and NarQ proteins the signal sensors, and NarL and NarP proteins their cognate response regulators, respectively (Stewart, 2003). The sensing mechanism of the kinase NarX has been recently established (Cheung and Hendrickson, 2009; Stewart and Chen, 2010). In *E. coli* NarL and NarP bind DNA to control induction of the *nar* and *nap* operons (Stewart, 2003; Darwin et al., 1998: Stewart and Bledsoe, 2005). The effects of nitrate and nitrite on the *E. coli* transcriptome during anaerobic growth have been investigated, revealing in a novel group of operons that are regulated by all FNR, NarL and NarP (Constantinidou et al., 2006). To date, *narXL* and *narQP* genes are confined to species classified in the γ and β subdivisions of the proteobacteria such as *Escherichia, Salmonella, Klebsiella, Yersinia, Burkholderia, Ralstonia, Neisseria* and *Pseudomonas* species among others. In *Ps. aeruginosa,* NarL in concert with the regulators Anr and Dnr and an integration host factor (IHF) activate transcription of the *narK1K2GHJI* operon encoding nitrate

reductase and two transporters in response to oxygen limitation, nitrate and N-oxides (Schreiber et al., 2007). Recently, it has been shown that during anaerobic growth of *Ps. aeruginosa* PAO1, NarL directly represses expression of periplasmic nitrate reductase, while induces maximal expression of membrane-bound nitrate reductase (van Alst et al., 2009).

NarR is a member of the CRP/FNR family of transcription activators, but it lacks a [4Fe-4S] cluster. Genes encoding NarR are found in the α-proteobacteria *Brucella suis, B. melitensis*, *Pa. denitrificans* and *Pa. pantotrophus*. In *Pa. pantotrophus* NarR controls expression of the *narKGHJI* genes encoding the respiratory nitrate reductase, NarGHI, and the nitrate transport system, NarK, in response to nitrate and/or nitrite (Wood et al., 2001). The mechanism of the response is not clear, but since NarR can also be activated by azide, which normaly binds to metal centres, it might be posible that NarR is a metalloprotein. There are no indications that they have counterparts of *narXL*. It therefore seems that NarR substitutes the NarXL system in the α-proteobacteria (for reviews see van Spanning et al., 2007; Bueno et al., 2012).

3.3.3 NO-response regulators

In addition to low oxygen conditions and nitrate/nitrite, expression of denitrification genes also requires a fine-tuned regulation in order to keep the free concentrations of NO_2^- and NO below cytotoxic levels. In this context, NO has been proposed as an additional key molecule that is involved in denitrification genes regulation (reviewed by Radionov et al., 2005; Spiro, 2011, Stern and Zhu, 2014). As yet, several NO-response transcription factors have been proposed to be involved in denitrification; NorR, NnrR, NsrR, and DNR (Fig. 7.9). Among them, NorR, and NsrR have been already described in section 2.1.2 as regulators of NO-detoxifying enzymes

such as the flavohemoglobin Hmp or the flavorubredoxin NorVW. NorR was first identified in *Ralstonia eutropha* (Pohlmann et al. 2000). This bacterium has two copies of the *norR* gene, both of which are located upstream of their *norAB* gene clusters where *norB* encodes a single-subunit NorB of the qNor type. In response to anaerobiosis and the presence of NO, NorR specifically activates transcription of the σ54-dependent *norAB* promoters (Büsch et al., 2005) (Fig. 7.9). NsrR has also a regulatory role in denitrifying bacteria coordinating production of Nir and Nor to prevent the build up of NO (reviewed by Tucker et al., 2010). Intriguingly, the same role is performed by Nnr homologs in denitrifying bacteria that do not contain NsrR. In the denitrifying pathogenic organisms *Neisseria meningitidis* and *Neisseria gonorrhoeae*, NsrR represses both the membrane-bound Nir (AniA) and the respiratory NorB expression in the absence of NO (Heurlier et al., 2008; Overton et al., 2006; Isabella et al., 2009). (Fig. 7.9). Exposure to NO inactivates this repressor by a NO-mediated modification of the protein-bound [Fe-S cluster] (for details see section 2.1.2).

NnrR (nitrite and nitric oxide reductase regulator) and DNR are members of the CRP/FNR family of transcription factors, but NnrR just like NarR, lacks the cysteines to incorporate a [4Fe-4S] cluster. NnrR and DNR orthologs, sometimes named as Nnr, or DnrR have been described in denitrifying bacteria including *Pa. denitrificans*, *Ps. stutzeri*, *Ps. aeruginose*, *Bradyrhizobium japonicum*, *Ensifer meliloti* and *Rhizobium etli* and they orchestrate the expression of the *nir* and *nor* gene clusters (Fig. 7.9) (reviewed by Radionov et al., 2005; Spiro, 2011; Stern and Zhu, 2014). The promoters of these operons contain NnrR binding sites that resemble the consensus FNR-box to a large extent. The mechanism of NO sensing by NnrR and DNR are less well defined than NorR and NsrR. The crystal structures of DNR have only been obtained without prosthetic groups, but reveal a hydrophobic pocket that might be a haem-binding site,

and purified apo-DNR can bind haem (Giardina et al., 2008). The current model proposes that DNA binding activity of DNR *in vitro* requires haem and NO, and perturbation of the haem synthesis capabilities of the cell reduced the capacity of DNR to activate transcription of the *nor* promoter (Castiglione et al., 2009; Rinaldo et al., 2012). In the case of NnrR, it has been proposed that NNR is activated *in vivo* by physiological (e.g. nitrate and nitrite) and non-physiological (e.g. nitroprusside) sources of NO (Hutchings *et al.*, 2000; van Spanning et al., 1999). Heterologous expression of the *Pa. denitrificans nnr* gene in *E. coli*, indicated that activation of NNR by NO does not require de novo synthesis of the NNR polypeptide. In anaerobic cultures, NNR is inactivated slowly following removal of the source of NO. In contrast, exposure of anaerobically grown cultures to oxygen causes rapid inactivation of NNR, suggesting that the protein is inactivated directly by oxygen (Lee *et al*., 2006). NNR site-directed mutagenesis and structural modelling suggested that an Arg-80 closed to the C-helix that forms the monomer–monomer interface in other members of the FNR/CRP family might play an important role in transducing the activating signal between the regulatory and DNA binding domains (Lee *et al*., 2006). Furthermore, assays of NNR activity in a haem-deficient mutant of *E. coli* provided preliminary evidence to indicate that NNR activity is haem dependent (Lee *et al*., 2006). However, the mechanism of NO or O_2 sensing by NNR has not been demonstrated *in vitro*.

In *Pa. denitrificans*, the global role of FnrP, NNR and NarR during the transition from aerobic to anaerobic respiration has been confirmed using proteomics, with data validation at the transcript and genome levels (Bouchal et al., 2010). Interestingly, these studies demonstrated that a mutation in the *fnrP* gene resulted in a significant decrease of the N_2OR level under semiaerobic conditions. The involvement of FnrP is also consistent with the presence of two FNR-binding sites TTGAGAATTGTCAA and

TTGACCTAAGTCAA in the *nos* promoter encoding nitrous oxide reductase. Another group of proteins controled by FnrP, NNR and NarR included SSU ribosomal protein S305 /σ54 modulation protein (Bouchal et al., 2010). Thus, in addition to transcription regulators, sigma (σ) factors may play an important role in the FNR-mediated regulatory network as well. In this context, it has been proposed that specific classes of σ-factor binding to promoter sites downstream of the FNR box may be essential for the observed specificity of any of the 3 FNR-type transcription activators *in Pa. denitrificans* (Veldman et al., 2006). Denitrification phenotypes of the *Pa. denitrificans* FnrP, NNR and NarR transcriptional regulators have been analyzed by using a robotized incubation system that monitor changes in concentrations of oxygen and nitrogen gases produced during the transition from oxic to anoxic respiration. These experiments have completed the current understanding about the involvement of these regulators in transcriptional activation of *nar, nir* and *nor* genes involved in N_2O production (Bergaust et al., 2012). With regard to the regulation of N_2O reduction, results from these studies indicate that N_2OR is subjected to a robust regulation being FnrP and NNR alternative and equally effective inducers in response to oxygen depletion (via FnrP) or an NO signal (via NNR) (Bergaust et al. 2012).

3.3.4 Redox-response regulators

Redox changes can regulate the expression of genes involved in denitrification (for reviews see van Spanning, 2011; Bueno et al., 2012). Redox-responsive two-component regulatory systems are present in a large number of Proteobacteria. These proteins are named RegBA in *R. capsulatus, Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* PrrBA in *R. sphaeroides*, ActSR in *E. meliloti* and

Agrobacterium tumefaciens, RegSR in *B. japonicum* and RoxSR in *Ps. Aeruginosa.* In *Rhodobacter* species, the RegBA/PrrBA regulon encodes proteins involved in numerous energy-generating and energy-utilizing processes such as photosynthesis, carbon fixation, nitrogen fixation, hydrogen utilization, aerobic respiration and denitrification, among others (reviewed by Elsen et al., 2004; Wu and Bauer, 2008; Bueno et al., 2012). The RegBA/PrrBA two-component systems comprise the membrane-associated RegB/PrrB histidine protein kinase, which senses changes in redox state, and its cognate PrrA/RegA response regulator. Under conditions where the redox state of the cell is altered due to generation of an excess of reducing potential, produced by either an increase in the input of reductants into the system (e.g. presence of reduced carbon source) or a shortage of the terminal respiratory electron acceptor (e.g. oxygen deprivation), the kinase activity of RegB/PrrB is stimulated relative to its phosphatase activity. This increases phosphorylation of the partner response regulators RegA/PrrA, which are transcription factors that bind DNA and activate or repress gene expression. The membrane-bound sensor kinase proteins RegB/PrrB contain an H-box site of autophosphorylation (His^{225}), a highly conserved quinone binding site (the heptapeptide consensus sequence GGXXNPF, which is totally conserved among all known RegB homologues), and a conserved redox-active cysteine (Cys^{265}, located in a "redox box"). The mechanism by which RegB controls kinase activity in response to redox changes has been an active area of investigation. A previous study demonstrated that RegB Cys^{265} is partially responsible for redox control of kinase activity. Under oxidizing growth conditions, Cys^{265} can form an intermolecular disulfide bond to convert active RegB dimers into inactive tetramers (Swem et al., 2003). The highly conserved sequence, GGXXNPF, located in a short periplasmic loop of the RegB transmembrane

domain has also being implicated in redox sensing by interacting with the ubiquinone pool (Swem et al., 2006).

RegA/PrrA contain conserved domains that are typical in two-component response regulators such as a phosphate accepting aspartate, an “acid box” containing two highly conserved aspartate residues and a helix-turn-helix (H-T-H) DNA-binding motif. The phosphorylated form of RegA/PrrA has increased DNA binding capacity (Laguri et al., 2006; Ranson-Olson et al., 2006). Under oxidizing conditions, RegB/PrrB shifts the relative equilibrium from the kinase to the phosphatase mode resulting in a dephosphorylated inactive RegA/PrrA form. Despite this evidence, it has been reported that inactivation of the *regA* gene affects expression of many different genes under oxidizing (aerobic) conditions suggesting that both, phosphorylated and unphosphorylated RegA/PrrA, may be active transcriptional regulators (Swem et al., 2001). In this context, it has been shown that both phosphorylated and unphosphorylated forms of RegA/PrrA are capable of binding DNA *in vitro* and activating transcription (Ranson-Olson et al., 2006).

The PrrBA from *R. sphaeroides* (Laratta et al., 2002), ActSR from *A. tumefaciens* (Baek et al., 2008), and RegSR from *B. japonicum* control denitrification (Torres et al., 2014a, see section 5.1). In *R. sphaeroides* 2.4.3, inactivation of *prrA* impaired ability to grow both photosynthetically and anaerobically in the dark on nitrite-amended medium (Laratta et al., 2002). The PrrA-deficient strain exhibited a severe decrease in both nitrite reductase activity and expression of a *nirK-lacZ* fusion when environmental oxygen tension was limited (Fig. 7.9). This regulation is not mediated by NnrR, since *nnrR* is fully expressed in a PrrA mutant background. Instead, Laratta and colleagues (2002) proposed a model where, under low-oxygen tension, the kinase activity of PrrB is increased relative to its phosphatase activity, resulting in an

increased concentration of PrrA-P. Thus, under microoxic conditions in the presence of NO, PrrA-P activates transcription of *nirK* in collaboration with NnrR. Insertional inactivation of the response regulator ActR in *A. tumefaciens* significantly reduced *nirK* expression and Nir activity but not *nnrR* expression (Fig. 7.9). In *A. tumefaciens*, a putative ActR binding site was identified in the *nirK* promoter region using mutational analysis and an *in vitro* binding assay (Baek et al., 2008). These studies also showed that purified ActR bound to the *nirK* promoter but not to the *nor* or *nnrR* promoter.

In addition to PrrBA, ActSR and RegSR (Fig. 7.9), it has been recently reported that the NtrYX two-component system of *Brucella* spp. acts as a redox sensor and regulates the expression of *nar*, *nir*, *nor* and *nos* operons in response to microoxic conditions (Fig. 7.9) (Roop and Caswell, 2012), and that PrrBA and NtrYX co-ordinately regulate the expression of denitrification (Carrica et al., 2013). NtrYX two-component system is also involved in the expression of respiratory nitrite reductase (AniA) and nitric oxide reductase (NorB) in the human pathogen *Neisseria gonorrhoeae* (Atack et al., 2013).

3.3.5 Copper and pH as emerging regulatory factors

The enzymes of denitrification are complex metalloenzymes that require a suite of redox-active cofactors including molybdenum, iron and/or copper for their respective activities. In particular, the reduction of nitrous oxide by denitrifying bacteria is heavily reliant on the availability of copper, a key constituent of nitrous oxide reductase. This phenomenon has been explored in detail in *Pa. denitrificans*, where bacterial cultures lacking the trace element copper accumulate significant amounts of nitrous oxide (Felgate et al 2012). Furthermore, mathematical models have been developed that quantitatively predict the levels of N_2O emitted by bacterial denitrification in response

to copper availability (Woolfenden et al. 2013). A recent global transcriptomic study by Sullivan and co-workers has revealed that copper-deficiency not only affects functional maturation of nitrous oxide reductase, but it has a important impact on gene expression in *Pa. denitrificans*, including expression of *nosZ* that is down-regulated during copper-limited growth (Sullivan et al., 2013). In addition, *nosZ* transcript levels in both a *Pa. denitrificans nosC* or *nosR* mutants were found to be similar in copper-limited or copper-sufficient growth conditions indicating that repression of *nosZ* during copper-limited growth was deregulated in response to metal availability. Therefore, these results strongly suggest a role of NosC and NosR in copper-regulation of *nosZ* expression, although the mechanism involved in this control remains to be established. Interestingly, these transcriptomics studies also revealed that the high levels of N_2O produced as a consequence of decreased NosZ activity lead to *Pa. denitrificans* switching from vitamin B_{12}-dependent to vitamin B_{12}-independent biosynthetic pathways through the transcriptional modulation of genes controlled by vitamin B_{12} riboswitches (Sullivan et al., 2013).

In addition to copper availability, pH is another key factor that has been demonstrated to significantly influence microbial N_2O emissions. Soil pH is known to be a major driver of denitrifier N_2O:N_2 ratios and numerous studies have shown that the reduction of N_2O to N_2 is impaired by low soil pH, suggesting that liming of acidic soils may be an effective strategy to lower N_2O emissions (Liu et al., 2010; Van den Heuveletal., 2011). A series of experiments involving *Pa. denitrificans* have shown that modulating pH has little effect on the transcription of the *nosZ* gene (Bergaust et al. 2010). Instead, the enzymatic rate of N_2O reduction was significantly attenuated at low pH levels, implying that environmental pH may have a direct post-translational effect on the assembly and/or activity of the N_2O reductase holoenzyme. Consistent with these

findings, spectroscopic and steady-state kinetics studies in N_2OR from *Achromobacter cycloclastes* suggest that [H^+] has multiple effects on both the activation and the catalytic reactions (Fujita and Dooley, 2007). One plausible explanation for these observations is that low pH may influence the assembly of the enzyme, which takes place in the periplasm. That said, a link between metal availability and pH has yet to be explored. Recent analyses of growth-linked NO, N_2O and N_2 profiles alongside relevant denitrification gene transcript levels (i.e. for *nirS*, *nirK* and *nosZ*), using cells extracted from soils with different pH values, suggests that low pH may interfere with the manufacture of N_2O reductase rather than the function of the enzyme once properly assembled (Liu *et al*., 2014).

5. *BRADYRHIZOBIUM JAPONICUM* AS A MODEL OF LEGUME-ASSOCIATED RHIZOBIAL DENITRIFIERS

Legume plants, which includes lentils, peas, beans, peanuts and soya, are hugely important as a source of food due to their high protein content. They are second only to cereals in agriculture importance, and many species as alfalfa are also used for forage, hay, silage and green manure, and it constitutes an important component for fodder animal feeding. Moreover, legume family has the unique ability to establish a N_2-fixing symbiotic association with soil bacteria collectively referred as rhizobia (Sprent, 2009). During this process, an exchange of molecular signals occurs between the two partners, leading to the formation of root nodules, where biological nitrogen fixation takes place by rhizobia (for a recent review see Udvardi and Poole, 2013). Legumes can safe huge amounts of environment polluting nitrogen fertilizers protecting ground water from toxicity while increasing soil fertility and contribute to the improvement of soil structure with a turn-over effects on the subsequent crops (Sprent, 2009). Thus, inoculation of legumes with rhizobia is an economical and environmental friendly

recommended worldwide agricultural practice to increase crop yield and to improve soil fertility without adding N fertilizers. More than 60% of N_2O emissions globally are emitted from agricultural soils due to the synthetic N addition into them. Thus, one strategy for N_2O mitigation is reducing the dependence on chemical fertilizers in agriculture enhancing biological nitrogen-fixation. However, legume crops also contribute to N_2O emissions by several ways: (i) biologically fixed N may be nitrified and denitrified, thus providing a source of N_2O (Inaba et al. 2012; Saggar et al., 2013); (ii) by providing N-rich residues for decomposition (Baggs et al. 2000) and (iii) directly by some rhizobia that are able to denitrify under free-living conditions or under symbiotic association with legume plants (Bedmar et al. 2005, 2013; Inaba et al., 2009; Hirayama et al., 2011; Inaba *et al.*, 2012).

Although denitrification among rhizobia is rare, several of the most agronomical interesting species contain denitrification genes in their genomes (Table 7.2). So, *Pseudomonas* sp. G-179 (actually *Rhizobium galegae*) (Bedzyk et al., 1999) has been shown to contain Nap, Nor and CuNir. *Rhizobium sullae* (formerly *R. hedysari*) only expresses CuNir (Toffanin et al., 1996). The genetic determinants for expression of CuNir and cNor are present in *Rhizobium etli* CFN42 (Bueno et al., 2005, Gómez-Hernández et al., 2011). *Ensifer meliloti* (formely *Sinorhizobium meliloti*) Galibert et al., 2001; Holloway et al., 1996; Torres et al., 2011a), and *Bradyrhizobium japonicum (*recently reclassified as *Bradyrhizobium diazoefficiens* USDA 110, Delamuta et al., 2013*)* (Kaneko et al., 2002; Bedmar et al., 2005) contain *nap*, *nirK*, *nor*, and *nos* genes (http://www.kazusa.or.jp/rhizobase). Among them, *B. japonicum* is the only rhizobial species that has the ability to grow under anoxic conditions with nitrate through denitrification pathway and where this process has been extensively investigated not

only under free-living but also under symbiotic conditions (for reviews see Bedmar et al., 2005; Delgado et al., 2007; Sanchez et al., 2011; Bedmar et al., 2013).

B. japonicum occupies two distinct niches: free-living in the soil and establishing symbiotic associations with soybean (*Glycine max*), siratro (*Macroptilium atropurpureum*), mung bean (*Vigna radiata*) and other *Vigna* species. Soybeans are unique in legumes with contents of 40% protein and 21% oil as well as isoflavones. Thus, soybean crops represent 50% of the total legume crop area and 68 % of global production, able to fix 16.4 Tg N annually, representing 77 % of the N fixed by legume crops (Herridge et al. 2008). Soybean has an industrial and economical interest for oil, food and protein, pharmaceuticals for protective coating or biodiesel production that represents the largest individual element of international oilseed production (59 %), with United States (34 %), Brazil (30 %) and Argentina (18 %) being the main contributers to world soybean production (SoyStats, 2015). Soybean is the first legume species with a complete genome sequence (Schmutz et al., 2010). It is, therefore, a key reference for the more than 20,000 legume species, and for the remarkable evolutionary innovation of nitrogen-fixing symbiosis. The genome sequence is also an essential framework for vast new experimental information such as tissue-specific expression and whole-genome association data. The genome sequence opens the door to crop improvements that are needed for sustainable human and animal food production, energy production and environmental balance in agriculture worldwide. *B. japonicum* strain USDA110 was originally isolated from soybean nodules in Florida, USA in 1957, and has been widely used for the purpose of molecular genetics, physiology, and ecology. Taken in consideration this background, *B. japonicum* USDA110 is considered a model rhizobial species for studying denitrification in legume-associated bacteria under both free-living and symbiotic conditions.

5.1. Regulation of *B. japonicum* denitrification

In *B. japonicum*, denitrification is dependent on the *napEDABC* (Delgado et al., 2003), *nirK* (Velasco et al., 2001), *norCBQD* (Mesa et al., 2002) and *nosRZDYFLX* genes (Velasco et al., 2004) that encode a periplasmic nitrate reductase, a Cu-containing nitrite reductase, a *c*-type nitric oxide-reductase and a nitrous oxide-reductase enzymes, respectively (Table 7.2). In addition, accessory cytochromes such as cytochrome c_{550}, encoded by *cyc*A, are necessary to support electron transport during denitrification being essential for the electron delivery to the CuNir (o NirK) reductase (Bueno et al., 2008). Neither azurin- nor pseudoazurin-like copper proteins have been annotated in the genome sequence of *B. japonicum* (http://www.kazusa.jp/rhizobase/).

Similarly to many other denitrifiers, expression of denitrification genes in *B. japonicum* requires both oxygen limitation and the presence of nitrate or a derived nitrogen oxide (Bedmar et al., 2005). In this bacterium, perception and transduction of the ‘‘low-oxygen signal’’ are mediated by two interlinked oxygen responsive regulatory cascades, the FixLJ-$FixK_2$-NnrR and the RegSR-NifA (reviewed by Torres et al., 2011; Bueno et al., 2012, Fig. 7.10). A moderate decrease in the oxygen concentration in the gas phase (≤5%) is sufficient to activate expression of FixLJ-$FixK_2$-dependent targets (Sciotti et al., 2003). The haem-based sensory kinase FixL senses this ‘‘low-oxygen’’ signal and auto-phosphorylates and transfers the phosphoryl group to the FixJ response regulator which then activates transcription of *fixK*$_2$ gen. In turn, the CRP/FNR-like transcriptional regulator $FixK_2$ induces expression of *nap*, *nirK*, and *nor* denitrification genes involved in N_2O production (Robles et al., 2006; Velasco et al., 2001; Mesa et al., 2002) as well as regulatory genes such as *rpoN*$_1$, *fixK*$_1$, and *nnrR* (Nellen-Anthamatten et al., 1998, Mesa et al., 2003, 2008). Thus, *B. japonicum* NnrR expands the FixLJ-$FixK_2$ regulatory cascade probably by an additional control level that

integrates the N oxide signal required for maximal induction of denitrification genes (Fig. 7.10). The NO-sensing mechanism by *B. japonicum* NnrR is still unknown. It has been recently found that *nap, nirK* or *nor* promoters exhibit differences with regard to their dependence on $FixK_2$ and NnrR. In fact, purified $FixK_2$ activates transcription from *nap*- or *nirK* dependent promoters but not from *nor*-dependent promoter. By contrast, NnrR bound to a specific DNA fragment from the promoter region of the *nor* genes, but not to those from the *nap* and *nirK* genes (Fig. 7.10) (E. Bueno, unpublished work).

In addition to FixLJ-$FixK_2$-NnrR, the second oxygen responsive regulatory cascade, RegSR/NifA, that respond to very low oxygen concentrations (≤ 0.5%), has been reported to be involved in the maximal induction of *B. japonicum* denitrification genes. In the RegSR-NifA cascade, the response regulator RegR of the RegSR two-component regulatory system induces expression of the *fixR-nifA* operon (Barrios et al., 1995, 1998; Bauer et al., 1998) under all oxygen conditions. Moreover, upon a switch to low oxygen or anoxic conditions, the redox-responsive NifA protein in concert with RNA polymerase containing RpoN (σ54) enhances its own synthesis. In *B. japonicum*, RpoN is encoded by the two highly similar and functionally equivalent genes ($rpoN_1$ and $rpoN_2$) (Kullik et al., 1991). Since $rpoN_1$ is under the control of $FixK_2$, this gene represents the link between the two regulatory cascades. Targets of NifA include *nif* and *fix* genes, which are directly or indirectly involved in nitrogen fixation (Nienaber et al., 2000; Hauser et al., 2007). Recent results from our group showed that NifA is also required for maximal expression of *nap, nirK*, and *nor* genes (Fig. 7.10) (Bueno et al., 2010). Whether or not these genes are direct or indirect targets of NifA is under investigation. In addition to NifA, it has been recently demonstrated the involvement of RegR in the control of denitrification genes in *B. japonicum* (Torres et al., 2014a). In this context, comparative transcriptomic analyses of wild-type and *regR* strains revealed

that almost 620 genes induced in the wild type under denitrifying conditions were regulated (directly or indirectly) by RegR, pointing out the important role of this protein as a global regulator of denitrification. Genes controlled by RegR included *nor* and *nos* structural genes encoding nitric oxide and nitrous oxide reductase, respectively (Fig. 7.10), as well as genes encoding electron transport proteins such as *cycA* or *cy2*, among others. It has also been demonstrated the capacity of purified RegR to interact with the promoters of *norC*, and *nosR* (Torres et al., 2014a). Expression studies with a *norC-lacZ* fusion, and heme *c*-staining analyses revealed that anoxia and nitrate are required for RegR-dependent induction of *nor* genes, and that this control is independent of the sensor protein RegS (Torres et al., 2014a).

Taken together, these results suggest the the existence of a complex regulatory network of the *B. japonicum* denitrification process (Fig. 7.10) and therefore, of N_2O emissions by soybean root nodules. While a progress on the knowledge about the regulation of *nap*, *nir*, and *nor* genes involved in N_2O synthesis has been made in *B. japonicum*, much remains to be discovered regarding the regulatory mechanisms and networks involved in the control of *nosRZDYFLX* genes involved in N_2O reduction to N_2, the key step to N_2O mitigation.

5.2. NO and N_2O metabolism in soybean nodules

Several studies have reported the evolution of N_2O from sliced or detached soybean nodules (Inaba et al. 2012, Mesa et al. 2004, Sameshima-Saito et al. 2006). It has been recently demonstrated that nitrate is essential for N_2O emissions from nodules of plants inoculated with *B. japonicum* USDA110 and its concentration enhanced N_2O fluxes showing a statistical linear correlation. In addition to nitrate, N_2O emission from soybean nodules is significantly induced when plants were subjected to flooding,

especially during long (7 days)-term flooding (Tortosa et al., 2015). In addition to nitrate and oxygen, other environmental factors might influence the production of N_2O in root nodules such as C availability, Cu, as well as soil pH. This knowledge will be very useful to setup management options by which soil conditions might be manipulated either to lower emission of N_2O, or to increase its reduction to N_2.

Flooding and nitrate also induce the formation in detached nodules of the precursor of N_2O, the cytotoxic and ozone depleting gas NO (Meakin et al. 2007; Sanchez et al. 2010). This molecule contributes to the formation of nitrosyl-leghaemoglobin (LbNO) complexes in soybean nodules (Fig. 7.11) (Sanchez et al., 2010) and is an inhibitor of nitrogenase activity (Kato et al. 2010; Sanchez et al., 2011a) and expression of the *nifH* and *nifD* genes (Sanchez et al. 2010).

The main process involved in NO and N_2O production in soybean nodules is *B. japonicum* denitrification (Fig. 7.11) (Inaba et al. 2012; Meakin et al. 2007; Sanchez et al. 2010). Thus, the main candidate for N_2O synthesis in nodules is the denitrification enzyme Nor which reduces NO to N_2O. It has also been demonstrated that the *B. japonicum* N_2OR is a key enzyme to mitigate N_2O emissions from soybean nodules (Horchani et al. 2011; Inaba et al. 2012; Tortosa et al., 2015). Based on this, Itakura et al. (2013) hypothesized and proved that N_2O emission from soil could be reduced by inoculating soybean plants with a *nosZ*-overexpressing strain of *Bradyrhizobium japonicum.* Thus, inoculation with *nosZ*$^+$ *B. japonicum* strains can be used as a strategy to mitigate N_2O emissions from increasing soybean fields.

5.3. A new system involved in NO and N_2O metabolism in *B. japonicum*

It is well established that *B. japonicum* denitrification is the main process involved in NO and N_2O production in soybean nodules. Nevertheless, basal levels of NO and N_2O

were recorded in nodules from soybean plants subjected to nitrate and flooding conditions and inoculated with a *napA* mutant where denitrification is blocked (Sanchez et al., 2010; Tortosa et al., 2015). These observations suggest that other mechanisms different to denitrification pathway could be involved in NO and N_2O production in nodules. In this context, it has been recently identified in *B. japonicum* a putative haemoglobin, Bjgb, implicated in NO detoxification (Cabrera et al. 2011; Sánchez et al. 2011b). Similarly to other bacterial haemoglobins, Bjgb might reduce NO to N_2O under anoxic free-living conditions or inside the nodules. In *B. japonicum*, the Bjgb is encoded in a gene cluster that also codes for a number of proteins with important roles in nitrate assimilation (Cabrera et al., 2015) including the large catalytic subunit of the assimilatory nitrate reductase (NasC), a major-facilitator superfamily (MFS)-type NO_3^- /NO_2^- transporter, an FAD-dependent NAD(P)H oxidoreductase (Fig. 7.12). A ferredoxin-dependent assimilatory NO_2^- reductase (NirA) is present a distinct locus on the chromosome. This *nirA* gene lies immediately downstream of genes recently reported to code for a NO_3^-/NO_2^- responsive regulatory system (NasS-NasT) in *B. japonicum* (Sanchez et al., 2014). This integrated system for NO detoxification and nitrate assimilation has been demonstrated to be another source of NO and probably to N_2O. In fact, the importance of NasC not only in NO_3^- assimilation but also in NO production has been demonstrated (Cabrera et al. 2015). Although, the biochemical basis for NO-formation during anaerobic bacterial respiration has been shown to result from NR-catalysed reduction of the pseudo substrate NO_2^-, by the respiratory membrane-bound Nar enzyme (Rowley et al., 2012; Gilberthorpe et al., 2008; Vine et al., 2011), to our knowledge, this is the first time where a combined NO_3^- assimilation/NO-detoxification system represents a novel method by which bacteria protect against cytoplasmic NO produced by NasC during anaerobic NO_3^--dependent

growth, where pathways for both respiratory denitrification and NO_3^-/NO_2^- assimilation are active (Fig. 7.12, Cabrera et al., 2015). These observations strongly suggest that in addition to denitrification, rhizobial nitrate assimilation might be another important source of NO and N_2O in nodules. Further investigations are being carried out to establish the role of this NO_3^- assimilation/NO-detoxification system in NO and N_2O metabolism in soybean nodules.

6. NO AND N_2O METABOLISM IN OTHER RHIZOBIA-LEGUME SYMBIOSIS

6.1. *Ensifer meliloti-Medicago truncatula*

E. meliloti is an aerobic soil bacterium which establishes symbiotic N_2-fixing associations with plants of the genera *Medicago*, *Melilotus* and *Trigonella*. *Medicago sativa* (also known as alfalfa or lucerne) is one of the most widely forage legume crops in the world. In addition to the traditional uses as an animal feed, alfalfa has a great potential as a bioenergy crop and different studies considered alfalfa (especially stems) as a good sustainable crop for second-generation bioethanol production. These plants also possess therapeutic virtues that have been used in veterinary and medicine. Among *Medicago* species, *Medicago truncatula* plays a prominent role in fundamental research on legume biology and symbiotic nitrogen fixation due to favorable characteristics including diploid genetics, small genome (~500 Mbp), ease of transformation, short life cycle and high levels of natural diversity (Cook, 1999). The genome of this model legume was sequenced in the first decade of the 21st century (Young et al. 2011). *Ensifer* (formerly *Sinorhizobium*) *meliloti* 1021 is a model rhizobial strain that has been extensively used to better understand the interaction between *E. meliloti* and *M. truncatula* that has been the subject of extensive biochemical, molecular, and genetic investigation (Jones et al., 2007; Young et al., 2011). Inspection of the *E. meliloti* 1021

genome sequence shows a composite architecture, consisting of three replicons with distinctive structural and functional: a 3.65 Mb chromosome and two megaplasmids, pSymA (1.35 Mb) and pSymB (1.68 Mb) (Galibert et al., 2001). pSymA contains a large fraction of the genes known to be specifically involved in symbiosis and genes likely to be involved in nitrogen and carbon metabolism, transport, stress and resistance responses that give *E. meliloti* an advantage in its specialized niche (Barnett et al., 2001). A 53 kb segment of pSymA is particularly rich in genes encoding proteins related to nitrogen metabolism, including *napEFDABC*, *nirK*, *norECBQD*, and *nosRZDFYLX* denitrification genes (Table 7.2). Transcriptomic analyses have shown that *E. meliloti* denitrification genes are induced in response to microoxic and symbiotic conditions (Becker et al., 2004). Under free-living microoxic conditions, the expression of denitrification genes is coordinated via the two-component regulatory system, FixLJ, and via the transcriptional regulator, FixK (Bobik et al., 2006). Furthermore, transcriptomic studies demonstrated that denitrification genes (*nirK* and *norC*) and other genes related to denitrification (*azu*1, *hemN*, *nnrU* and *nnrS*) are also induced in response to NO and that the regulatory protein NnrR is involved in the control of this process (Meilhock et al., 2010). However, and despite possessing and expressing the complete set of denitrification genes, *E. meliloti* has been considered a partial denitrifier due to its inability to grow under anaerobic conditions with nitrate or nitrite as terminal electron acceptors. Despite the inability of *E. meliloti* to grow under denitrifying conditions, *napA*, *nirK*, *norC*, and *nosZ* structural genes are functional since they are involved in the expression of denitrification enzymes under specific growth conditions (initial oxygen concentrations of 2%)(Torres et al., 2014b). By using a robotized incubation system it has been recently confirmed the incapacity of *E. meliloti* to respire nitrate and reduce it to N_2O or N_2 under anoxic conditions (Bueno et al., 2015). By

contrary, in the latter studies the capacity of *E. meliloti* to grow through anaerobic respiration of N_2O to N_2 was demonstrated. N_2OR activity was not dependent on the presence of nitrogen oxyanions or NO, thus the expression could be induced by oxygen depletion alone. When incubated at pH 6, the capacity of *E. meliloti* to reduce N_2O was severely impair, corroborating previous observations found in both, extracted soil bacteria and *Pa. denitrificans* pure cultures, where expression of functional N_2OR is difficult at low pH (Bergaust et al., 2010; Liu et al., 2014). Furthermore, the presence in the medium of highly reduced C-substrates, such as butyrate, negatively affected N_2OR activity. The emission of N_2O from soils can be lowered if legumes plants are inoculated with rhizobial strains overexpressing N_2O reductase. This study demonstrates that strains like *E. meliloti* 1021, which do not produce N_2O from nitrate respiration but are able to reduce the N_2O emitted by other organisms, could act as potential N_2O sinks. These results could be expanded to competitive and efficient N_2-fixers *E. meliloti* strains in order to develop strategies to reduce N_2O emissions from alfalfa crops.

6.1.1. NO in M. truncatula nodules

It is well known that NO is produced at various stages of *E. meliloti-M. truncatula* simbiosis and this molecules has a beneficial role during infection, nodule development and mature nodule functioning (for a recent review see Hichriand et al., 2015). On the other hand NO was also shown to have inhibitory effects on nitrogenase, induces senescence, and it has been recently reported to contribute to the plant glutamine synthetase (GS) post-translational modification in nitrogen fixing nodules (Blanquet et al., 2015). In the nodules, both the plant and the bacterial partners should be considered as potential sources of NO. In plants, beside a nonenzymatic conversion of NO_2^- to NO in the apoplast (Bethke *et al.*, 2004), seven enzymatic pathways for NO production have

been described (Gupta *et al.*, 2011). In the reductive pathways, NO_2^- can be reduced to NO through the action of either nitrate reductase (NR), plasma membrane bound nitrite:NO reductase, xanthine oxido-reductase, or the mitochondrial electron-transport chain (ETC), particularly in a low-O_2 environment (Gupta *et al.*, 2011; Mur *et al.*, 2013) (Fig. 7.11). Oxidative pathways that lead to NO production depend on arginine, polyamines or hydroxylamine as primary substrates. This oxidative NO production, mediated by still uncharacterized enzymes [NO synthase (NOS)-like, polyamine oxidase (PAOx)], occurs under normoxic conditions (Gupta et al., 2011; Mur et al., 2013). In addition to plant sources, *E. meliloti napA* and *nirK* denitrification genes were shown to participate significantly in NO synthesis, at least in mature nodules (Fig. 7.11) (Horchani et al., 2011). Given the clear evidences of NO production in *M. truncatula* nodules, NO-detoxification systems in nodules are essential in maintaining a balanced NO concentration and an efficient simbiosis. In this context, plant hemoglobins (non-symbiotic hemoglobins but also leghemoglobins or truncated hemoglobins) have been shown to be involved in NO degradation (Gupta *et al.*, 2011). From the bacterial side, two *E. meliloti* proteins, Hmp and Nor are the major NO detoxifying enzymes essential in maintaining a balanced NO concentration and an efficient symbiosis (Cam *et al.*, 2012; Meilhoc *et al.*, 2013) (Fig. 7.11). Furthermore, it has been recently demonstrated the involvement of *E. meliloti nnrS*$_1$ and *nnrS*$_2$ in NO degradation under both in free-living and symbiotic conditions (Blanquet et al., 2015) (Fig. 7. 11). $NnrS_1$ and $NnrS_2$, are haem and copper containing membrane proteins whose homologues in *Vibrio cholerae* and *Rhodobacter sphaeroides* 2.4.1 have been shown to be important in resisting to nitrosative stress in culture (Stern et al., 2012, Arai et al., 2013). Hence, *E. meliloti* possesses at least four systems (Hmp, Nor, $NnrS_1$ and $NnrS_2$) to detoxify NO, which belong to the NO stimulon (Meilhoc et al., 2010) and their expression is

dependent upon the NO-specific regulator NnrR. These proteins might not have the same role and/or not function in the same conditions inside nodules. Indeed they have different localization in the bacterial cell and on the other hand they display a different expression pattern within the different zones of the nodules (Meilhoc *et al.*, 2013; Roux *et al.*, 2014). Although the involvement of Hmp, Nor, $NnrS_1$ and $NnrS_2$ in NO detoxificacion has been demonstrated, the potential impact of those NO consuming proteins on the emission of the greenhouse gas N_2O by alfalfa nodules is poorly investigated.

6.2. *Rhizobium etli-Phaseolus vulgaris*

Rhizobium etli fixes nitrogen in association with *Phaseolus vulgaris* L., or common bean which is the most important legume for human consumption. This crop is the principal source of protein for hundreds of millions of people and more than 18 million tonnes of dry common bean are produced annually (Broughton et al., 2003). *P. vulgaris* is also a model species for the study of symbiosis in association with nitrogen-fixing bacteria from the genus *Rhizobium*. The genome sequence of *P. vulgaris* has been recently released (Schmutz et al., 2014). *Rhizobium etli* is the natural microsymbiont of *P. vulgaris* that has been isolated from diverse geographical regions across Latin America given the strong integration of beans into the diet of this continent. *R. etli* CFN42 was originally isolated from bean nodules in México, and since its sequence is known (Gonzalez et al., 2006), this strain has been widely used for molecular genetics, physiology, and ecology studies. *R. etli* CFN42 contains a chromosome and six large plasmids (pCFN42a to pCFN42f) whose sizes range from 184.4 to 642.5 kb (Gonzalez et al. 2006). In *R. etli* CFN42, genes encoding denitrification enzymes were identified on plasmid pCFN42f. Genes located in this region include those encoding proteins with significant similarity to Cu-containing nitrite reductase (*nirK*), cytochrome-*c* type nitric

oxide reductase (*norCBQD*) (Table 7.2), and pseudoazurin (*azuP*f). Neither genes encoding for a respiratory nitrate reductase (*nap* or *nar* genes) nor for the respiratory nitrous oxide reductase (*nos* genes) were found in the *R. etli* genome. Plasmid pCFN42f also includes regulatory genes such as *fixK* and *fixL*. In contrast to *E. meliloti* or *B. japonicum*, the transcriptional activator with functional homology with FixJ is absent in *R. etli*. Instead, it has been recently identified FxkR as the missing regulator that allows the trunsduction of the microaerobic signal for the activation of the FixKf regulon (Zamorano-Sanchez et al., 2012). In the *nirK–norC* region of pCFN42f is also located the *nnrR* gene which encodes NnrR, the FNR type transcriptional regulator of denitrification genes. Although *R. etli* is unable to respire nitrate and to perform a complete denitrification pathway, the presence of NirK and NorC-coding regions in this bacterium suggests an NO detoxifying role for these enzymes, preventing accumulation of NO inside the free-living cells or in the nodules. In fact, *in vivo* experiments demonstrated that NirK is required for nitrite reduction to NO and that NorC is required to detoxify NO under free-living conditions (Bueno et al., 2005; Gómez-Hernández et al., 2010). In *R. etli*, microaerobic expression of *nirK* and *norC* promoters requires a functional FixKf, whereas the response to NO is mediated by NnrR. As reported in *B. japonicum*, microaerobic expression of *R. etli nnrR* is controlled by FixKf. By contrary, in *E. meliloti* NnrR and FixK are part of two different regulatory pathways (for a review see Cabrera et al., 2011). Additionally, the N_2-fixation regulator NifA has a negative effect on the transcription of the *nirK* operon (Gómez Hernández et al., 2011). This finding contradicts those reported in *B. japonicum* where NifA is involved in maximal expression of *nap*, *nirK* and *norC* denitrification genes (Bueno et al., 2010).

R. etli nirK and *norC* denitrification genes are also functional in common bean nodules. NirK is an important contributor to the formation of NO in response to NO_3^-,

since levels of LbNO complexes in nodules exposed to NO_3^- increased in those produced by the *norC* mutant, but decreased in *nirK* nodules compared with LbNO levels detected in wild-type nodules (Gomez-Hernandez et al., 2011) (Fig. 7.11). Interestingly, the presence of NO_3^- in the plant nutrient solution declined nitrogenase-specific activity in both the wild-type and the *norC* nodules. However, the inhibition of nitrogenase activity by NO_3^- was not detected in *nirK* nodules (Gómez-Hernández et al., 2010). Taken together, these results clearly demonstrate the capacity of common bean nodules to produce NO from nitrate present in the nutrient solution. *R. etli* lacks genes encoding Nap or Nar, but have a gene (RHE_CH01780) that encodes a putative assimilatory nitrate reductase (Nas) (http://genome.microbedb.jp/rhizobase/). In addition to the bacterial Nas, NO_3^- can be reduced to NO_2 in the nodule through the action of the plant nitrate reductase (NR) that has been reported to be a source of NO in nodules (see section 6.1.1). Thus, plant NR or *R. etli* Nas are candidates to reduce NO_3^- to NO_2^- inside the nodules. Thus, both enzymes should be considered as potential sources of NO_3^--dependent NO production. However, the contribution of these enzymes to NO formation in *P. vulgaris* nodules is unknown. While a progress has been made on the study of NO metabolism in *R. etli* free-living cells as well as in common bean nodules, very llitle is known about N_2O metabolism in the *R. etli-P. vulgaris* simbiosis.

7. CONCLUSIONS

The negative impact of N_2O on climate change and stratospheric ozone has been clearly reported. It is currently believed that microbial denitrification and nitrification are the most important biological pathways for N_2O emission from soils mainly due to the application of synthetic nitrogen-based fertlizers as part of the agricultural practices. One important strategy to ameliorate N_2O emission would be an increased

understanding of the environmental and molecular factors which contribute to the biological generation and consumption of N_2O. Denitrification and dissimilatory nitrate reduction to ammonia (DNRA) are the major microbial processes in soil that are capable of removing NO_3^- through the reduction of NO_3^-/NO_2^- to N_2 or NH_4 respectively. Both energy-conserving processes compete for nitrate since they share NO_3^- reduction to NO_2^-. While denitrification causes N loss from terrestrial and aquatic environments and releases N_2O and N_2 to the atmosphere, DNRA retains NH_4 in soils and sediments and has a higher tendency for incorporation into microbial or plant biomass. Hence, the relative contributions of denitrification versus respiratory ammonification activities have important consequences for N retention, plant growth and climate. In addition to denitrifiers, recent studies in *E. coli* and *S.* Typhimurium propose the involvement of nitrate-ammonifying bacteria in N_2O emissions, however the metabolism of N_2O in these organisms is poorly understood. Nitrate-ammonifying bacteria usually lack both the respiratory Cu-containing (NirK) and cd_1-type (NirS) nitrite reductases as well as typical membrane-bound respiratory NO reductases (*c*Nor and qNor enzymes) found in denitrifiers. Instead, *E. coli* produces NO during NO_3^-/NO_2^- reduction to NH_4 catalysed by the periplasmic Nap/Nrf and the cytosolic Nar/Nir nitrate reductase and nitrite reductase complexes (Fig. 7.2). By contrast to *E. coli*, NO formation from NO_2^- reduction by Nrf or Nir does not ocurr in *S.* Typhimurium. Interestingly, a new enzyme, the membrane-bound nitrate reductase NarG has been proposed as one major source of NO in *E. coli* and *S.* Typhimurium (Fig. 7.2). Given the high toxicity of NO, this molecule has to be removed in order to avoid a nitrosative stress. Since, nitrate-ammonifiers do not have the typical NO reductases found in denitrifiers, other enzymes need to overcome the NO-detoxification role. In this context, NrfA and NorVW are considered the main candidates to function as NO reductases *in*

vivo and in *vitro*. While NrfA reduces NO to NH_4, NorVW reduces NO to N_2O (Fig. 7.2). The key molecules that act as signals for the regulation of NO-production (Nap/Nrf, Nar/Nir, NarG) and NO-detoxification (Nrf, NorVW) proteins are oxygen, and a NOx (nitrate, nitrite, or NO). These environmental signals are perceived by a diversed number of transcriptional regulators (NarXL/QP, FNR, NorR and NsrR) that integrate them into regulatory networks in order to allow the cells to respire nitrate/nitrite and avoid NO accumulation as by-product of the reduction process.

It was believed for long time that respiratory nitrate ammonification is typical from Gamma-, Delta- and Epsilonproteobacteria and denitrification from Alpha-, Beta, and Gammaproteobacteria, and both pathways do not coexist within a single organism. However, it has been recently demonstrated the functionality of both the denitrification and the respiratory ammonification pathways in the Gammaproteobacterium *Shewanella loihica* strain PV-4.

Epsilonproteobacteria is another interesting grupo of ammonifiers where cells employ a periplasmic nitrate reductase (Nap) for nitrate reduction to nitrite and the latter is subsequently reduced to ammonium by cytochrome *c* nitrite reductase (Nrf). The capacity of the epsilonbacterium *W. succinogenes* to produce N_2O during growth by nitrate ammonification has been recently demonstrated. However, the question remains how NO is generated from nitrite by *W. succinogenes* since NapA and NrfA are unlikely to release NO as a by-product (as opposed to the *E. coli* NrfA and NarG enzymes). In addition to respire nitrite, *W. succinogenes* NrfA has a detoxifying function in cell physiology given its demonstrated capacity to mediate the stress response to NO_2^-, NO, hydroxylamine and hydrogen peroxide. In contrast to *E. coli* or *S.* Typhimurim, *W. succinogenes* lacks NorVW, however a cytoplasmic flavodiiron protein (Fdp) and an hybrid cluster protein (Hcp) homologous to *Helicobacter pylori*

NorH have been proposed to be involved in nitrosative stress defence in *W. succinogenes.* The contribution of these proteins to N_2O production, however, has to be clarified in the future.

Given the capacity of nitrate-ammonifying bacteria to produce N_2O during growth by nitrate respiration, it seems reasonable to assume that these bacteria are also capable to reduce N_2O formed as a product of NO detoxification. However, the capacity to reduce N_2O is restricted to Epsilonproteobacteria and some nitrate-ammonifying *Bacillus* species. In fact it has been recently reported in *W. succinogenes*, *A. dehalogenans* and *B. vireti* the capacity to grow by anaerobic N_2O respiration using N_2O as sole electron acceptor. These ammonifiers as well as some other non-denitrifiers contain a *nos* gene cluster encoding the "atypical" nitrous oxide reductase NosZ and some of them even a cytochrome *c* nitrous oxide reductase (*c*NosZ) (Table 7.1, Fig. 7.5). By contrary, other nitrate-ammonifying bacteria including enterobacteria such as *E. coli* or *S.* Typhimurium that also can produce N_2O do not have an enzyme that can consume it. Thus, these bacteria might contribute significantly to global N_2O emissions.

In the model Epsilonproteobacterium *W. succinogenes*, the respiratory Nap, Nrf, and cNosZ enzymes are up-regulated by low oxygen, and nitrogen oxides. In addition to nitrate, and NO, N_2O is also a key molecule that act as signal for the regulation of cNosZ. In contrast to *E. coli* and other nitrate-ammonifying bacteria, *W. succinogenes* lacks the typical nitrate- or NO-responsive proteins such as NarXL/NarQP, NsrR and NorR. Instead, *W. succinogenes* cells employ three transcription regulators of the Crp-FNR superfamily designated NssA, NssB and NssC, to mediate up-regulation of Nap, Nrf and *c*Nos via dedicated signal transduction routes (Fig. 7.6).

Denitrification is currently considered to be the largest source of N_2O in soils. In addition to free-living soil bacteria, legume-associated endosymbiotic denitrifiers also contribute to N_2O emissions in free-living conditions as well as inside the root nodules. The environmental signals as well as the regulatory networks involved in the control of denitrification are well known. In addition to oxygen, a NOx (nitrate, nitrite, or NO), and the redox state of the cell, new factors such as pH and Cu have been identified recently to be involved in the control of denitrification and more precisely in the regulation of the *nos* genes encoding the nitrous oxide reductase, NosZ. In contrast to the atypical *c*NosZ from *W. succinogenes* that responds to N_2O, there is an absence of regulation of the typical NosZ by this molecule. The well established regulatory mechanisms and networks involved in the control of denitrification (see Fig. 7.9) become more complex in rhizobial denitrifiers where denitrification and nitrogen fixation processes share common regulators (FixK, NifA, RegR, see Fig. 7.10).

In denitrifiers, it has been well established the role of the Cu-containing (NirK) and cd_1-type (NirS) nitrite reductases as well as the membrane-bound respiratory NO reductases (*c*Nor and qNor enzymes) in NO and N_2O formation. However, new enzymes are emerging as candidates to be involved in NO and N_2O metabolism in denitrifiers. Particulary, it has been recently demonstrated that the assimilatory nitrate reductase (NasC) from *B. japonicum* is important not only in NO_3^- assimilation but also in NO production. In this context, it has been recently identified in *B. japonicum* a putative haemoglobin, Bjgb, implicated in NO detoxification. Similarly to other bacterial haemoglobins, Bjgb might reduce NO to N_2O under anoxic free-living conditions or inside the nodules. Furthermore, *E. meliloti* possesses, in addition to Nor, at least three systems (Hmp, $NnrS_1$ and $NnrS_2$) to detoxify NO under free-living conditions which are also essential in maintaining a balanced NO concentration in

nodules and an efficient simbiosis. However the potential impact of those new NO consuming proteins on the emission of the greenhouse gas N_2O by root nodules has to be demonstrated.

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REFERENCES

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

Arai, H., Roh, J. H., Eraso, J. M., & Kaplan, S. (2013). Transcriptome response to nitrosative stress in *Rhodobacter sphaeroides* 2.4.1. *Biosci Biotechnol Biochem, 77*, 111-118.

Arkenberg, A., Runkel, S., Richardson, D. J., & Rowley, G. (2011). The production and detoxification of a potent cytotoxin, nitric oxide, by pathogenic enteric bacteria. *Biochem Soc Trans, 39*, 1876-1879.

Atack, J. M., Srikhanta, Y. N., Djoko, K. Y., Welch, J. P., Hasri, N. H., Steichen, C. T., et al. (2013). Characterization of an *ntrX* mutant of *Neisseria gonorrhoeae* reveals a response regulator that controls expression of respiratory enzymes in oxidase-positive proteobacteria. *J Bacteriol, 195*, 2632-2641.

Baar, C., Eppinger, M., Raddatz, G., Simon, J., Lanz, C., Klimmek, O., et al. (2003). Complete genome sequence and analysis of *Wolinella succinogenes*. *Proc Natl Acad Sci U S A, 100*, 11690-11695.

Baek, S. H., Hartsock, A., & Shapleigh, J. P. (2008). *Agrobacterium tumefaciens* C58 uses ActR and FnrN to control *nirK* and *nor* expression. *J Bacteriol, 190*, 78-86.

Baggs, E. M., Rees, R. M., Smith, K. A., & Vinten, A. J. A. (2000). Nitrous oxide emission from soils after incorporation of crop residues. *Soil Use Mgmt., 16*, 82-87.

Bamford, V. A., Angove, H. C., Seward, H. E., Thomson, A. J., Cole, J. A., Butt, J. N., et al. (2002). Structure and spectroscopy of the periplasmic cytochrome *c* nitrite reductase from *Escherichia coli*. *Biochemistry, 41*, 2921-2931.

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. *Proc Natl Acad Sci U S A, 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. *J Bacteriol, 177*, 1760-1765.

Barrios, H., Grande, R., Olvera, L., & Morett, E. (1998). In vivo genomic footprinting analysis reveals that the complex *Bradyrhizobium japonicum fixRnifA* promoter region is differently occupied by two distinct RNA polymerase holoenzymes. *Proc Natl Acad Sci U S A, 95*, 1014-1019.

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. *J Bacteriol, 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. *Mol Plant Microbe Interact, 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. In B. Rodelas & J. Gonzalez-López (Eds.), *Beneficial Plant-microbial Interactions: Ecology and Applications* (pp. 164-182). Boca Ratón, Florida, USA: CRC Press.

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

Bedzyk, L., Wang, T., & Ye, R. W. (1999). The periplasmic nitrate reductase in *Pseudomonas* sp. strain G-179 catalyzes the first step of denitrification. *J Bacteriol, 181*, 2802-2806.

Bell, L. C., Richardson, D. J., & Ferguson, S. J. (1992). Identification of nitric oxide reductase activity in *Rhodobacter capsulatus*: the electron transport pathway can either use or bypass both cytochrome c_2 and the cytochrome bc_1 complex. *J Gen Microbiol, 138*, 437-443.

Bergaust, L., Mao, Y., Bakken, L. R., & Frostegard, A. (2010). Denitrification response patterns during the transition to anoxic respiration and posttranscriptional effects of suboptimal pH on nitrous oxide reductase in *Paracoccus denitrificans*. *Appl Environ Microbiol, 76*, 6387-6396.

Bergaust, L., van Spanning, R. J., Frostegard, A., & Bakken, L. R. (2012). Expression of nitrous oxide reductase in *Paracoccus denitrificans* is regulated by oxygen and nitric oxide through FnrP and NNR. *Microbiology, 158*, 826-834.

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*. *J Bacteriol, 196*, 2190-2200.

Bertero, M. G., Rothery, R. A., Palak, M., Hou, C., Lim, D., Blasco, F., et al. (2003). Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. *Nat Struct Biol, 10*, 681-687.

Bethke, P. C., Badger, M. R., & Jones, R. L. (2004). Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell, 16*, 332-341.

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. *Mol Plant Microbe Interact, 28*, 1353-1363.

Blasco, F., Iobbi, C., Ratouchniak, J., Bonnefoy, V., & Chippaux, M. (1990). Nitrate reductases of *Escherichia coli*: sequence of the second nitrate reductase and comparison with that encoded by the *narGHJI* operon. *Mol Gen Genet, 222*, 104-111.

Bleakley, B. H., & Tiedje, J. M. (1982). Nitrous oxide production by organisms other than nitrifiers or denitrifiers. *Appl Environ Microbiol, 44*, 1342-1348.

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

Bodenmiller, D. M., & Spiro, S. (2006). The *yjeB* (*nsrR*) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol, 188*, 874-881.

Bokranz, M., Katz, J., Schröder, I., Robertson, A. M., & Kröger, A. (1983). Energy metabolism and biosynthesis of *Vibrio succinogenes* growing with nitrate or nitrite as terminal electron acceptor. *Arch Microbiol, 135*, 36-41.

Bothe, J. R., & Beyer, K. D. (2007). Experimental determination of the $NH_4NO_3/(NH_4)_2SO_4/H_2O$ phase diagram. *J Phys Chem A, 111*, 12106-12117.

Bouchal, P., Struharova, I., Budinska, E., Sedo, O., Vyhlidalova, T., Zdrahal, Z., et al. (2010). Unraveling an FNR based regulatory circuit in *Paracoccus denitrificans* using a proteomics-based approach. *Biochim Biophys Acta, 1804*, 1350-1358.

Bricio, C., Alvarez, 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. *Appl Environ Microbiol, 80*, 4871-4878.

Bronder, M., Mell, H., Stupperich, E., & Kroger, A. (1982). Biosynthetic Pathways of *Vibrio succinogenes* growing with fumarate as terminal electron acceptor and sole carbon source. *Arch Microbiol, 131*, 216-223.

Broughton, W. J., Hernández, G., Blair, M., Beebe, S., Gepts, P., & Vanderleyden, J. (2003). Beans (*Phaseolus* spp.) - Model food legumes. *Plant and Soil 252*, 55–128.

Browning, D. F., Lee, D. J., Spiro, S., & Busby, S. J. (2010). Down-regulation of the *Escherichia coli* K-12 *nrf* promoter by binding of the NsrR nitric oxide-sensing transcription repressor to an upstream site. *J Bacteriol, 192*, 3824-3828.

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 Microbiol Lett, 279*, 188-194.

Bueno, E., Gomez-Hernandez, N., Girard, L., Bedmar, E. J., & Delgado, M. J. (2005). Function of the *Rhizobium etli* CFN42 *nirK* gene in nitrite metabolism. *Biochem Soc Trans, 33*, 162-163.

Bueno, E., Mesa, S., Bedmar, E. J., Richardson, D. J., & Delgado, M. J. (2012). Bacterial adaptation of respiration from oxic to microoxic and anoxic conditions: redox control. *Antioxid Redox Signal, 16*, 819-852.

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

Burgess, B. K., & Lowe, D. J. (1996). Mechanism of molybdenum nitrogenase. *Chem Rev, 96*, 2983-3012.

Busch, A., Strube, K., Friedrich, B., & Cramm, R. (2005). Transcriptional regulation of nitric oxide reduction in *Ralstonia eutropha* H16. *Biochem Soc Trans, 33*, 193-194.

Bush, M., Ghosh, T., Tucker, N., Zhang, X., & Dixon, R. (2010). Nitric oxide-responsive interdomain regulation targets the σ54-interaction surface in the enhancer binding protein NorR. *Mol Microbiol, 77*, 1278-1288.

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. *J Bacteriol, 183*, 189-199.
Cabrera, J. J., Salas, A., Torres, M. J., Bedmar, E. J., Richardson, D. J., Gates, A. J., et al. (2015). An integrated biochemical system for nitrate assimilation and nitric oxide detoxification in *Bradyrhizobium japonicum*. *Biochem J*.
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 Phytol, 196*, 548-560.
Campbell, B. J., Engel, A. S., Porter, M. L., & Takai, K. (2006). The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nat Rev Microbiol, 4*, 458-468.
Campbell, M. A., Nyerges, G., Kozlowski, J. A., Poret-Peterson, A. T., Stein, L. Y., & Klotz, M. G. (2011). Model of the molecular basis for hydroxylamine oxidation and nitrous oxide production in methanotrophic bacteria. *FEMS Microbiol Lett, 322*, 82-89.
Carrica, M. D., Fernandez, I., Sieira, R., Paris, G., & Goldbaum, F. A. (2013). The two-component systems PrrBA and NtrYX co-ordinately regulate the adaptation of *Brucella abortus* to an oxygen-limited environment. *Mol Microbiol*.
Castiglione, N., Rinaldo, S., Giardina, G., & Cutruzzola, F. (2009). The transcription factor DNR from *Pseudomonas aeruginosa* specifically requires nitric oxide and haem for the activation of a target promoter in *Escherichia coli*. *Microbiology, 155*, 2838-2844.
Chen, S., Cao, L., Intengan, H. D., Humphreys, M., & Gardner, D. G. (2002). Osmoregulation of endothelial nitric-oxide synthase gene expression in inner medullary collecting duct cells. Role in activation of the type A natriuretic peptide receptor. *J Biol Chem, 277*, 32498-32504.
Cheung, J., & Hendrickson, W. A. (2009). Structural analysis of ligand stimulation of the histidine kinase NarX. *Structure, 17*, 190-201.
Chhabra, S., & Spiro, S. (2015). Inefficient translation of *nsrR* constrains behaviour of the NsrR regulon in *Escherichia coli*. *Microbiology, 161*, 2029-2038.
Clarke, T. A., Cole, J. A., Richardson, D. J., & Hemmings, A. M. (2007). The crystal structure of the pentahaem *c*-type cytochrome NrfB and characterization of its solution-state interaction with the pentahaem nitrite reductase NrfA. *Biochem J, 406*, 19-30.
Clarke, T. A., Mills, P. C., Poock, S. R., Butt, J. N., Cheesman, M. R., Cole, J. A., et al. (2008). *Escherichia coli* cytochrome *c* nitrite reductase NrfA. *Methods Enzymol, 437*, 63-77.
Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. A., et al. (2006). A reassessment of the FNR regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL, and NarQP as *Escherichia coli* K12 adapts from aerobic to anaerobic growth. *J Biol Chem, 281*, 4802-4815.
Cook, D. R. (1999). *Medicago truncatula*--a model in the making! *Curr Opin Plant Biol, 2*, 301-304.
Corker, H., & Poole, R. K. (2003). Nitric oxide formation by *Escherichia coli.* Dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohemoglobin Hmp. *J Biol Chem, 278*, 31584-31592.
Costa, C., Macedo, A., Moura, I., Moura, J. J., Le Gall, J., Berlier, Y., et al. (1990). Regulation of the hexaheme nitrite/nitric oxide reductase of *Desulfovibrio desulfuricans, Wolinella succinogenes* and *Escherichia coli.* A mass spectrometric study. *FEBS Lett, 276*, 67-70.
Coyle, C. L., Zumft, W. G., Kroneck, P. M., Korner, H., & Jakob, W. (1985). Nitrous oxide reductase from denitrifying *Pseudomonas perfectomarina*. Purification

and properties of a novel multicopper enzyme. *Eur J Biochem, 153*, 459-467.
Crack, J. C., Green, J., Hutchings, M. I., Thomson, A. J., & Le Brun, N. E. (2012). Bacterial iron-sulfur regulatory proteins as biological sensor-switches. *Antioxid Redox Signal, 17*, 1215-1231.
Crack, J. C., Munnoch, J., Dodd, E. L., Knowles, F., Al Bassam, M. M., Kamali, S., et al. (2015). NsrR from *Streptomyces coelicolor* is a nitric oxide-sensing [4Fe-4S] cluster protein with a specialized regulatory function. *J Biol Chem, 290*, 12689-12704.
Crack, J. C., Stapleton, M. R., Green, J., Thomson, A. J., & Le Brun, N. E. (2013). Mechanism of [4Fe-4S](Cys)4 cluster nitrosylation is conserved among NO-responsive regulators. *J Biol Chem, 288*, 11492-11502.
Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M. N., Scott, C., Thomson, A. J., et al. (2002). NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. *EMBO J, 21*, 3235-3244.
Darwin, A. J., Ziegelhoffer, E. C., Kiley, P. J., & Stewart, V. (1998). Fnr, NarP, and NarL regulation of *Escherichia coli* K-12 *napF* (periplasmic nitrate reductase) operon transcription in vitro. *J Bacteriol, 180*, 4192-4198.
Daskalakis, V., Ohta, T., Kitagawa, T., & Varotsis, C. (2015). Structure and properties of the catalytic site of nitric oxide reductase at ambient temperature. *Biochim Biophys Acta, 1847*, 1240-1244.
D'Autreaux, B., Tucker, N. P., Dixon, R., & Spiro, S. (2005). A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature, 437*, 769-772.
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*. *Eur J Biochem, 242*, 592-600.
de Vries, S., Strampraad, M. J., Lu, S., Moenne-Loccoz, P., & Schroder, I. (2003). Purification and characterization of the MQH_2:NO oxidoreductase from the hyperthermophilic archaeon *Pyrobaculum aerophilum*. *J Biol Chem, 278*, 35861-35868.
De Vries, S., Suharti, & Pouvreau, L. A. M. (2007). Nitric oxide reductase: structural variations and catalytic mechanism. In H. Bothe, Ferguson, S.J., Newton, W.E. (Ed.), *Biology of the Nitrogen Cycle* (pp. 57-67). Amsterdam: Elservier Science.
Delamuta, J. R., Ribeiro, R. A., Ormeno-Orrillo, E., Melo, I. S., Martinez-Romero, E., & Hungria, M. (2013). Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. *Int J Syst Evol Microbiol, 63*, 3342-3351.
Delgado, M. J., Casella, S., Bedmar, E.J. (2007). Denitrification in rhizobia-legume symbiosis. In H. Bothe, Ferguson, S.J., Newton, W.E. (Ed.), *Biology of the Nitrogen Cycle.* (pp. 83-93). Amsterdam: Elsevier Science.
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.
Desloover, J., Roobroeck, D., Heylen, K., Puig, S., Boeckx, P., Verstraete, W., et al. (2014). Pathway of nitrous oxide consumption in isolated *Pseudomonas stutzeri* strains under anoxic and oxic conditions. *Environ Microbiol, 16*, 3143-3152.
Dreusch, A., Burgisser, D. M., Heizmann, C. W., & Zumft, W. G. (1997). Lack of copper insertion into unprocessed cytoplasmic nitrous oxide reductase generated by an R20D substitution in the arginine consensus motif of the signal peptide.

Biochim Biophys Acta, 1319, 311-318.
Einsle, O. (2011). Structure and function of formate-dependent cytochrome *c* nitrite reductase, NrfA. *Methods Enzymol, 496*, 399-422.
Einsle, O., Messerschmidt, A., Huber, R., Kroneck, P. M., & Neese, F. (2002). Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome *c* nitrite reductase. *J Am Chem Soc, 124*, 11737-11745.
Elsen, S., Swem, L. R., Swem, D. L., & Bauer, C. E. (2004). RegB/RegA, a highly conserved redox-responding global two-component regulatory system. *Microbiol Mol Biol Rev, 68*, 263-279.
Elvers, K. T., Turner, S. M., Wainwright, L. M., Marsden, G., Hinds, J., Cole, J. A., et al. (2005). NssR, a member of the Crp-Fnr superfamily from *Campylobacter jejuni*, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin. *Mol Microbiol, 57*, 735-750.
Felgate, H., Giannopoulos, G., Sullivan, M. J., Gates, A. J., Clarke, T. A., Baggs, E., et al. (2012). The impact of copper, nitrate and carbon status on the emission of nitrous oxide by two species of bacteria with biochemically distinct denitrification pathways. *Environ Microbiol, 14*, 1788-1800.
Filenko, N., Spiro, S., Browning, D. F., Squire, D., Overton, T. W., Cole, J., et al. (2007). The NsrR regulon of *Escherichia coli* K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J Bacteriol, 189*, 4410-4417.
Forrester, M. T., & Foster, M. W. (2012). Protection from nitrosative stress: a central role for microbial flavohemoglobin. *Free Radic Biol Med, 52*, 1620-1633.
Fujita, K., & Dooley, D. M. (2007). Insights into the mechanism of N_2O reduction by reductively activated N_2O reductase from kinetics and spectroscopic studies of pH effects. *Inorg Chem, 46*, 613-615.
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, A. M., & Gardner, P. R. (2002). Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, *Escherichia coli*. Evidence for a novel inducible anaerobic nitric oxide-scavenging activity. *J Biol Chem, 277*, 8166-8171.
Gardner, A. M., Gessner, C. R., & Gardner, P. R. (2003). Regulation of the nitric oxide reduction operon (*norRVW*) in *Escherichia col*i. Role of NorR and σ54 in the nitric oxide stress response. *J Biol Chem, 278*, 10081-10086.
Gardner, A. M., Helmick, R. A., & Gardner, P. R. (2002). Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. *J Biol Chem, 277*, 8172-8177.
Gardner, P. R. (2005). Nitric oxide dioxygenase function and mechanism of flavohemoglobin, hemoglobin, myoglobin and their associated reductases. *J Inorg Biochem, 99*, 247-266.
Giardina, G., Rinaldo, S., Johnson, K. A., Di Matteo, A., Brunori, M., & Cutruzzola, F. (2008). NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *J Mol Biol, 378*, 1002-1015.
Gilberthorpe, N. J., & Poole, R. K. (2008). Nitric oxide homeostasis in *Salmonella typhimurium*: roles of respiratory nitrate reductase and flavohemoglobin. *J Biol Chem, 283*, 11146-11154.
Goddard, A. D., Moir, J. W., Richardson, D. J., & Ferguson, S. J. (2008). Interdependence of two NarK domains in a fused nitrate/nitrite transporter. *Mol Microbiol, 70*, 667-681.

Gomes, C. M., Giuffre, A., Forte, E., Vicente, J. B., Saraiva, L. M., Brunori, M., et al. (2002). A novel type of nitric-oxide reductase. *Escherichia coli* flavorubredoxin. *J Biol Chem, 277*, 25273-25276.

Gomez-Hernandez, N., Reyes-Gonzalez, A., Sanchez, C., Mora, Y., Delgado, M. J., & Girard, L. (2011). Regulation and symbiotic role of *nirK* and *norC* expression in *Rhizobium etli*. *Mol Plant Microbe Interact, 24*, 233-245.

Gonzalez, P. J., Correia, C., Moura, I., Brondino, C. D., & Moura, J. J. (2006). Bacterial nitrate reductases: Molecular and biological aspects of nitrate reduction. *J Inorg Biochem, 100*, 1015-1023.

Grahl, S., Maillard, J., Spronk, C. A., Vuister, G. W., & Sargent, F. (2012). Overlapping transport and chaperone-binding functions within a bacterial twin-arginine signal peptide. *Mol Microbiol, 83*, 1254-1267.

Gupta, K. J., Bauwe, H., & Mur, L. A. (2011). Nitric oxide, nitrate reductase and UV-B tolerance. *Tree Physiol, 31*, 795-797.

Haltia, T., Brown, K., Tegoni, M., Cambillau, C., Saraste, M., Mattila, K., et al. (2003). Crystal structure of nitrous oxide reductase from *Paracoccus denitrificans* at 1.6 A resolution. *Biochem J, 369*, 77-88.

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

Hauser, F., Pessi, G., Friberg, M., Weber, C., Rusca, N., Lindemann, A., et al. (2007). Dissection of the *Bradyrhizobium japonicum* NifA+σ_{54} regulon, and identification of a ferredoxin gene (*fdxN*) for symbiotic nitrogen fixation. *Mol Genet Genomics, 278*, 255-271.

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

Hendriks, J., Warne, A., Gohlke, U., Haltia, T., Ludovici, C., Lubben, M., et al. (1998). The active site of the bacterial nitric oxide reductase is a dinuclear iron center. *Biochemistry, 37*, 13102-13109.

Hermann, B., Kern, M., La Pietra, L., Simon, J., & Einsle, O. (2015). The octahaem MccA is a haem *c*-copper sulfite reductase. *Nature, 520*, 706-709.

Heurlier, K., Thomson, M. J., Aziz, N., & Moir, J. W. (2008). The nitric oxide (NO)-sensing repressor NsrR of *Neisseria meningitidis* has a compact regulon of genes involved in NO synthesis and detoxification. *J Bacteriol, 190*, 2488-2495.

Heylen, K., & Keltjens, J. (2012). Redundancy and modularity in membrane-associated dissimilatory nitrate reduction in *Bacillus*. *Front Microbiol, 3*, 371.

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

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.

Hirayama, J., Eda, S., Mitsui, H., & Minamisawa, K. (2011). Nitrate-dependent N_2O emission from intact soybean nodules via denitrification by *Bradyrhizobium japonicum* bacteroids. *Appl Environ Microbiol, 77*, 8787-8790.

Hole, U. H., Vollack, K. U., Zumft, W. G., Eisenmann, E., Siddiqui, R. A., Friedrich, B., et al. (1996). Characterization of the membranous denitrification enzymes nitrite reductase (cytochrome *cd*$_1$) and copper-containing nitrous oxide reductase from *Thiobacillus denitrificans*. *Arch Microbiol, 165*, 55-61.

Holloway, P., McCormick, W., Watson, R. J., & Chan, Y. K. (1996). Identification and analysis of the dissimilatory nitrous oxide reduction genes, *nosRZDFY*, of *Rhizobium meliloti*. *J Bacteriol, 178*, 1505-1514.

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. *J Bacteriol, 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 Physiol, 155*, 1023-1036.

Hutchings, M. I., Mandhana, N., & Spiro, S. (2002). The NorR protein of *Escherichia coli* activates expression of the flavorubredoxin gene *norV* in response to reactive nitrogen species. *J Bacteriol, 184*, 4640-4643.

Hutchings, M. I., & Spiro, S. (2000). The nitric oxide regulated nor promoter of *Paracoccus denitrificans*. *Microbiology, 146 (Pt 10)*, 2635-2641.

Inaba, S., Ikenishi, F., Itakura, M., Kikuchi, M., Eda, S., Chiba, N., et al. (2012). N_2O emission from degraded soybean nodules depends on denitrification by *Bradyrhizobium japonicum* and other microbes in the rhizosphere. *Microbes Environ, 27*, 470-476.

Inaba, S., Tanabe, K., Eda, S., Ikeda, S., Higashitani, A., Mitsui, H., et al. (2009). Nitrous oxide emission and microbial community in the rhizosphere of nodulated soybeans during the late growth period. *Microbes Environ, 24*, 64-67.

IPCC. (2014). *Climate change 2014: synthesis report. Contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change.* Geneva, Switzerland: IPCC.

Itakura, M., Uchida, Y., Akiyama, H., Hoshino, Y. T., Shimomura, Y., Morimoto, S., et al. (2013). Mitigation of nitrous oxide emissions from soils by *Bradyrhizobium japonicum* inoculation. [10.1038/nclimate1734]. *Nature Clim. Change, 3*, 208-212.

Jensen, B. B., & Burris, R. H. (1986). N_2O as a substrate and as a competitive inhibitor of nitrogenase. *Biochemistry, 25*, 1083-1088.

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*. *J Biol Chem, 282*, 6425-6437.

Jetten, M. S. (2008). The microbial nitrogen cycle. *Environ Microbiol, 10*, 2903-2909.

Johnston, E. M., Dell'Acqua, S., Pauleta, S. R., Moura, I., & Solomon, E. I. (2015). Protonation state of the CuS Cu site in nitrous oxide reductase: redox dependence and insight into reactivity. *Chem Sci, 6*, 5670-5679.

Johnston, E. M., Dell'Acqua, S., Ramos, S., Pauleta, S. R., Moura, I., & Solomon, E. I. (2014). Determination of the active form of the tetranuclear copper sulfur cluster in nitrous oxide reductase. *J Am Chem Soc, 136*, 614-617.

Jones, C. M., Graf, D. R., Bru, D., Philippot, L., & Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME J, 7*, 417-426.

Jones, J. R., Tsigkou, O., Coates, E. E., Stevens, M. M., Polak, J. M., & Hench, L. L. (2007). Extracellular matrix formation and mineralization on a phosphate-free porous bioactive glass scaffold using primary human osteoblast (HOB) cells. *Biomaterials, 28*, 1653-1663.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., & Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat Rev*

Microbiol, 5, 619-633.
Jormakka, M., Richardson, D., Byrne, B., & Iwata, S. (2004). Architecture of NarGH reveals a structural classification of Mo-bisMGD enzymes. *Structure, 12*, 95-104.
Justino, M. C., Ecobichon, C., Fernandes, A. F., Boneca, I. G., & Saraiva, L. M. (2012). *Helicobacter pylori* has an unprecedented nitric oxide detoxifying system. *Antioxid Redox Signal, 17*, 1190-1200.
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 Res, 9*, 189-197.
Kaspar, H. F., & Tiedje, J. M. (1981). Dissimilatory reduction of nitrate and nitrite in the bovine rumen: nitrous oxide production and effect of acetylene. *Appl Environ Microbiol, 41*, 705-709.
Kato, K., Kanahama, K., & Kanayama, Y. (2010). Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in *Lotus* root nodules. *J Plant Physiol, 167*, 238-241.
Kern, M., Eisel, F., Scheithauer, J., Kranz, R. G., & Simon, J. (2010). Substrate specificity of three cytochrome *c* haem lyase isoenzymes from *Wolinella succinogenes*: unconventional haem *c* binding motifs are not sufficient for haem *c* attachment by NrfI and CcsA1. *Mol Microbiol, 75*, 122-137.
Kern, M., Mager, A. M., & Simon, J. (2007). Role of individual *nap* gene cluster products in NapC-independent nitrate respiration of *Wolinella succinogenes*. *Microbiology, 153*, 3739-3747.
Kern, M., & Simon, J. (2008). Characterization of the NapGH quinol dehydrogenase complex involved in *Wolinella succinogenes* nitrate respiration. *Mol Microbiol, 69*, 1137-1152.
Kern, M., & Simon, J. (2009). Electron transport chains and bioenergetics of respiratory nitrogen metabolism in *Wolinella succinogenes* and other Epsilonproteobacteria. *Biochim Biophys Acta, 1787*, 646-656.
Kern, M., & Simon, J. (2009). Periplasmic nitrate reduction in *Wolinella succinogenes*: cytoplasmic NapF facilitates NapA maturation and requires the menaquinol dehydrogenase NapH for membrane attachment. *Microbiology, 155*, 2784-2794.
Kern, M., & Simon, J. (2016). Three transcription regulators of the Nss family mediate the adaptive response induced by nitrate, nitric oxide or nitrous oxide in *Wolinella succinogenes*. *Environ Microbiol*.
Kern, M., Klotz, M. G., & Simon, J. (2011a). The *Wolinella succinogenes mcc* gene cluster encodes an unconventional respiratory sulphite reduction system. *Mol Microbiol, 82*, 1515-1530.

Kern, M., Volz, J., & Simon, J. (2011b). The oxidative and nitrosative stress defence network of *Wolinella succinogenes*: cytochrome *c* nitrite reductase mediates the stress response to nitrite, nitric oxide, hydroxylamine and hydrogen peroxide. *Environ Microbiol, 13*, 2478-2494.
Kern, M., Winkler, C., & Simon, J. (2011c). Respiratory nitrogen metabolism and nitrosative stress defence in -proteobacteria: the role of NssR-type transcription regulators. *Biochem Soc Trans, 39*, 299-302.
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 Lett, 445*, 389-394.
Kobayashi, M., & Shoun, H. (1995). The copper-containing dissimilatory nitrite

reductase involved in the denitrifying system of the fungus *Fusarium oxysporum*. *J Biol Chem, 270*, 4146-4151.
Kraft, B., Strous, M., & Tegetmeyer, H. E. (2011). Microbial nitrate respiration--genes, enzymes and environmental distribution. *J Biotechnol, 155*, 104-117.
Kroger, A., Biel, S., Simon, J., Gross, R., Unden, G., & Lancaster, C. R. (2002). Fumarate respiration of *Wolinella succinogenes*: enzymology, energetics and coupling mechanism. *Biochim Biophys Acta, 1553*, 23-38.
Kroneck, P. M., Antholine, W. A., Riester, J., & Zumft, W. G. (1989). The nature of the cupric site in nitrous oxide reductase and of CuA in cytochrome *c* oxidase. *FEBS Lett, 248*, 212-213.
Kullik, I., Fritsche, S., Knobel, H., Sanjuan, J., Hennecke, H., & Fischer, H. M. (1991). *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the σ_{54} gene (*rpoN*). *J Bacteriol, 173*, 1125-1138.
Laguri, C., Stenzel, R. A., Donohue, T. J., Phillips-Jones, M. K., & Williamson, M. P. (2006). Activation of the global gene regulator PrrA (RegA) from *Rhodobacter sphaeroides*. *Biochemistry, 45*, 7872-7881.
Lancaster, C. R., Sauer, U. S., Gross, R., Haas, A. H., Graf, J., Schwalbe, H., et al. (2005). Experimental support for the "E pathway hypothesis" of coupled transmembrane e^- and H^+ transfer in dihemic quinol:fumarate reductase. *Proc Natl Acad Sci U S A, 102*, 18860-18865.
Laratta, W. P., Choi, P. S., Tosques, I. E., & Shapleigh, J. P. (2002). Involvement of the PrrB/PrrA two-component system in nitrite respiration in *Rhodobacter sphaeroides* 2.4.3: evidence for transcriptional regulation. *J Bacteriol, 184*, 3521-3529.
Lee, Y. Y., Shearer, N., & Spiro, S. (2006). Transcription factor NNR from *Paracoccus denitrificans* is a sensor of both nitric oxide and oxygen: isolation of *nnr** alleles encoding effector-independent proteins and evidence for a haem-based sensing mechanism. *Microbiology, 152*, 1461-1470.
Liu, B., Frostegård, Å., & Bakken, L. R. (2014). Impaired reduction of N_2O to N_2 in acid soil is due to a posttranscriptional interference with the expression of *nosZ*. *MBio, 5*, e01383–e01314.
Liu, B., Morkved, P. T., Frostegard, A., & Bakken, L. R. (2010). Denitrification gene pools, transcription and kinetics of NO, N_2O and N_2 production as affected by soil pH. *FEMS Microbiol Ecol, 72*, 407-417.
Liu, G. H., Liu, B., Wang, J. P., Che, J. M., Chen, Q. Q., & Chen, Z. (2015). High-quality genome sequence of *Bacillus vireti* DSM 15602T for setting up phylogenomics for the genomic taxonomy of *Bacillus*-like bacteria. *Genome Announc, 3*.
Liu, J. J., Wu, W. X., Ding, Y., Shi, D. Z., & Chen, Y. X. (2010). Ammonia-oxidizing archaea and their important roles in nitrogen biogeochemical cycling: a review. *Ying Yong Sheng Tai Xue Bao, 21*, 2154-2160.
Lockwood, C. W., Burlat, B., Cheesman, M. R., Kern, M., Simon, J., Clarke, T. A., et al. (2015). Resolution of key roles for the distal pocket histidine in cytochrome *c* nitrite reductases. *J Am Chem Soc, 137*, 3059-3068.
Luckmann, M., Mania, D., Kern, M., Bakken, L. R., Frostegard, A., & Simon, J. (2014). Production and consumption of nitrous oxide in nitrate-ammonifying *Wolinella succinogenes* cells. *Microbiology, 160*, 1749-1759.
Lukat, P., Rudolf, M., Stach, P., Messerschmidt, A., Kroneck, P. M., Simon, J., et al. (2008). Binding and reduction of sulfite by cytochrome *c* nitrite reductase. *Biochemistry, 47*, 2080-2086.

Maia, L. B., & Moura, J. J. (2014). How biology handles nitrite. *Chem Rev, 114*, 5273-5357.

Mania, D., Heylen, K., van Spanning, R. J., & Frostegard, A. (2014). The nitrate-ammonifying and *nosZ*-carrying bacterium *Bacillus vireti* is a potent source and sink for nitric and nitrous oxide under high nitrate conditions. *Environ Microbiol, 16*, 3196-3210.

Mania, D., Heylen, K., van Spanning, R. J., & Frostegard, A. (2016). Regulation of nitrogen metabolism in the nitrate-ammonifying soil bacterium *Bacillus vireti* and evidence for its ability to grow using NO as electron acceptor. *Environ Microbiol*.

Martinez-Espinosa, R. M., Dridge, E. J., Bonete, M. J., Butt, J. N., Butler, C. S., Sargent, F., et al. (2007). Look on the positive side! The orientation, identification and bioenergetics of 'Archaeal' membrane-bound nitrate reductases. *FEMS Microbiol Lett, 276*, 129-139.

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*. *Nat Struct Mol Biol, 19*, 238-245.

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., Blanquet, P., Cam, Y., & Bruand, C. (2013). Control of NO level in rhizobium-legume root nodules: not only a plant globin story. *Plant Signal Behav, 8*, doi: 10 4161/psb 25923.

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

Mesa, S., Alche Jd, J. D., Bedmar, E., & Delgado, M. J. (2004). Expression of *nir*, *nor* and *nos* denitrification genes from *Bradyrhizobium japonicum* in soybean root nodules. *Physiol Plant, 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-FixK2 regulatory cascade. *J Bacteriol, 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*. *J Bacteriol, 190*, 6568-6579.

Mesa, S., Velasco, L., Manzanera, M. E., Delgado, M. J., & Bedmar, E. J. (2002). Characterization of the *norCBQD* genes, encoding nitric oxide reductase, in the nitrogen fixing bacterium *Bradyrhizobium japonicum*. *Microbiology, 148*, 3553-3560.

Mettert, E. L., & Kiley, P. J. (2015). Fe-S proteins that regulate gene expression. *Biochim Biophys Acta, 1853*, 1284-1293.

Mills, C. E., Sedelnikova, S., Soballe, B., Hughes, M. N., & Poole, R. K. (2001). *Escherichia coli* flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a low affinity for dioxygen in the absence or presence of nitric oxide. *Biochem J, 353*, 207-213.

Mills, P. C., Rowley, G., Spiro, S., Hinton, J. C., & Richardson, D. J. (2008). A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar *Typhimurium* against killing by NO in anoxic environments. *Microbiology, 154*, 1218-1228.

Miyahara, M., Kim, S. W., Fushinobu, S., Takaki, K., Yamada, T., Watanabe, A., et al. (2010). Potential of aerobic denitrification by *Pseudomonas stutzeri* TR2 to reduce nitrous oxide emissions from wastewater treatment plants. *Appl Environ Microbiol, 76*, 4619-4625.

Monk, C. E., Pearson, B. M., Mulholland, F., Smith, H. K., & Poole, R. K. (2008). Oxygen- and NssR-dependent globin expression and enhanced iron acquisition in the response of campylobacter to nitrosative stress. *J Biol Chem, 283*, 28413-28425.

Morley, N., Baggs, E. M., Dorsch, P., & Bakken, L. (2008). Production of NO, N_2O and N_2 by extracted soil bacteria, regulation by NO_2^- and O_2 concentrations. *FEMS Microbiol Ecol, 65*, 102-112.

Muhlig, A., Kabisch, J., Pichner, R., Scherer, S., & Muller-Herbst, S. (2014). Contribution of the NO-detoxifying enzymes HmpA, NorV and NrfA to nitrosative stress protection of *Salmonella Typhimurium* in raw sausages. *Food Microbiol, 42*, 26-33.

Mur, L. A., Mandon, J., Persijn, S., Cristescu, S. M., Moshkov, I. E., Novikova, G. V., et al. (2013). Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants, 5*, pls052.

Myers, K. S., Yan, H., Ong, I. M., Chung, D., Liang, K., Tran, F., et al. (2013). Genome-scale analysis of *Escherichia coli* FNR reveals complex features of transcription factor binding. *PLoS Genet, 9*, e1003565.

Nellen-Anthamatten, D., Rossi, P., Preisig, O., Kullik, I., Babst, M., Fischer, H. M., et al. (1998). *Bradyrhizobium japonicum* FixK2, a crucial distributor in the FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen levels. *J Bacteriol, 180*, 5251-5255.

Nienaber, A., Huber, A., Gottfert, M., Hennecke, H., & Fischer, H. M. (2000). Three new NifA-regulated genes in the *Bradyrhizobium japonicum* symbiotic gene region discovered by competitive DNA-RNA hybridization. *J Bacteriol, 182*, 1472-1480.

Nilavongse, A., Brondijk, T. H., Overton, T. W., Richardson, D. J., Leach, E. R., & Cole, J. A. (2006). The NapF protein of the *Escherichia coli* periplasmic nitrate reductase system: demonstration of a cytoplasmic location and interaction with the catalytic subunit, NapA. *Microbiology, 152*, 3227-3237.

Overton, T. W., Whitehead, R., Li, Y., Snyder, L. A., Saunders, N. J., Smith, H., et al. (2006). Coordinated regulation of the *Neisseria gonorrhoeae*-truncated denitrification pathway by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP. *J Biol Chem, 281*, 33115-33126.

Partridge, J. D., Bodenmiller, D. M., Humphrys, M. S., & Spiro, S. (2009). NsrR targets in the *Escherichia coli* genome: new insights into DNA sequence requirements for binding and a role for NsrR in the regulation of motility. *Mol Microbiol, 73*, 680-694.

Partridge, J. D., Sanguinetti, G., Dibden, D. P., Roberts, R. E., Poole, R. K., & Green, J. (2007). Transition of *Escherichia coli* from aerobic to micro-aerobic conditions involves fast and slow reacting regulatory components. *J Biol Chem, 282*, 11230-11237.

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*. *Mol Microbiol, 45*, 1303-1314.

Payne, W. J., Grant, M. A., Shapleigh, J., & Hoffman, P. (1982). Nitrogen oxide reduction in *Wolinella succinogenes* and *Campylobacter* species. *J Bacteriol,*

152, 915-918.
Peakman, T., Busby, S., & Cole, J. (1990). Transcriptional control of the *cysG* gene of *Escherichia coli* K-12 during aerobic and anaerobic growth. *Eur J Biochem, 191*, 325-331.
Peakman, T., Crouzet, J., Mayaux, J. F., Busby, S., Mohan, S., Harborne, N., et al. (1990). Nucleotide sequence, organisation and structural analysis of the products of genes in the *nirB-cysG* region of the *Escherichia coli* K-12 chromosome. *Eur J Biochem, 191*, 315-323.
Pisa, R., Stein, T., Eichler, R., Gross, R., & Simon, J. (2002). The *nrfI* gene is essential for the attachment of the active site haem group of *Wolinella succinogenes* cytochrome *c* nitrite reductase. *Mol Microbiol, 43*, 763-770.
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 Comput Biol, 8*, e1002674.
Pohlmann, A., Cramm, R., Schmelz, K., & Friedrich, B. (2000). A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. *Mol Microbiol, 38*, 626-638.
Pomowski, A., Zumft, W. G., Kroneck, P. M., & Einsle, O. (2011). N_2O binding at a [4Cu:2S] copper-sulphur cluster in nitrous oxide reductase. *Nature, 477*, 234-237.
Poock, S. R., Leach, E. R., Moir, J. W., Cole, J. A., & Richardson, D. J. (2002). Respiratory detoxification of nitric oxide by the cytochrome *c* nitrite reductase of *Escherichia coli*. *J Biol Chem, 277*, 23664-23669.
Poole, R. K. (2005). Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans, 33*, 176-180.
Potter, L., Angove, H., Richardson, D., & Cole, J. (2001). Advances in Microbial Physiology. In R. K. Poole (Ed.), (Vol. 45, pp. 51–112). London: Academic Press Ltd.
.
Potter, L., Angove, H., Richardson, D., & Cole, J. (2001). Nitrate reduction in the periplasm of gram-negative bacteria. *Adv Microb Physiol, 45*, 51-112.
Potter, L., Angove, H., Richardson, D., & Cole, J. (2001). Nitrate reduction in the periplasm of gram-negative bacteria. *Adv Microb Physiol, 45*, 51-112.
Prendergast-Miller, M. T., Baggs, E. M., & Johnson, D. (2011). Nitrous oxide production by the ectomycorrhizal fungi *Paxillus involutus* and *Tylospora fibrillosa*. *FEMS Microbiol Lett, 316*, 31-35.
Qu, Z., Bakken, L. R., Molstad, L., Frostegard, A., & Bergaust, L. (2015). Transcriptional and metabolic regulation of denitrification in *Paracoccus denitrificans* allows low but significant activity of nitrous oxide reductase under oxic conditions. *Environ Microbiol*.
Ranson-Olson, B., Jones, D. F., Donohue, T. J., & Zeilstra-Ryalls, J. H. (2006). In vitro and in vivo analysis of the role of PrrA in *Rhodobacter sphaeroides* 2.4.1 *hemA* gene expression. *J Bacteriol, 188*, 3208-3218.
Rasmussen, T., Berks, B. C., Sanders-Loehr, J., Dooley, D. M., Zumft, W. G., & Thomson, A. J. (2000). The catalytic center in nitrous oxide reductase, Cu_Z, is a copper-sulfide cluster. *Biochemistry, 39*, 12753-12756.
Reents, H., Munch, R., Dammeyer, T., Jahn, D., & Hartig, E. (2006). The Fnr regulon of *Bacillus subtilis*. *J Bacteriol, 188*, 1103-1112.
Richardson, D., Felgate, H., Watmough, N., Thomson, A., & Baggs, E. (2009). Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle -

could enzymic regulation hold the key? *Trends Biotechnol, 27*, 388-397.
Richardson, D., & Sawers, G. (2002). Structural biology. PMF through the redox loop. *Science, 295*, 1842-1843.
Richardson, D. J. (2000). Bacterial respiration: a flexible process for a changing environment. *Microbiology, 146 (Pt 3)*, 551-571.
Richardson, D. J. (2011). Redox complexes of the nitrogen cycle. In J. W. B. Moir (Ed.), *Nitrogen Cycling in Bacteria* (pp. 23-39). Norkfolk, UK: 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. *Cell Mol Life Sci, 58*, 165-178.
Richardson, D. J., van Spanning, R. J., & Ferguson, S. J. (2007). The prokaryotic nitrate reductases. In B. H., F. S.J. & N. W.E. (Eds.), *Biology of the Nitrogen Cycle* (pp. 21-35). The Nerthelands: Elservier.
Rinaldo, S., Arcovito, A., Brunori, M., & Cutruzzola, F. (2007). Fast dissociation of nitric oxide from ferrous *Pseudomonas aeruginosa* cd_1 nitrite reductase. A novel outlook on the catalytic mechanism. *J Biol Chem, 282*, 14761-14767.
Rinaldo, S., Arcovito, A., Giardina, G., Castiglione, N., Brunori, M., & Cutruzzola, F. (2008). New insights into the activity of *Pseudomonas aeruginosa* cd_1 nitrite reductase. *Biochem Soc Trans, 36*, 1155-1159.
Rinaldo, S., Brunori, M., & Cutruzzola, F. (2007). Nitrite controls the release of nitric oxide in P*seudomonas aeruginosa* cd_1 nitrite reductase. *Biochem Biophys Res Commun, 363*, 662-666.
Rinaldo, S., Castiglione, N., Giardina, G., Caruso, M., Arcovito, A., Longa, S. D., et al. (2012). Unusual heme binding properties of the dissimilative nitrate respiration regulator, a bacterial nitric oxide sensor. *Antioxid Redox Signal, 17*, 1178-1189.
Rinaldo, S., & Cutruzzolá, F. (2007). Nitrite reductases in denitrification In H. Bothe, S. J. Ferguson & W. E. Newton (Eds.), *Biology of the Nitrogen Cycle* (pp. 37-56). The Netherlands: Elservier.
Robles, E. F., Sanchez, 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. *Biochem Soc Trans, 34*, 108-110.
Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., & Gelfand, M. S. (2005). Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput Biol, 1*, e55.
Rodrigues, M. L., Oliveira, T. F., Pereira, I. A., & Archer, M. (2006). X-ray structure of the membrane-bound cytochrome *c* quinol dehydrogenase NrfH reveals novel haem coordination. *EMBO J, 25*, 5951-5960.
Rolfe, M. D., Ocone, A., Stapleton, M. R., Hall, S., Trotter, E. W., Poole, R. K., et al. (2012). Systems analysis of transcription factor activities in environments with stable and dynamic oxygen concentrations. *Open Biol, 2*, 120091.
Roop, R. M., 2nd, & Caswell, C. C. (2012). Redox-responsive regulation of denitrification genes in *Brucella*. *Mol Microbiol, 85*, 5-7.
Roux, B., Rodde, N., Jardinaud, M. F., Timmers, T., Sauviac, L., Cottret, L., et al. (2014). An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plant J, 77*, 817-837.
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*. *Biochem J, 441*, 755-762.
Saggar, S., Jha, N., Deslippe, J., Bolan, N. S., Luo, J., Giltrap, D. L., et al. (2013). Denitrification and N_2O:N_2 production in temperate grasslands: processes, measurements, modelling and mitigating negative impacts. *Sci Total Environ, 465*, 173-195.
Sameshima-Saito, R., Chiba, K., Hirayama, J., Itakura, M., Mitsui, H., Eda, S., et al. (2006). Symbiotic *Bradyrhizobium japonicum* reduces N_2O surrounding the soybean root system via nitrous oxide reductase. *Appl Environ Microbiol, 72*, 2526-2532.
Sánchez, C., Bedmar, E.J. Delgado, M.J. (2011). Denitrification in Legume-associated endosymbiotic *Bacteria*. In J. W. B. Moir (Ed.), *Nitrogen cycling in Bacteria* (pp. 197-210). Norfolk, UK: Caister Academic Press.
Sanchez, 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. *Mol Plant Microbe Interact, 23*, 702-711.
Sanchez, 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*. *Environ Microbiol, 16*, 3263-3274.
Sanford, R. A., Wagner, D. D., Wu, Q., Chee-Sanford, J. C., Thomas, S. H., Cruz-Garcia, C., et al. (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc Natl Acad Sci U S A, 109*, 19709-19714.
Saraiva, L. M., Vicente, J. B., & Teixeira, M. (2004). The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv Microb Physiol, 49*, 77-129.
Saunders, N. F., Hornberg, J. J., Reijnders, W. N., Westerhoff, H. V., de Vries, S., & van Spanning, R. J. (2000). The NosX and NirX proteins of *Paracoccus denitrificans* are functional homologues: their role in maturation of nitrous oxide reductase. *J Bacteriol, 182*, 5211-5217.
Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., et al. (2010). Genome sequence of the palaeopolyploid soybean. *Nature, 463*, 178-183.
Schmutz, J., McClean, P. E., Mamidi, S., Wu, G. A., Cannon, S. B., Grimwood, J., et al. (2014). A reference genome for common bean and genome-wide analysis of dual domestications. *Nat Genet, 46*, 707-713.
Schreiber, F., Wunderlin, P., Udert, K. M., & Wells, G. F. (2012). Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies. *Front Microbiol, 3*, 372.
Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M., et al. (2007). The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol, 189*, 4310-4314.
Schumacher, W., & Kroneck, P. M. H. (1992). Anaerobic energy metabolism of the sulfur-reducing bacterium "*Spirillum*" 5175 during dissimilatory nitrate reduction to ammonia. *Arch Microbiol, 157*, 464-470.
Sciotti, M. A., Chanfon, A., Hennecke, H., & Fischer, H. M. (2003). Disparate oxygen responsiveness of two regulatory cascades that control expression of symbiotic genes in *Bradyrhizobium japonicum*. *J Bacteriol, 185*, 5639-5642.
Shapleigh, J. P. (2006). The denitrifying prokaryotes. In M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt (Eds.), *The Prokaryotes: a*

handbook on the biology of Bacteria (3 ed., pp. 769–792). New York: Springer Science+Business Media.

Shapleigh, J. P. (2011). Oxygen control of nitrogen oxide respiration, focusing on *alpha*-proteobacteria. *Biochem Soc Trans, 39*, 179-183.

Shiro, Y., Sugimoto, H., Tosha, T., Nagano, S., & Hino, T. (2012). Structural basis for nitrous oxide generation by bacterial nitric oxide reductases. *Philos Trans R Soc Lond B Biol Sci, 367*, 1195-1203.

Simon, J. (2002). Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiol Rev, 26*, 285-309.

Simon, J., Einsle, O., Kroneck, P. M., & Zumft, W. G. (2004). The unprecedented nos gene cluster of *Wolinella succinogenes* encodes a novel respiratory electron transfer pathway to cytochrome *c* nitrous oxide reductase. *FEBS Lett, 569*, 7-12.

Simon, J., Gross, R., Einsle, O., Kroneck, P. M., Kroger, A., & Klimmek, O. (2000). A NapC/NirT-type cytochrome *c* (NrfH) is the mediator between the quinone pool and the cytochrome *c* nitrite reductase of *Wolinella succinogenes*. *Mol Microbiol, 35*, 686-696.

Simon, J., & Hederstedt, L. (2011). Composition and function of cytochrome *c* biogenesis System II. *FEBS J, 278*, 4179-4188.

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

Simon, J., & Kroneck, P. M. (2013). Microbial sulfite respiration. *Adv Microb Physiol, 62*, 45-117.

Simon, J., & Kroneck, P. M. (2014). The production of ammonia by multiheme cytochromes *c*. *Met Ions Life Sci, 14*, 211-236.

Simon, J., van Spanning, R. J., & Richardson, D. J. (2008). The organisation of proton motive and non-proton motive redox loops in prokaryotic respiratory systems. *Biochim Biophys Acta, 1777*, 1480-1490.

Smith, H. K., Shepherd, M., Monk, C., Green, J., & Poole, R. K. (2011). The NO-responsive hemoglobins of *Campylobacter jejuni*: concerted responses of two globins to NO and evidence in vitro for globin regulation by the transcription factor NssR. *Nitric Oxide, 25*, 234-241.

Smith, K. A., Mosier, A. R., Crutzen, P. J., & Winiwarter, W. (2012). The role of N_2O derived from crop-based biofuels, and from agriculture in general, in Earth's climate. *Philos Trans R Soc Lond B Biol Sci, 367*, 1169-1174.

Smith, M. S. (1983). Nitrous oxide production by *Escherichia coli* is correlated with nitrate reductase activity. *Appl Environ Microbiol, 45*, 1545-1547.

Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., et al. (2008). Greenhouse gas mitigation in agriculture. *Philos Trans R Soc Lond B Biol Sci, 363*, 789-813.

Snyder, S. W., & Hollocher, T. C. (1987). Purification and some characteristics of nitrous oxide reductase from *Paracoccus denitrificans*. *J Biol Chem, 262*, 6515-6525.

Solomon, E. I., Heppner, D. E., Johnston, E. M., Ginsbach, J. W., Cirera, J., Qayyum, M., et al. (2014). Copper active sites in biology. *Chem Rev, 114*, 3659-3853.

Spector, M. P., Garcia del Portillo, F., Bearson, S. M., Mahmud, A., Magut, M., Finlay, B. B., et al. (1999). The *rpoS*-dependent starvation-stress response locus *stiA* encodes a nitrate reductase (*narZYWV*) required for carbon-starvation-inducible thermotolerance and acid tolerance in *Salmonella typhimurium*. *Microbiology, 145 (Pt 11)*, 3035-3045.

Spiro, S. (2007). Regulators of bacterial responses to nitric oxide. *FEMS Microbiol Rev, 31*, 193-211.
Spiro, s. (2011). Nitric oxide metabolism: physiology and regulatory mechanisms. In J. W. Moir (Ed.), *Nitrogen Cycling In Bacteria* (pp. 177-197). Norfolk, UK: Caister Academic Press.
Spiro, S. (2012). Nitrous oxide production and consumption: regulation of gene expression by gas-sensitive transcription factors. *Philos Trans R Soc Lond B Biol Sci, 367*, 1213-1225.
Sprent, J. I. (2009). An interdisciplinary look at legumes and their bacterial symbionts: some thoughts from Big Sky. *New Phytol, 184*, 15-17.
Stach, P., Einsle, O., Schumacher, W., Kurun, E., & Kroneck, P. M. (2000). Bacterial cytochrome *c* nitrite reductase: new structural and functional aspects. *J Inorg Biochem, 79*, 381-385.
Stein, L. Y. (2011). Surveying N_2O-producing pathways in bacteria. *Methods Enzymol, 486*, 131-152.
Stern, A. M., Hay, A. J., Liu, Z., Desland, F. A., Zhang, J., Zhong, Z., et al. (2012). The NorR regulon is critical for *Vibrio cholerae* resistance to nitric oxide and sustained colonization of the intestines. *MBio, 3*, e00013-00012.
Stern, A. M., & Zhu, J. (2014). An introduction to nitric oxide sensing and response in bacteria. *Adv Appl Microbiol, 87*, 187-220.
Stewart, V. (2003). Biochemical Society Special Lecture. Nitrate- and nitrite-responsive sensors NarX and NarQ of proteobacteria. *Biochem Soc Trans, 31*, 1-10.
Stewart, V., & Bledsoe, P. J. (2005). Fnr-, NarP- and NarL-dependent regulation of transcription initiation from the *Haemophilus influenzae* Rd *napF* (periplasmic nitrate reductase) promoter in *Escherichia coli* K-12. *J Bacteriol, 187*, 6928-6935.
Stewart, V., & Chen, L. L. (2010). The S helix mediates signal transmission as a HAMP domain coiled-coil extension in the NarX nitrate sensor from *Escherichia coli* K-12. *J Bacteriol, 192*, 734-745.
Stieglmeier, M., Mooshammer, M., Kitzler, B., Wanek, W., Zechmeister-Boltenstern, S., Richter, A., et al. (2014). Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing *archaea*. *ISME J, 8*, 1135-1146.
Streminska, M. A., Felgate, H., Rowley, G., Richardson, D. J., & Baggs, E. M. (2012). Nitrous oxide production in soil isolates of nitrate-ammonifying bacteria. *Environ Microbiol Rep, 4*, 66-71.
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. *Proc Natl Acad Sci U S A, 110*, 19926-19931.
Swem, L. R., Elsen, S., Bird, T. H., Swem, D. L., Koch, H. G., Myllykallio, H., et al. (2001). The RegB/RegA two-component regulatory system controls synthesis of photosynthesis and respiratory electron transfer components in *Rhodobacter capsulatus*. *J Mol Biol, 309*, 121-138.
Swem, L. R., Gong, X., Yu, C. A., & Bauer, C. E. (2006). Identification of a ubiquinone-binding site that affects autophosphorylation of the sensor kinase RegB. *J Biol Chem, 281*, 6768-6775.
Swem, L. R., Kraft, B. J., Swem, D. L., Setterdahl, A. T., Masuda, S., Knaff, D. B., et al. (2003). Signal transduction by the global regulator RegB is mediated by a redox-active cysteine. *EMBO J, 22*, 4699-4708.
Takaya, N. (2002). Dissimilatory nitrate reduction metabolisms and their control in

fungi. *J Biosci Bioeng, 94*, 506-510.
ter Beek, J., Krause, N., Reimann, J., Lachmann, P., & Adelroth, P. (2013). The nitric-oxide reductase from *Paracoccus denitrificans* uses a single specific proton pathway. *J Biol Chem, 288*, 30626-30635.
Teraguchi, S., & Hollocher, T. C. (1989). Purification and some characteristics of a cytochrome *c*-containing nitrous oxide reductase from *Wolinella succinogenes*. *J Biol Chem, 264*, 1972-1979.
Thomson, A. J., Giannopoulos, G., Pretty, J., Baggs, E. M., & Richardson, D. J. (2012). Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philos Trans R Soc Lond B Biol Sci, 367*, 1157-1168.
Toffanin, A., Wu, Q., Maskus, M., Caselia, S., Abruna, H. D., & Shapleigh, J. P. (1996). Characterization of the gene encoding nitrite reductase and the physiological consequences of its expression in the nondenitrifying *Rhizobium "hedysari"* strain HCNT1. *Appl Environ Microbiol, 62*, 4019-4025.
Tolla, D. A., & Savageau, M. A. (2011). Phenotypic repertoire of the FNR regulatory network in *Escherichia coli*. *Mol Microbiol, 79*, 149-165.
Torres, M. J., Argandona, 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., Bueno, E., Mesa, S., Bedmar, E. J., & Delgado, M. J. (2011b). Emerging complexity in the denitrification regulatory network of *Bradyrhizobium japonicum*. *Biochem Soc Trans, 39*, 284-288.
Torres, M. J., Hidalgo-Garcia, A., Bedmar, E. J., & Delgado, M. J. (2013). Functional analysis of the copy 1 of the *fixNOQP* operon of *Ensifer meliloti* under free-living micro-oxic and symbiotic conditions. *J Appl Microbiol*.
Torres, M. J., Rubia, M. I., Bedmar, E. J., & Delgado, M. J. (2011a). Denitrification in *Sinorhizobium meliloti*. *Biochem Soc Trans, 39*, 1886-1889.
Torres, M. J., Rubia, M. I., de la Pena, T. C., Pueyo, J. J., Bedmar, E. J., & Delgado, M. J. (2014). Genetic basis for denitrification in *Ensifer meliloti*. *BMC Microbiol, 14*, 142.
Tortosa, G., Hidalgo, A., Salas, A., Bedmar, E., Mesa, S., & Delgado, M. (2015). Nitrate and flooding induce N_2O emissions from soybean nodules. *Symbiosis*1-9.
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. *Environ Microbiol, 7*, 1985-1995.
Tucker, N. P., Ghosh, T., Bush, M., Zhang, X., & Dixon, R. (2010). Essential roles of three enhancer sites in σ54-dependent transcription by the nitric oxide sensing regulatory protein NorR. *Nucleic Acids Res, 38*, 1182-1194.
Tucker, N. P., Hicks, M. G., Clarke, T. A., Crack, J. C., Chandra, G., Le Brun, N. E., et al. (2008). The transcriptional repressor protein NsrR senses nitric oxide directly via a [2Fe-2S] cluster. *PLoS One, 3*, e3623.
Tucker, N. P., Le Brun, N. E., Dixon, R., & Hutchings, M. I. (2010). There's NO stopping NsrR, a global regulator of the bacterial NO stress response. *Trends Microbiol, 18*, 149-156.
Tyson, K. L., Cole, J. A., & Busby, S. J. (1994). Nitrite and nitrate regulation at the promoters of two *Escherichia coli* operons encoding nitrite reductase: identification of common target heptamers for both NarP- and NarL-dependent regulation. *Mol Microbiol, 13*, 1045-1055.
Udvardi, M., & Poole, P. S. (2013). Transport and metabolism in legume-rhizobia

symbioses. *Annu Rev Plant Biol, 64*, 781-805.
van Alst, N. E., Sherrill, L. A., Iglewski, B. H., & Haidaris, C. G. (2009). Compensatory periplasmic nitrate reductase activity supports anaerobic growth of *Pseudomonas aeruginosa* PAO1 in the absence of membrane nitrate reductase. *Can J Microbiol, 55*, 1133-1144.
Van den Heuvel, R. N., Bakker, S. E., Jetten, M. S., & Hefting, M. M. (2011). Decreased N_2O reduction by low soil pH causes high N_2O emissions in a riparian ecosystem. *Geobiology, 9*, 294-300.
van Spanning, R. J., Delgado, M.J., Richardson, D.J. (2005). The nitrogen cycle: denitrification and its relationship to N_2 fixation. In D. Werner, Newton,W.E (Ed.), *Nitrogen Fixation in Agriculture, Forestry, Ecology and the Environment.* (pp. 277-342). Netherlands: Springer.
van Spanning, R. J., Richardson, D.J., Ferguson, S.J. (2007). Introduction to the biochemistry and molecular biology of denitrification. In H. Bothe, Ferguson, S.J., Newton, W.E. (Ed.), *Biology of the Nitrogen Cycle* (Vol. 3-20). Amsterdam: Elsevier Science
van Spanning, R. J. (2011). Structure, function, regulation and evolution of the nitrite and nitrous oxide reductase: denitrification enzymes with a *b*-propeller fold. In J. W. B. Moir (Ed.), *Nitrogen Cycling in Bacteria* (pp. 135-161). Norkfolk, UK: Caister Academic Press.
Van Spanning, R. J., De Boer, A. P., Reijnders, W. N., Spiro, S., Westerhoff, H. V., Stouthamer, A. H., et al. (1995). Nitrite and nitric oxide reduction in *Paracoccus denitrificans* is under the control of NNR, a regulatory protein that belongs to the FNR family of transcriptional activators. *FEBS Lett, 360*, 151-154.
Van Spanning, R. J., De Boer, A. P., Reijnders, W. N., Westerhoff, H. V., Stouthamer, A. H., & Van Der Oost, J. (1997). FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. *Mol Microbiol, 23*, 893-907.
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*. *Biochim Biophys 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.
Veldman, R., Reijnders, W. N., & van Spanning, R. J. (2006). Specificity of FNR-type regulators in *Paracoccus denitrificans*. *Biochem Soc Trans, 34*, 94-96.
Vine, C. E., & Cole, J. A. (2011). Nitrosative stress in *Escherichia coli:* reduction of nitric oxide. *Biochem Soc Trans, 39*, 213-215.
Vinogradov, S. N., Tinajero-Trejo, M., Poole, R. K., & Hoogewijs, D. (2013). Bacterial and archaeal globins - a revised perspective. *Biochim Biophys Acta, 1834*, 1789-1800.
Weiss, B. (2006). Evidence for mutagenesis by nitric oxide during nitrate metabolism in *Escherichia coli*. *J Bacteriol, 188*, 829-833.
Wood, N. J., Alizadeh, T., Bennett, S., Pearce, J., Ferguson, S. J., Richardson, D. J., et al. (2001). Maximal expression of membrane-bound nitrate reductase in *Paracoccus* is induced by nitrate via a third FNR-like regulator named NarR. *J Bacteriol, 183*, 3606-3613.
Wood, N. J., Alizadeh, T., Richardson, D. J., Ferguson, S. J., & Moir, J. W. (2002).

Two domains of a dual-function NarK protein are required for nitrate uptake, the first step of denitrification in *Paracoccus pantotrophus*. *Mol Microbiol, 44*, 157-170.

Woolfenden, H. C., Gates, A. J., Bocking, C., Blyth, M. G., Richardson, D. J., & Moulton, V. (2013). Modeling the effect of copper availability on bacterial denitrification. *Microbiologyopen, 2*, 756-765.

Wu, J., & Bauer, C. E. (2008). RegB/RegA, a global redox-responding two-component system. *Adv Exp Med Biol, 631*, 131-148.

Wunsch, P., Herb, M., Wieland, H., Schiek, U. M., & Zumft, W. G. (2003). Requirements for Cu_A and Cu-S center assembly of nitrous oxide reductase deduced from complete periplasmic enzyme maturation in the nondenitrifier *Pseudomonas putida*. *J Bacteriol, 185*, 887-896.

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

Wust, A., Schneider, L., Pomowski, A., Zumft, W. G., Kroneck, P. M., & Einsle, O. (2012). Nature's way of handling a greenhouse gas: the copper-sulfur cluster of purple nitrous oxide reductase. *Biol Chem, 393*, 1067-1077.

Yamazaki, T., Yoshida, N., Wada, E., & Matsuo, S. (1987). N_2O reduction by Azotobacter Vinelandii with emphasis on kinetic nitrogen isotope effects. *Plant Cell Physiol 28*, 263–271

Yin, S. X., Chen, D., Chen, L. M., & Edis, R. (2002). Dissimilatory nitrate reduction to ammonium and responsible microorganisms in two Chinese and Australian paddy soil. *Soil Biol Biochem 34*, 1131–1137.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., et al. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell, 3*, 593-603.

Yoshinari, T. (1980). N_2O reduction by *Vibrio succinogenes*. *Appl Environ Microbiol, 39*, 81-84.

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., Elbaz, M. A., Nakano, M. M., & Moenne-Loccoz, P. (2008). Transcription factor NsrR from *Bacillus subtilis* senses nitric oxide with a 4Fe-4S cluster (dagger). *Biochemistry, 47*, 13084-13092.

Zamorano-Sanchez, D., Reyes-Gonzalez, A., Gomez-Hernandez, N., Rivera, P., Georgellis, D., & Girard, L. (2012). FxkR provides the missing link in the *fixL-fixK* signal transduction cascade in *Rhizobium etli* CFN42. *Mol Plant Microbe Interact, 25*, 1506-1517.

Zhang, B., Crack, J. C., Subramanian, S., Green, J., Thomson, A. J., Le Brun, N. E., et al. (2012). Reversible cycling between cysteine persulfide-ligated [2Fe-2S] and cysteine-ligated [4Fe-4S] clusters in the FNR regulatory protein. *Proc Natl Acad Sci U S A, 109*, 15734-15739.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev, 61*, 533-616.

Zumft, W. G. (2005). Biogenesis of the bacterial respiratory Cu_A, Cu-S enzyme nitrous oxide reductase. *J Mol Microbiol Biotechnol, 10*, 154-166.

Zumft, W. G., Dreusch, A., Lochelt, S., Cuypers, H., Friedrich, B., & Schneider, B. (1992). Derived amino acid sequences of the *nosZ* gene (respiratory N_2O

reductase) from *Alcaligenes eutrophus, Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper-binding residues. Implications for the Cu_A site of N_2O reductase and cytochrome-*c* oxidase. *Eur J Biochem, 208*, 31-40.

Zumft, W. G., & Kroneck, P. M. (2007). Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by *Bacteria* and *Archaea*. *Adv Microb Physiol, 52*, 1

Figure legends:

Figure 7.1. Biological pathways of N_2O metabolism in nitrate-reducing bacteria. The major processes involved in nitrate transformation to N_2O are denitrification, dissimilatory nitrate reduction to ammonium (DNRA), assimilation, and detoxification. The main enzymes involved are; NarG, membrane-bound dissimilatory nitrate reductase, NapA, periplasmic dissimilatory nitrate reductase, NasC, assimilatory nitrate reductase, NirB, siroheme containing nitrite reductase, NrfA, cytochrome c nitrite reductase, NirK/NirS, Cu-containing/cd_1-type nitrite reductase, NorB, nitric oxide reductase, NorVW, anaerobic nitric oxide reductase flavorubredoxin, NosZ, nitrous oxide reductase, Nif, nitrogenase.

Figure 7.2. Enzymes and regulators involved in NO and N_2O metabolism in *E. coli* and *Salmonella* Thyphimurium. A. Enzymes involved in nitrate reduction (NapABC, NapGH and NarGHI), nitrite reduction (NrfA, NirBD), NO production (NrfA/NirB, NarG), and in N_2O production (NorVW) are shown. For Nrf system, only catalytic subunit NrfA is shown. B. Regulators involved in NO production (NarXL/NarQP, FNR, NsrR) and N_2O production (NorR) are also indicated. Positive regulation is denoted by arrows, and negative regulation is indicated by perpendicular lines. See text for details.

Figure 7.3. **Model of respiratory Nrf systems**. **A.** Nrf system of *E. coli*. **B.** Nrf system of *W. succinogenes*. See text for details. For simplicity, only monomeric enzyme forms are shown. Fe-S, iron-sulfur center; MK, menaquinone; MKH_2, menaquinol.

Figure 7.4. Compilation of selected *nos* gene clusters in epsilonproteobacterial genomes. The *nosZ* genes encode cytochrome *c* nitrous oxide reductases (*c*NosZ enzymes) that belong to the so-called atypical N_2O reductases. The presence of *nosB*, -*G*, -*H*, -*C1* and -*C2* genes as well as the absence of *nosR* and -*X* genes is indicative for atypical *nos* gene clusters. Undesignated genes shown in white encode hypothetical proteins.

Figure 7.5. Putative electron transport pathways connecting the membranous menaquinone/menaquinol pool with periplasmic *c*NosZ in Epsilonproteobacteria. **D**ashed arrows indicate speculative reactions, interactions or proton pathways. See Fig. 1 for the organization of genes encoding NosGH, NosC1, NosC2 and NosB. Protons shown in green or red contribute to electroneutral or electrogenic reactions. Note that

epsilonproteobacterial cytochrome bc_1 complexes are predicted to contain a dihaem cytochrome *c* that is thought to interact with the cytochrome *c* domain of *c*NosZ or another cytochrome *c*. In *W. succinogenes*, a suitable candidate for such a small soluble cytochrome *c* is Ws0700. MK, menaquinone; MKH_2, menaquinol.

Figure 7.6. Working model depicting the dissimilatory/detoxifying metabolism of nitrogen compounds in *W. succinogenes* cells and the predicted roles of the NssA, NssB and NssC proteins. NO and N_2O are thought to passively cross the cell membrane whereas ammonium is probably taken up by an Amt-type transporter. There are no obvious candidates for nitrate or nitrite uptake systems encoded in the genome. Externally supplied nitrate, NO or N_2O were found to be capable of inducing each of the three respiratory systems (bottom). The assumed interaction of either NssA, NssB and NssC with regulatory elements of the *nap*, *nrf* and *nos* gene clusters is shown and the encircled + denotes that an Nss protein is required to up-regulate the corresponding enzyme system. Question marks denote that the signal transduction pathways for NssA, NssB (responsive to NO) and NssC (responsive to N_2O) are not known. It cannot be excluded, however, that NO and N_2O directly interact with NssB and NssC, respectively. Adapted from Kern & Simon (2015).

Figure 7.7. Topological organization of denitrification enzymes. The membrane-bound (NarGHI), and periplasmic, (NapABC) nitrate reductases as well as the nitrite reductases (Cu-type or cd_1-type), nitric oxide reductases (cNor, qNor, and qCuANor), and nitrous oxide reductase (NosZ) are shown. Adapted from Bueno et al., (2012).

Figure 7.8. The typical *nosRZDFYLX* gene cluster from denitrifiers, *nos* gene products and proposed operating electron transfer pathways from quinol (QH_2) to NosZ via the cytochrome bc_1-complex (cytbc_1) and cytochrome *c* (cyt*c*) or pseudoazurin, the other providing electrons to Cu_Z via NosR and NosX FMN-proteins. NosDFYL required for Cu_Z assemblage in NosZ is also shown. IM; inner membrane, OM; outer membrane. Adapted from van Spanninng (2011).

Figure 7.9. Regulatory network of denitrification in response to O_2 concentration, nitrate/nitrite (NO_3^-/NO_2^-), nitric oxide (NO) and redox conditions. Positive regulation is denoted by arrows, and negative regulation is indicated by perpendicular lines. Adapted from Bueno et al., (2012).

Figure 7.10. Regulatory network of *B. japonicum* denitrification. Positive regulation is denoted by arrows, and unknown control mechanisms are indicated by dashed lines. Adapted from Bueno et al., (2012).

Figure 7.11. Schematic representation of NO and N_2O metabolism in root nodules from *Glycine max-B. japonicum*, *Medicago truncatula-Ensifer meliloti* and *Phaseolus vulgaris-Rhizobium etli* symbiosis. The large grey square represents the plant cell and the small grey squares represent the bacteroids.

Figure 7.12. A) Organisation of regulatory and structural genes for the assimilatory NO_3^- /NO_2^- pathway in *B. japonicum*. B) Proposed biochemical pathway for NO_3^- -assimilation and NO detoxification system, alongside well-characterised denitrification pathway in *B. japonicum*. Assimilatory reduction of NO_3^- to NH4 is performed by sequential action of the NO_3^--reductase NasC and Ferredoxin (Fd)-dependent NO_2^- -reductase NirA. Electrons from NAD(P)H are supplied to NasC and also Bjgb by Flp. During assimilatory NO_3^- reduction, cytoplasmic NO_2^- may accumulate and be further reduced, by NasC, to generate cytotoxic NO. NarK can ounteract accumulation of NO_2^- by exporting it to the periplasm. Bjgb might detoxify NO to N_2O in the absence of O_2. Adapted from Cabrera et al., (2015).

1

Table 7.1. Denitrification genes in rhizobia

Species and Strain	Denitrification genes				Reference
	nap	***nirK***	***nor***	***nos***	
Rhizobium galegae (formerly *Pseudomonas* sp. G-179*)*	*EFDABC*	*nirK*	*EFCBQD*	—	Bedzyk et al., 1999
Rhizobium sullae (formerly *R. hedysari*)	—	*niK*	—	—	Toffanin et al., 1996
Rizobium etli CFN42	—	*nirK*	*ECBQD*	—	Gomez-Hernandez et al., 2011
Ensifer meliloti 1021 (formerly *Sinorhizobium meliloti*)	*EFDABC*	*nirK*	*ECBQD*	*nosRZDFYLX*	Torres et al., 2011
Bradyrhizobium japonicum USDA110	*EDABC*	*nirK*	*CBQD*	*nosRZDFYLX*	Bedmar et al., 2005
Rhizobium sp NGR234	*EFDABC*	*nirK*	*CBQD*	—	http://genome.microbedb.jp/rhizobase/

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Table 7.2. Properties of typical and atypical N_2O reductases and *nos* gene clusters

	Typical	Atypical
Metabolic type of the host cell	denitrifier	non-denitrifier[1]
NosZ signal peptide	Tat-dependent	Sec-dependent
Characteristic motifs of Cu_Z center ligands	DXHHXH, EPHD	DXHH, EPH
Haem *c* domain (*c*NosZ-type enzymes)	absent	often present
nosB, -G, *-C1*, *-C2*, *-H* genes	absent	present
nosR, *-X* genes	present	absent
Representative model organisms	*Paracoccus denitrificans*	*Wolinella succinoges*
	Bradyrhizobium japonicum	*Anaeromyxobacter dehalogenans*

Figure 7.1

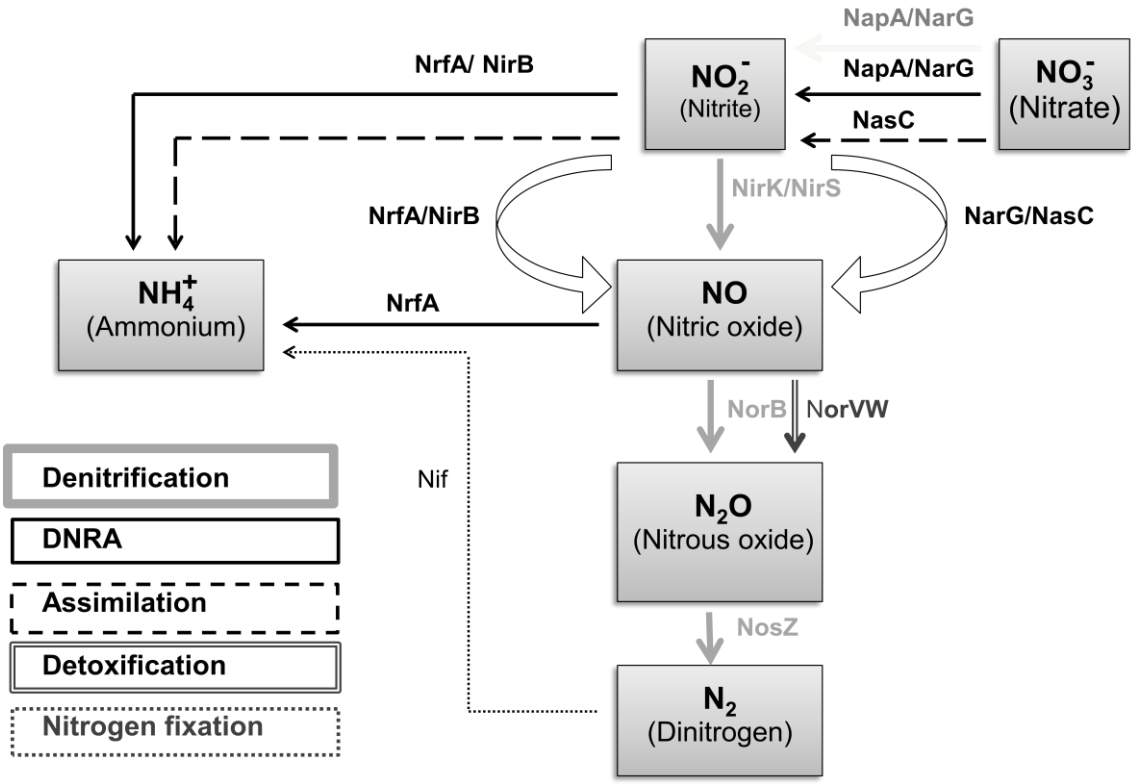

NapA/NarG
NrfA/ NirB
NapA/NarG
NasC
NO_2^- (Nitrite)
NO_3^- (Nitrate)
NirK/NirS
NrfA/NirB
NarG/NasC
NH_4^+ (Ammonium)
NrfA
NO (Nitric oxide)
NorB
NorVW
Denitrification
DNRA
Assimilation
Detoxification
Nitrogen fixation
Nif
N_2O (Nitrous oxide)
NosZ
N_2 (Dinitrogen)

Figure 7.2

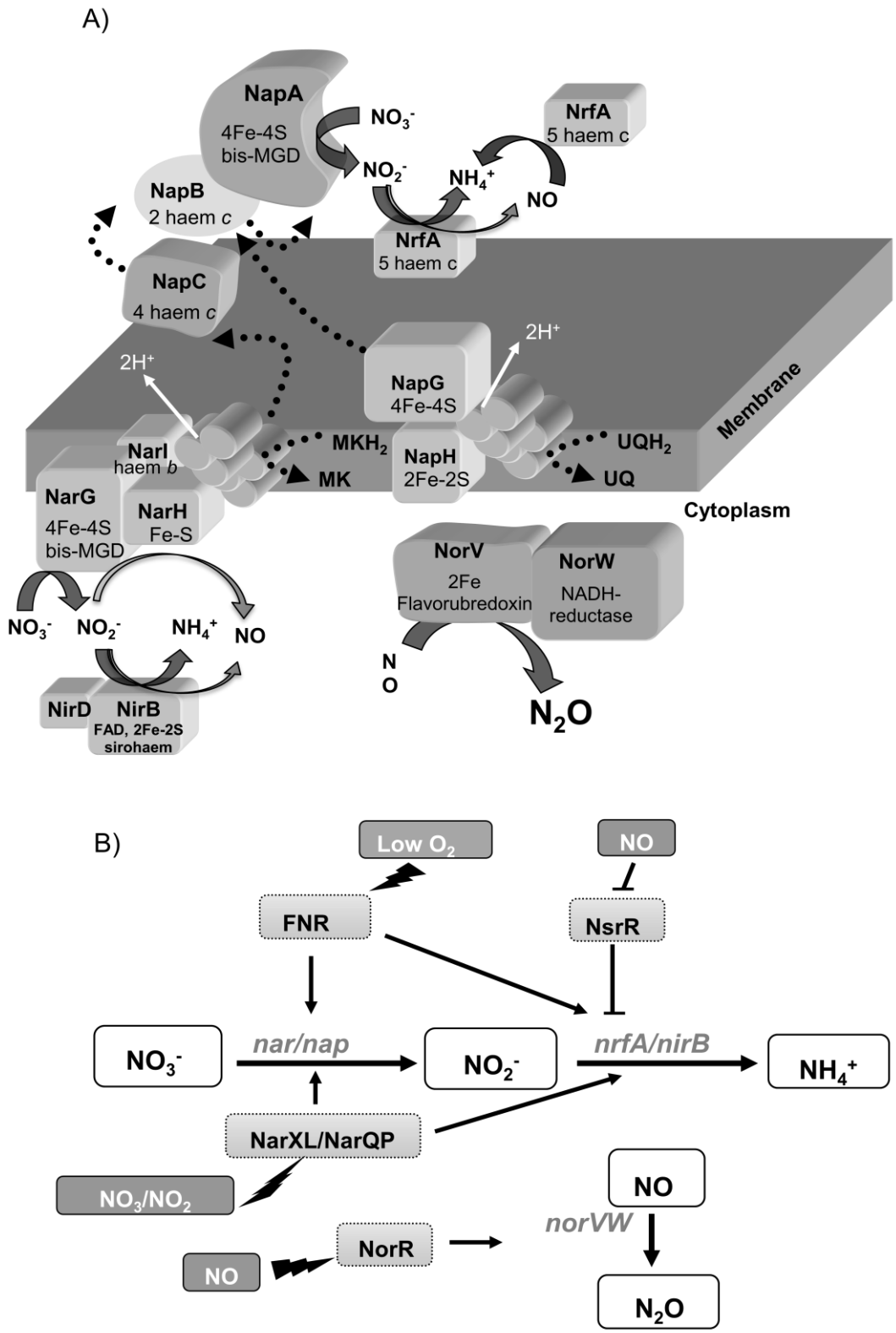

A)
NapA
4Fe-4S
bis-MGD
NapB
2 haem c
NapC
4 haem c
NrfA
5 haem c
NrfA
5 haem c
NapG
4Fe-4S
NapH
2Fe-2S
NarI
haem b
NarG
4Fe-4S
bis-MGD
NarH
Fe-S
NirD
NirB
FAD, 2Fe-2S
sirohaem
NorV
2Fe
Flavorubredoxin
NorW
NADH-
reductase
Membrane
Cytoplasm
NO_3^-
NO_2^-
NH_4^+
NO
$2H^+$
MKH_2
MK
UQH_2
UQ
N
O
N_2O
B)
Low O_2
NO
FNR
NsrR
NO_3^-
nar/nap
NO_2^-
nrfA/nirB
NH_4^+
NarXL/NarQP
NO_3/NO_2
NO
norVW
NorR
NO
N_2O

Figure 7.3

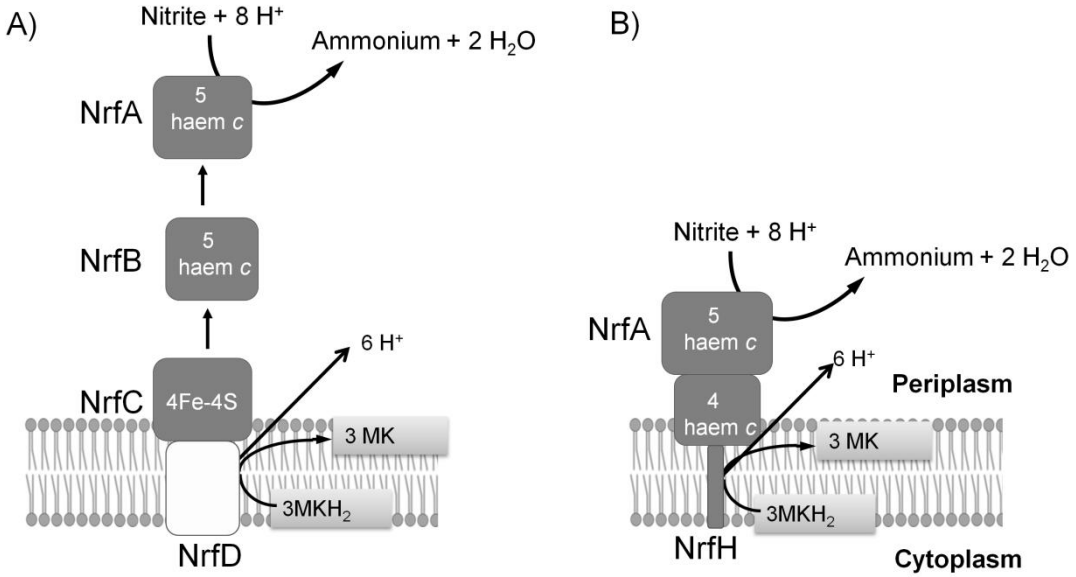

A)
Nitrite + 8 H+
Ammonium + 2 H2O
NrfA
5 haem c
NrfB
5 haem c
NrfC
4Fe-4S
6 H+
3 MK
3MKH2
NrfD
B)
Nitrite + 8 H+
Ammonium + 2 H2O
NrfA
5 haem c
6 H+
Periplasm
4 haem c
3 MK
3MKH2
NrfH
Cytoplasm

Figure 7.4

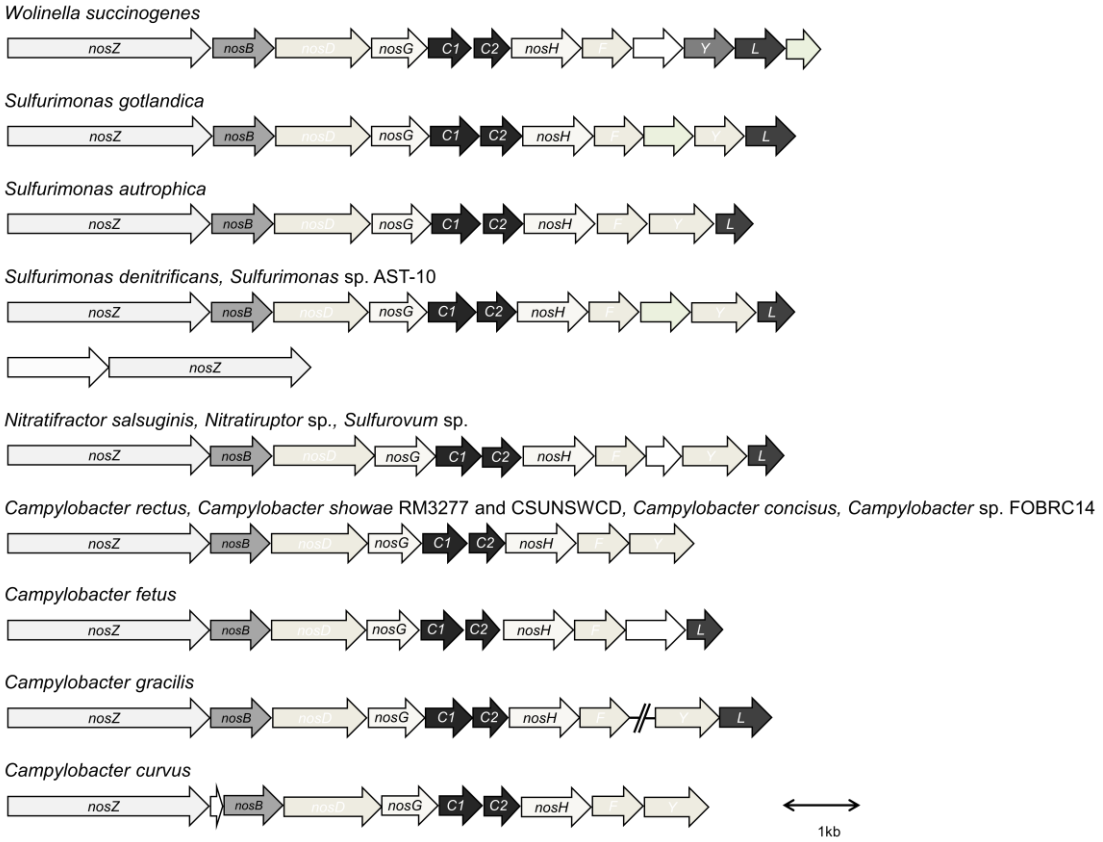

Wolinella succinogenes
nosZ
nosB
nosD
nosG
C1
C2
nosH
F
Y
L
Sulfurimonas gotlandica
Sulfurimonas autrophica
Sulfurimonas denitrificans, Sulfurimonas sp. AST-10
Nitratifractor salsuginis, Nitratiruptor sp., Sulfurovum sp.
Campylobacter rectus, Campylobacter showae RM3277 and CSUNSWCD, Campylobacter concisus, Campylobacter sp. FOBRC14
Campylobacter fetus
Campylobacter gracilis
Campylobacter curvus
1kb

Figure 7.5

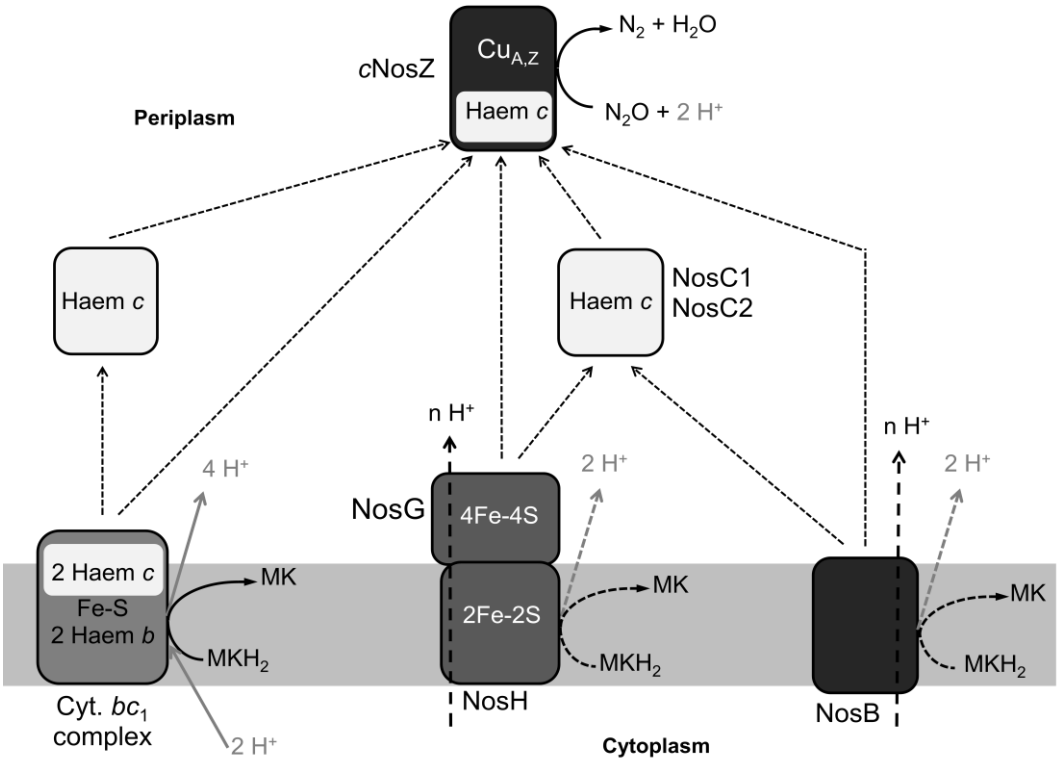

cNosZ
$Cu_{A,Z}$
Haem *c*
$N_2 + H_2O$
$N_2O + 2\ H^+$
Periplasm
Haem *c*
Haem *c*
NosC1
NosC2
n H^+
n H^+
4 H^+
2 H^+
2 H^+
NosG
4Fe-4S
2 Haem *c*
Fe-S
2 Haem *b*
MK
MKH_2
2Fe-2S
MK
MKH_2
MK
MKH_2
Cyt. bc_1
complex
2 H^+
NosH
NosB
Cytoplasm

Figure 7.6

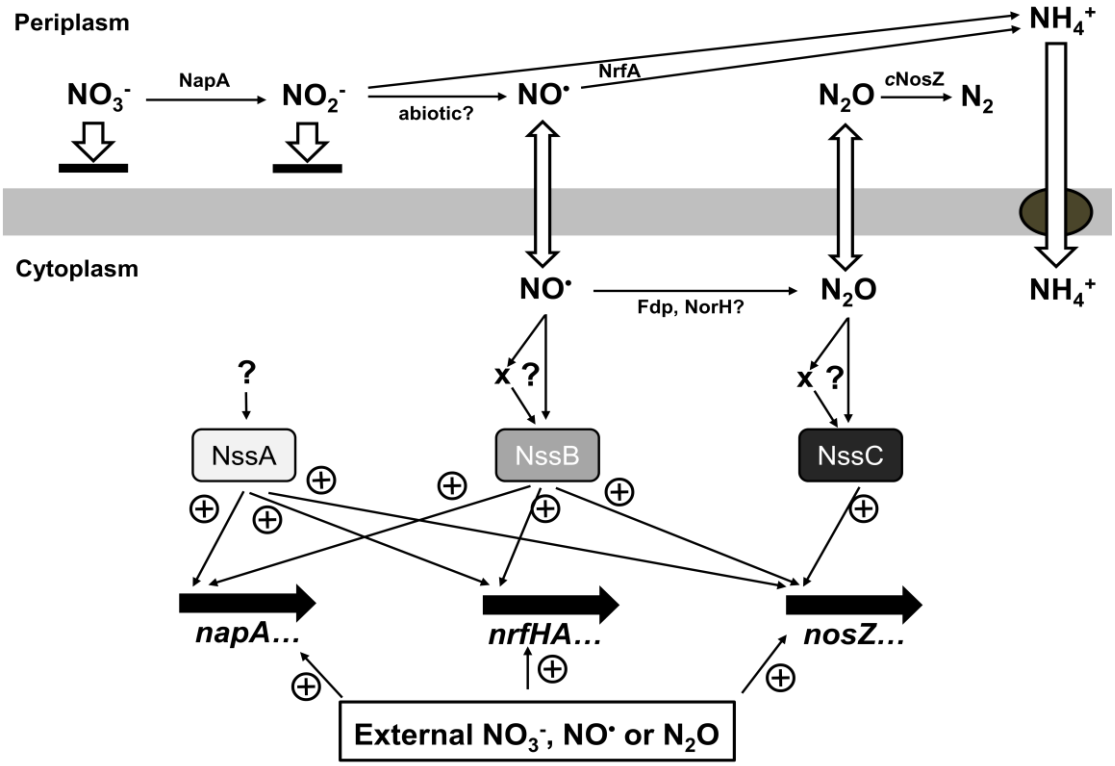

Periplasm
NO_3^-
NapA
NO_2^-
abiotic?
NO·
NrfA
NH_4^+
N_2O
cNosZ
N_2
Cytoplasm
NO·
Fdp, NorH?
N_2O
NH_4^+
?
x ?
x ?
NssA
NssB
NssC
napA…
nrfHA…
nosZ…
External NO_3^-, NO· or N_2O

Figure 7.7

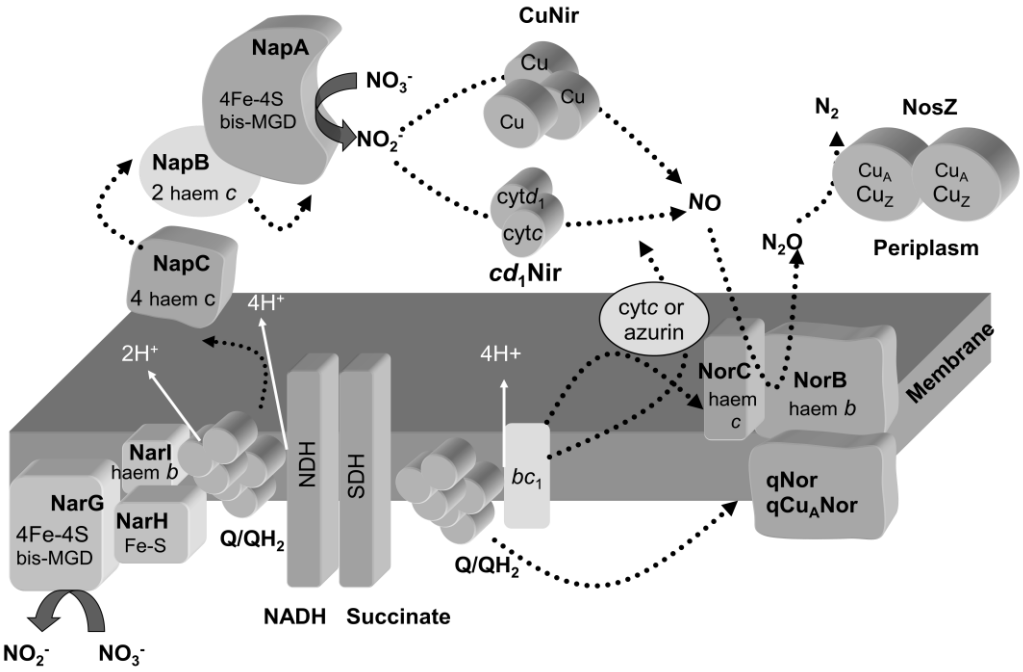

CuNir
NapA
4Fe-4S
bis-MGD
NO_3^-
NO_2^-
Cu
Cu
Cu
NapB
2 haem *c*
$cytd_1$
cytc
cd_1Nir
NO
N_2
NosZ
Cu_A Cu_Z
Cu_A Cu_Z
N_2O
Periplasm
NapC
4 haem c
$4H^+$
cytc or
azurin
$2H^+$
4H+
NorC
haem
c
NorB
haem *b*
Membrane
NarI
haem *b*
NarG
4Fe-4S
bis-MGD
NarH
Fe-S
NDH
SDH
bc_1
qNor
qCu_ANor
Q/QH_2
Q/QH_2
NADH
Succinate
NO_2^-
NO_3^-

Figure 7.8

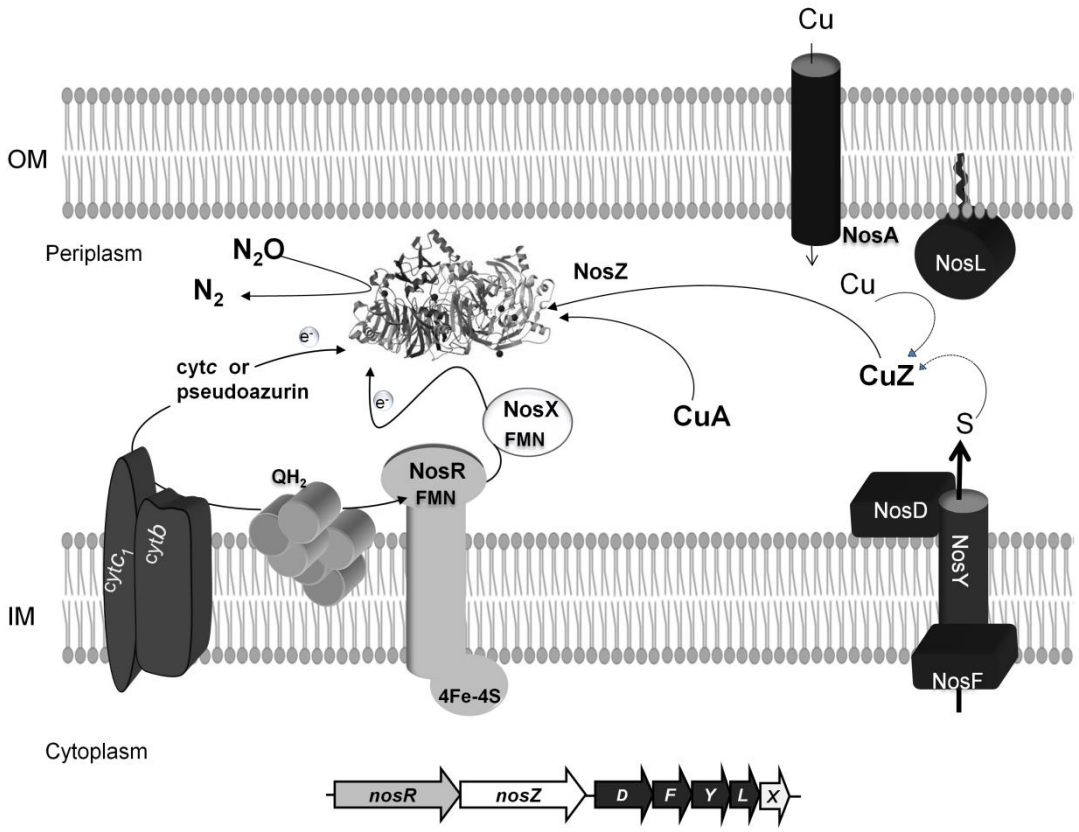

Cu
OM
Periplasm
N_2O
N_2
NosZ
NosA
NosL
Cu
cytc or
pseudoazurin
e⁻
e⁻
NosX
FMN
CuA
CuZ
S
QH_2
NosR
FMN
NosD
NosY
$cytc_1$
cytb
IM
4Fe-4S
NosF
Cytoplasm
nosR
nosZ
D
F
Y
L
X

Figure 7.9

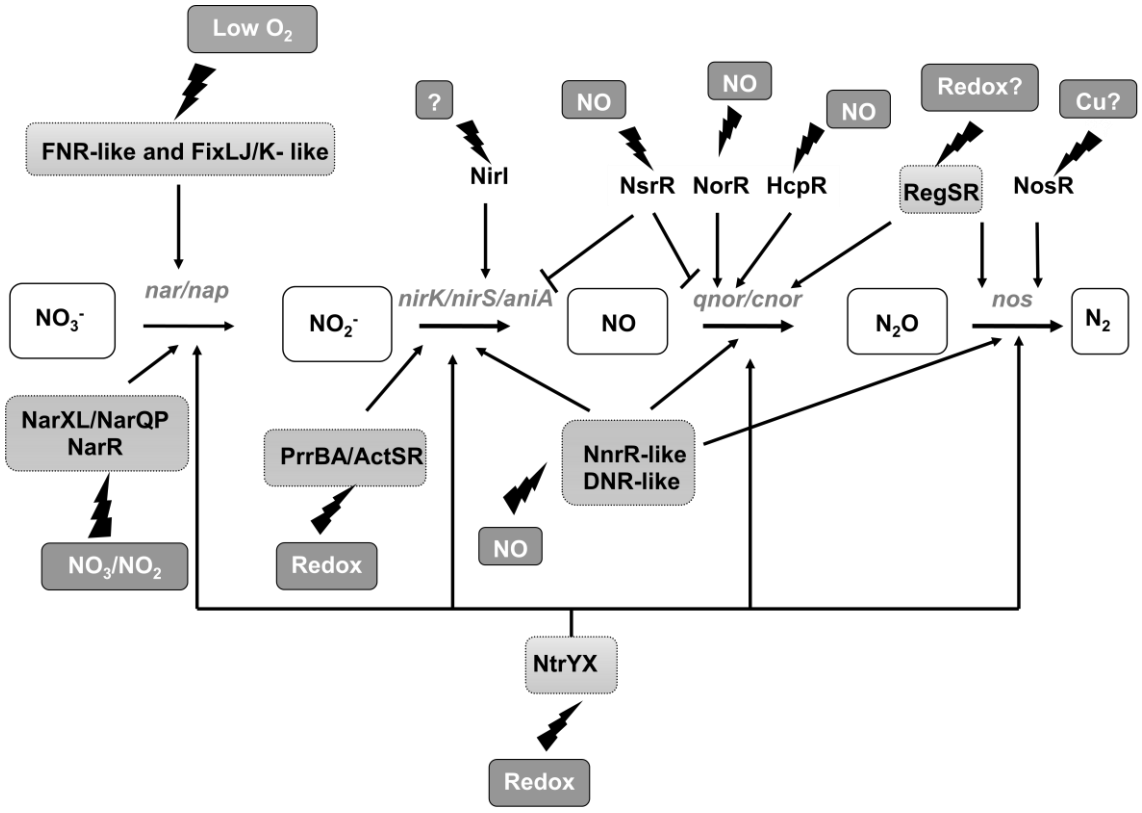

Low O_2
FNR-like and FixLJ/K- like
?
NirI
NO
NsrR
NO
NorR
NO
HcpR
Redox?
RegSR
Cu?
NosR
NO_3^-
nar/nap
NO_2^-
nirK/nirS/aniA
NO
qnor/cnor
N_2O
nos
N_2
NarXL/NarQP
NarR
NO_3/NO_2
PrrBA/ActSR
Redox
NO
NnrR-like
DNR-like
NtrYX
Redox

Figure 7.10

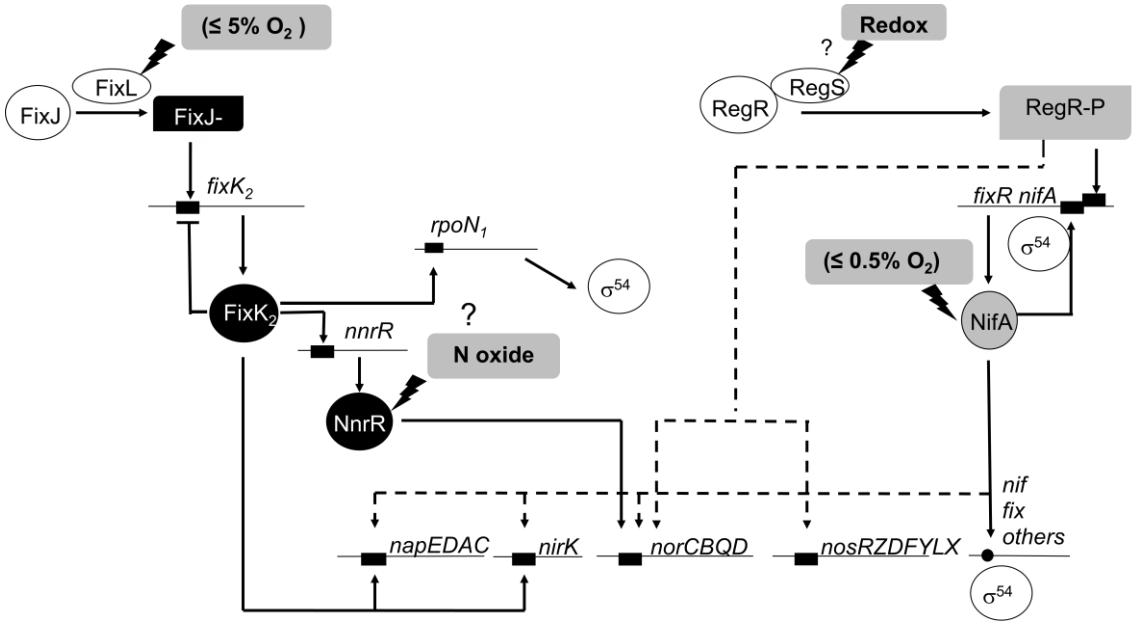

(≤ 5% O2)
FixL
FixJ
FixJ-
fixK2
rpoN1
σ54
FixK2
?
nnrR
N oxide
NnrR
Redox
?
RegS
RegR
RegR-P
fixR nifA
σ54
(≤ 0.5% O2)
NifA
nif
fix
others
σ54
napEDAC
nirK
norCBQD
nosRZDFYLX

Figure 7.11

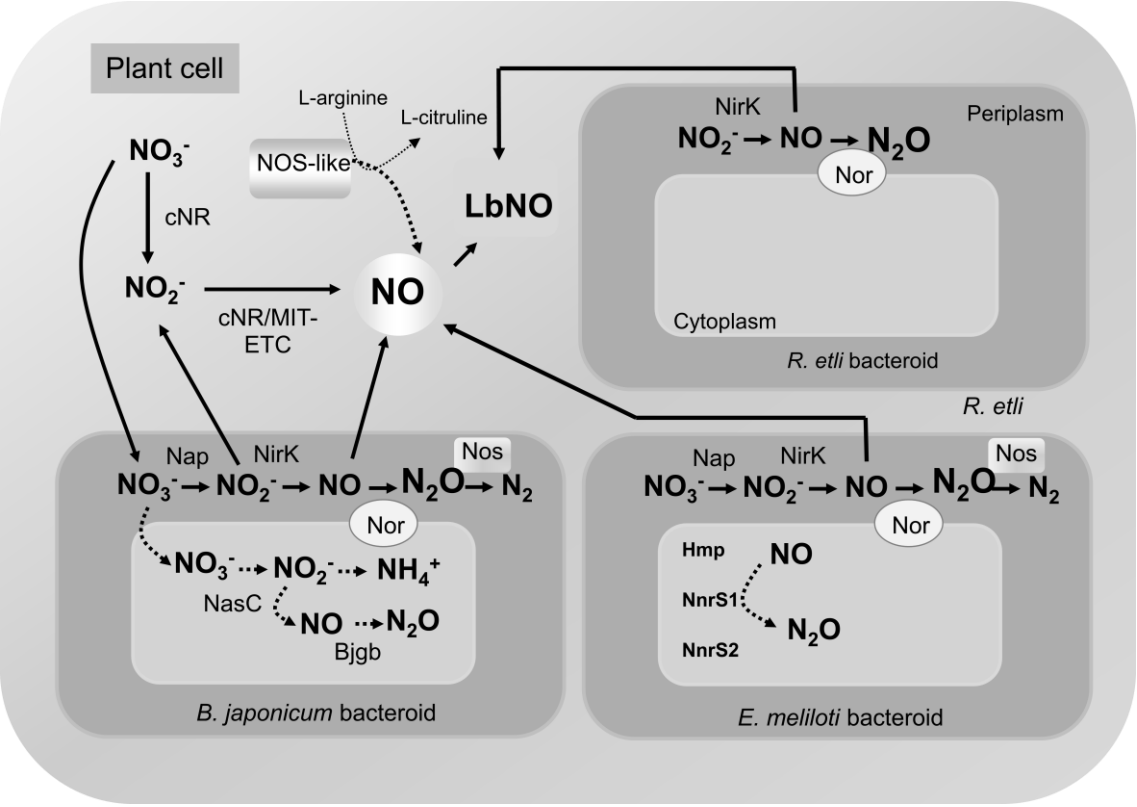
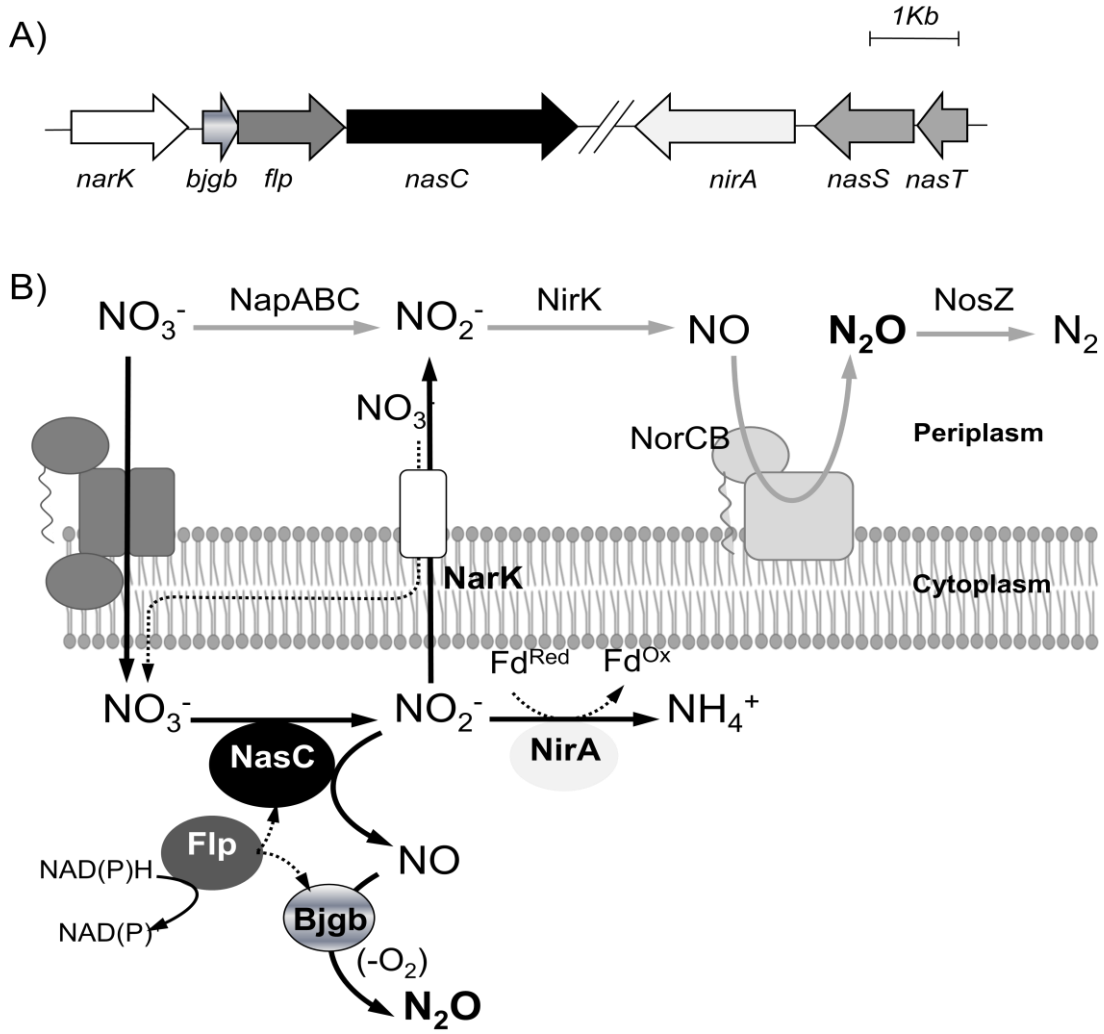

Plant cell
L-arginine
L-citruline
NOS-like
LbNO
NO
cNR
cNR/MIT-ETC
Periplasm
NirK
Nor
Cytoplasm
R. etli bacteroid
R. etli
Nap
NirK
Nos
Nor
NasC
Bjgb
B. japonicum bacteroid
Hmp
NnrS1
NnrS2
E. meliloti bacteroid

Figure 7.12


A)
1Kb
narK
bjgb
flp
nasC
nirA
nasS
nasT
B)
NO3-
NapABC
NO2-
NirK
NO
N2O
NosZ
N2
NO3-
NorCB
Periplasm
NarK
Cytoplasm
FdRed
FdOx
NO3-
NO2-
NH4+
NirA
NasC
Flp
NAD(P)H
NAD(P)+
NO
Bjgb
(-O2)
N2O