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The nitric oxide response in plant-associated endosymbiotic bacteria

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Abstract

Nitric oxide (NO) is a gaseous signalling molecule which becomes very toxic due to its ability to react with multiple cellular targets in biological systems. Bacterial cells protect against NO through the expression of enzymes that detoxify this molecule by oxidizing it to nitrate or reducing it to nitrous oxide or ammonia. These enzymes are haemoglobins, *c*-type nitric oxide reductase, flavorubredoxins and the cytochrome *c* respiratory nitrite reductase. Expression of the genes encoding these enzymes is controlled by NO-sensitive regulatory proteins. The production of NO in rhizobia–legume symbiosis has been demonstrated recently. In functioning nodules, NO acts as a potent inhibitor of nitrogenase enzymes. These observations have led to the question of how rhizobia overcome the toxicity of NO. Several studies on the NO response have been undertaken in two non-dentrifying rhizobial species, *Sinorhizobium meliloti* and *Rhizobium etli*, and in a denitrifying species, *Bradyrhizobium japonicum*. In the present mini-review, current knowledge of the NO response in those legume-associated endosymbiotic bacteria is summarized.

Introduction

Nitric oxide (NO) is a reactive free radical that plays important roles in diverse physiological processes, serving as a signalling molecule in response to biotic and abiotic stresses. At the ecological level, it has an important role as an obligatory intermediate in the respiratory pathway denitrification. NO reacts with a wide range of cellular targets, including lipids, metal centres in proteins, protein tyrosines and thiols, and DNA [1]. Thus organisms that are exposed to NO require activities that detoxify NO. In bacteria, several enzymes have been shown to detoxify NO by oxidizing it to nitrate or reducing it to ammonia or nitrous oxide (N_2O). These enzymes are Nor (nitric oxide reductase), sdHbs (single-domain haemoglobins), trHbs (truncated haemoglobins), FHbs (flavohaemoglobins), FlRd (flavorubredoxin) and cytochrome *c* respiratory nitrite reductase (Nrf) [2–7].

In bacteria, the existence of regulatory proteins that, in the presence of NO, switch on the expression of genes encoding enzymes that detoxify NO has also been demonstrated. Among them, the transcription factors of the FNR (fumarate and nitrate reductase regulatory protein)/CRP (cAMP receptor protein) family, such as the Dnr and NnrR (nitrite and nitric oxide reductase regulator)-like proteins respond to NO and control expression of *nir* and *nor* denitrification genes [6,8]. In the non-denitrifying bacterium *Escherichia coli*, FNR is a sensor of molecular O_2 that can be inactivated by NO by the formation of dinitrosyl–iron complexes as result of the reaction of NO with the [Fe–S] cluster [9]. NorR is another regulator that activates transcription of three different enzymes that act on NO detoxification (Nor, FlRd and FHbs) [6,8,10,11]. NsrR is a NO-sensitive repressor that, like NorR, regulates expression of the three dedicated NO-metabolizing enzymes (Nor, FlRd and FHbs) in different species [6,8,12].

Bacterial members of the order Rhizobiales, collectively referred to as rhizobia, have the ability to establish a dinitrogen (N_2)-fixing symbiosis on legume roots and on the stems of some aquatic legumes producing specialized structures called nodules [13]. Inside these nodules, bacteria differentiate into bacteroids which induce the synthesis of the nitrogenase complex that catalyses the biological reduction of N_2 to ammonia. Many rhizobial species have genes for enzymes of some or all of the reactions for denitrification which comprises the reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to N_2, via the gaseous intermediates NO and N_2O. The enzymes involved in denitrification are nitrate, nitrite, nitric oxide and nitrous oxide reductases encoded by *nar*/*nap*, *nir*, *nor* and *nos* genes respectively. In fact, denitrification can be observed in free-living rhizobia forms and in legume root nodules (for reviews, see [14–17]).

NO is produced in functional nodules of legume plants [18–21]. NO synthesis in plants has been reported to occur via different routes such as NR (nitrate reductase), NO_2^-–NO reductase, mitochondrial ETC (electron-transport chain),

Key words: *Bradyrhizobium japonicum*, denitrification, nitric oxide, *Rhizobium etli*, *Sinorhizobium meliloti*.

Abbreviations used: cNor, cytochrome *c*-type nitric oxide reductase; CRP, cAMP receptor protein; ETC, electron-transport chain; FHb, flavohaemoglobin; FlRd, flavorubredoxin; FNR, fumarate and nitrate reductase regulatory protein; LbNO, nitrosyl-leghaemoglobin; Nor, nitric oxide reductase; NOS, nitric oxide synthase; NR, nitrate reductase; sdHb, single-domain haemoglobin; SNP, sodium nitroprusside; trHb, truncated haemoglobin.

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NOS (nitric oxide synthase)-like, non-enzymatic reduction and, potentially, as yet unidentified polyamine oxidation pathway (for reviews, see [22,23]). In legume plants, a NOS-like activity has been identified in nodules of *Lupinus albus* [24]. Furthermore, a NOS inhibitor (*N*-methyl-L-arginine) impaired NO detection in *Medicago truncatula* nodules [18], suggesting that a NOS-like enzyme may participate to NO production in nodules. Recent studies have found that plant NR and the ETC are involved in NO synthesis in *M. truncatula* nitrogen-fixing nodules ([25], reviewed in [16]). Rhizobial denitrification in the bacteroids is also a likely source of NO in nodules [19,21,25]. There is increasing evidence of the requirement of NO for an optimal establishment of the *M. truncatula–Sinorhizobium meliloti* symbiotic interaction [20,26]. In N_2-fixing nodules, a direct inhibition of nitrogenase activity by NO has been demonstrated [21,27,28], which suggests that modulation of NO levels in rhizobia might be necessary for an efficient symbiosis. In contrast with all that has been carried out in other bacterial species, the response of rhizobia to the presence of NO has been poorly documented. The present article provides a brief review of what is known so far about the response to NO in *S. meliloti* (Ensifer), *Rhizobium etli* and *Bradyrhizobium japonicum*.

Sinorhizobium meliloti

S. meliloti is an alphaproteobacterium able to establish symbiotic associations with legume plants of the genera *Medicago*, *Melilotus* and *Trigonella*, which includes species of great agronomic interest such as alfalfa or the model legume *M. truncatula*. The *S. meliloti* genome consists of three replicons: a 3.65 Mb chromosome, and 1.35 Mb pSymA and 1.68 Mb pSymB megaplasmids. *S. meliloti* contains the complete set of denitrification genes (*nap*, *nirK*, *nor* and *nos*) in pSymA. However, it is considered a partial denitrifying bacterium since it is not able to grow anaerobically with NO_3^- or NO_2^- as respiratory substrates. In symbiotic *M. truncatula* nodules, recent findings have demonstrated that *S. meliloti napA* and *nirK* denitrification genes contribute to nitric oxide production [25]. However, the role of *S. meliloti* denitrification genes under free-living conditions is not known.

A powerful approach to investigating bacterial responses to nitrosative stress is to measure the global changes in gene expression that occur upon exposure to such stress. This has been used to study the nitrosative stress responses of *S. meliloti* [29]. Transcriptomic analyses showed that approximately 100 genes were up-regulated by NO. At least 70% of those genes up-regulated by NO had been described previously as being induced under microaerobic conditions [30]. Most of the genes common to the NO and micro-oxia stimulons are regulated by the two-component system FixLJ (i.e. *nifA*, *fixK* and *smc03253*) (Figure 1). The low O_2 signal is perceived by the FixL histidine kinase that autophosphorylates and then transfers the phosphoryl group to the response regulator FixJ which activates transcription of FixK and NifA regulators responsible for the activation of *fix* genes (respiration) and *nif* genes (N_2 fixation) respectively [30]. In *S. meliloti*, FixLJ responds to both micro-oxic conditions and the presence of NO (Figure 1). It has been shown that FixL can bind ligands other than O_2 such as NO or CO. Although the affinity of FixL is higher for NO than for O_2, the only molecule able to abolish FixL kinase activity is O_2 [31]. The higher affinity of FixL for NO than for O_2 could explain the on-state of the kinase in the presence of NO. However, the biological relevance of the FixLJ response to NO in *S. meliloti* remains to be established. *S. meliloti* NnrR is also involved in the NO response. In this bacterium, the FNR/CRP regulators NnrR and FixK are part of two different NO-responsive signalling pathways (Figure 1). In the presence of NO, NnrR regulates an operon (*sma1289–1294*) whose function is yet unknown [32]. Recent transcriptomic studies confirmed those findings and identified denitrification genes (*nirK* and *norC*), as well as other genes related to denitrification (*azu1*, *hemN*, *nnrU* and *nnrS*) as NnrR targets [29].

Among the genes induced by NO, it is interesting to mention the *hmp* gene encoding a FHb (Hmp) [29]. Microarray analyses showed that *hmp* is a target of FixJ. However, the involvement of FixK as an intermediate transcriptional activator of this gene needs to be demonstrated. In *S. meliloti*, *hmp* is clearly involved in NO response, because null mutants in this gene displayed a higher sensitivity to NO compared with the wild-type, whereas overexpression of *hmp* enhanced NO resistance [29]. Furthermore, this gene and NO play a role during the symbiotic interaction between *S. meliloti* and *M. truncatula* because nodules produced by *hmp*-null mutants displayed lower N_2-fixation efficiency than those produced by the wild-type strain. The Hmp enzyme consists of two domains, an N-terminal haem-binding globin domain and a C-terminal FAD- and NAD(P)H-binding reductase domain, which are both required for NO detoxification. Hmp protects against NO both aerobically and anaerobically. Under anaerobiosis, FHbs have a slow NO reductase activity which converts NO into N_2O and might not be sufficient to protect against NO [33]. In the presence of O_2, the enzyme, using an electron from NAD(P)H delivered via the flavin protein, catalyses a denitrosylase ('dioxygenase') reaction in which NO is stoichiometrically converted into NO_3^- [34,35]. Because micro-oxic conditions prevail inside nodules, detoxification of NO by Hmp could lead to the formation of either NO_3^- or N_2O, which may enter the denitrification pathway. These results propose the *S. meliloti* FHb as a significant NO-detoxifying protein in *M. truncatula* nodules which, together with plant haemoglobins, might participate in limiting NO toxicity inside nodules.

Rhizobium etli

R. etli establishes symbiotic associations with plants of the *Phaseolus* genus. The genome of *R. etli* CFN42 contains a chromosome and six large plasmids (pCFN42a to pCFN42f)

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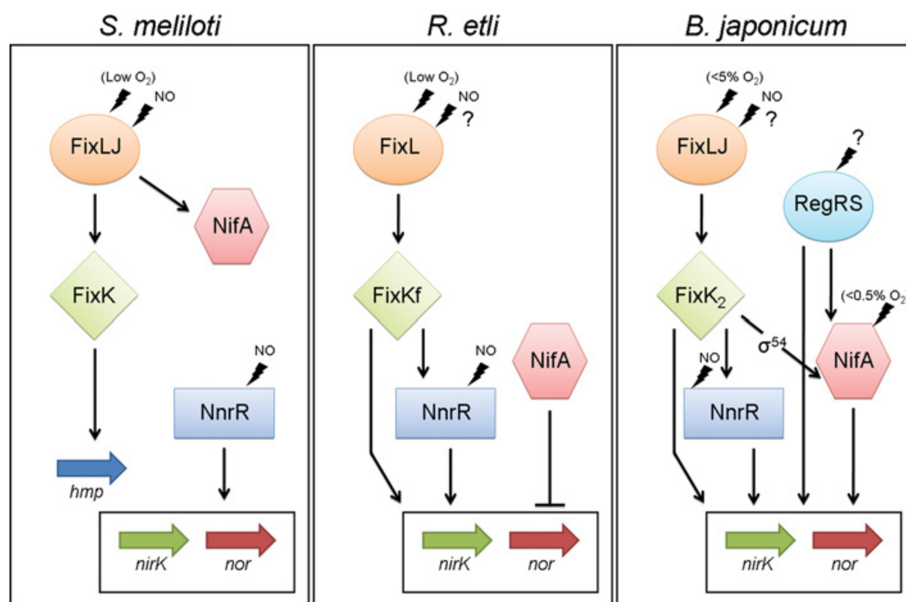
Figure 1 | NO signalling regulatory cascades in *S. meliloti*, *R. etli* and *B. japonicum*

Schemes are based on data from [16].



whose sizes range from 184.4 to 642.5 kb [36]. Plasmid d corresponds to the symbiotic one (pSym) and contains several genes involved in nodulation and N_2-fixation processes. However, some important regulatory elements, such as *fixL* and *fixJ*, are not encoded on this replicon. In *R. etli*, it is the largest plasmid, pCFN42f, which includes regulatory genes such as *fixK* and the *fixL*. In contrast with *S. meliloti* or *B. japonicum*, the transcriptional activator with functional homology with FixJ remains to be described in *R. etli*. Plasmid pCFN42f also contains a gene cluster with some denitrification genes such as *nirK*, *norCBQD* and *azuPf* coding for a copper-containing nitrite reductase, a cNor (cytochrome *c*-type nitric oxide reductase) and pseudoazurin respectively [36,37]. In the *nirK*–*norC* region is also located the *nnrR* gene which encodes NnrR, the FNR-type transcriptional regulator of denitrification genes.

R. etli lacks part of the denitrification pathway (*nap* or *nar* and *nos* genes) and is unable to use NO_3^- for respiration and lacks NR activity. In contrast, it shows NR activity and NO_2^- uptake under anaerobic conditions which are highly diminished in a *nirK* mutant [38]. 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.

Recently, Gómez-Hernández et al. [39] have demonstrated the *in vivo* relevance of the enzymes encoded by the *nirK* and *norC* genes by exploring the response of the corresponding mutants to both NO_2^- and to NO generators, such as SNP (sodium nitroprusside). Under microaerobic conditions, a *nirK*-deficient strain was unable to produce NO from NO_2^- and a *norC* mutant was defective in NO consumption activity, compared with wild-type cells. Moreover, the *norC* mutant failed to grow under microaerobic conditions in the presence of 100 μM SNP or 30 μM $NaNO_2$. These data clearly indicate that *R. etli* NorC is required *in vivo* to detoxify NO under free-living conditions.

In *R. etli*, microaerobic expression of *nirK* and *norC* promoters requires a functional FixKf, whereas the response to NO is mediated by NnrR (Figure 1). In contrast with *S. meliloti*, where *nirK* and *norC* are fully dependent on NnrR, in *R. etli* full expression of *norC* in response to nitrogen oxides and microaerobiosis requires the presence of FixKf and NnrR, but microaerobic expression of *nirK* is still observed in an *nnrR* mutant background. Although NnrR and FixK are part of two different NO-responsive signalling pathways in *S. meliloti*, in *R. etli* microaerobic expression of *nnrR* is controlled by FixKf (Figure 1). Additionally, the N_2-fixation regulator NifA has a negative effect on the transcription of the *nirK* operon [39].

Expression of *nirK* and *norC* genes has been observed in common bean (*Phaseolus vulgaris*) nodules by using qRT-PCR (quantitative real-time PCR). NO production has been shown in common bean nodules by measuring LbNO (nitrosyl-leghaemoglobin) complexes. As has been demonstrated previously in soya bean (*Glycine max*) nodules, bacterial NirK is an important contributor to the formation of NO in common bean nodules 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 [39]. Interestingly, the NO_3^--induced decline in nitrogenase-specific activity observed in both the wild-type and the *norC* nodules was not detected in *nirK* nodules.

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Bradyrhizobium japonicum

B. japonicum is able to establish symbiotic associations with leguminous plants, including soya bean. Unlike the previous two examples, *B. japonicum* itself is a denitrifying organism, capable of growing anaerobically with NO_3^- as a terminal electron acceptor and of reducing NO_3^- to N_2. In this bacterium, denitrification depends on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDFYLX* genes encoding the nitrate, nitrite, nitric oxide and nitrous oxide reductases respectively [14]. *B. japonicum norC* or *norB* mutants are defective in anaerobic growth under NO_3^--respiring conditions and accumulate NO [21,40], suggesting that the cNor is physiologically important for NO detoxification.

In *B. japonicum,* expression of *nor* genes in response to microaerobic conditions and NO_3^- or a derived nitrogen oxide is controlled by a sophisticated regulatory network consisting of two linked regulatory cascades, the FixLJ/FixK$_2$–NnrR and the RegSR/NifA systems [41]. In common with *R. etli*, but in contrast with *S. meliloti*, *B. japonicum* NnrR is under the control of FixK$_2$ [42]. Thus NnrR expands the FixLJ/FixK$_2$ regulatory cascade by an additional control level, which integrates the nitrogen oxide signal required for maximal induction of denitrification genes (Figure 1). As observed in *R. etli*, the *B. japonicum nirK* and *nor* promoters exhibit slight differences with regard to their dependence on NnrR [42]. Whereas induction of *nor* genes is abolished completely in the absence of a functional *nnrR* gene, microaerobic induction of *nirK* is retained in a *nnrR* mutant. Maximal expression of *nirK* and *nor* genes in *B. japonicum* also needs the NifA regulatory protein [43]. In contrast, this regulator in *R. etli* has a negative effect on the expression of the *nirK* operon. These observations indicate that modulation of denitrification genes by NifA is different in denitrifying and non-denitrifying rhizobial species. Very recently, it has been shown that expression of *B. japonicum norC* is controlled by the response regulator RegR, a member of the family of two-component regulatory redox-responsive proteins present in a large number of Alphaproteobacteria [41]. However, the mechanism involved in the control of *B. japonicum* denitrification by RegSR is yet unknown.

In contrast with *P. vulgaris*–*R. etli* symbiosis, inoculation of NO_3^--treated soya bean plants with a *B. japonicum norC* mutant does not affect the level of NO and LbNO complexes within nodules, so there must be other systems that remove NO in the nodule [44]. Recent findings have demonstrated that hypoxic conditions provoked by flooding are required to demonstrate a role of *B. japonicum* cNor in NO reduction in nodules [21].

To identify other NO-detoxifying systems in *B. japonicum*, a search on the *B. japonicum* USDA110 genome sequence (http://genome.kazusa.or.jp/rhizobase/) was performed. This revealed a putative sdHb, Bjgb, encoded by the gene *blr2807*, which has a high identity with sdHbs from *Vitreoscilla stercoraria* and *Campylobacter jejuni* which are implicated in NO detoxification [2,45,46]. *B. japonicum blr2807* is included in a gene cluster containing a putative ABC (ATP-binding cassette)-nitrate transporter, (*blr2803*-*5*), a nitrite extrusion protein (*blr2806*), a putative FAD- and NAD(P)H-binding reductase protein (*blr2808*) and the large catalytic subunit, NasA, from the assimilatory nitrate reductase (*blr2809*) [47]. In order to investigate the involvement of Bjgb in the NO response, we constructed a deletion mutant lacking the *blr2807* gene and tested its sensitivity to the NO donor SNP. The sensitivity of the *bjgb* mutant to SNP was compared with that of a *norC* mutant and the wild-type strain. Addition of SNP after 24 h of growth under microaerobic conditions (2% O_2) led to a transient arrest of cell growth in wild-type cells which was restored after 24 h (Figure 2A). However, the *norC* or the *bjgb* mutants showed a substantially longer period of microaerobic growth inhibition of ~72 h after addition of either 0.5 or 1 mM SNP to the cultures (Figures 2B and 2C). These results revealed

Figure 2 | Growth curves of wild-type and mutant *B. japonicum* under microaerobic conditions

Microaerobic (2% O_2) growth curves of wild-type *B. japonicum* USDA110 (**A**), and the *norC* (**B**) and *bjgb* (**C**) mutants in Bergersen minimum medium in the absence (◆), and the presence of 0.5 mM SNP (□) or 1 mM SNP (Δ). NO was added at the time indicated by the arrow and the results are means ± S.D. representative of three independent experiments. O.D. = attenuance (D_{600}).

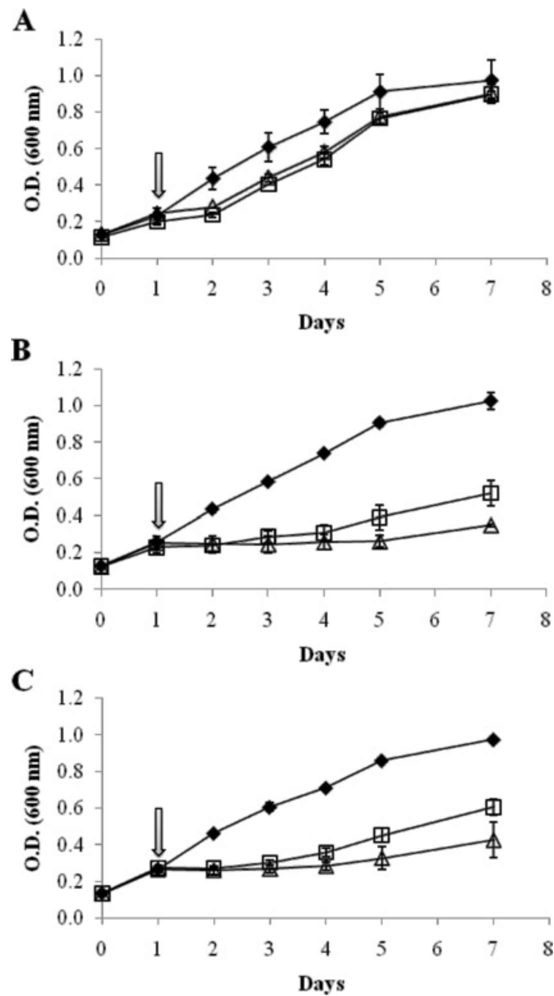

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Table 1 | **Expression data of *B. japonicum blr2803–blr2809* genes in cells grown under different O_2 concentrations**

Gene nomenclature and protein description is according to Kaneko et al. [51]. Normalized signal intensities were detected in transcriptomics experiments of *B. japonicum* cells grown under free-living, oxic (21% O_2), micro-oxic (0.5% O_2) and anoxic conditions with nitrate [48,50].

		Signal intensities (mean ± S.D.)		
Gene	**Description**	**Oxic**	**Micro-oxic**	**Anoxic**
blr2803	ABC transporter nitrate-binding protein	152 ± 57	129 ± 43	364 ± 97
blr2804	ABC transporter permease protein	160 ± 75	158 ± 40	287 ± 73
blr2805	ABC transporter ATP-binding protein	152 ± 64	129 ± 32	312 ± 86
blr2806	Nitrite extrusion protein	76 ± 21	78 ± 32	780 ± 287
blr2807	SdHb (Bjgb)	98 ± 28	124 ± 56	390 ± 111
blr2808	FAD- and NAD(P)H-binding reductase	20 ± 9	21 ± 36	179 ± 66
blr2809	Assimilatory nitrate reductase large subunit	105 ± 52	74 ± 96	309 ± 82

the importance of cNor and Bjgb for NO detoxification in *B. japonicum* under free-living conditions. The symbiotic properties of the *bjgb* mutant on soya bean plants grown under normoxic or hypoxic conditions are currently under investigation.

Similarly to other bacterial Hbs, Bjgb might express NO reductase activity to N_2O or denitrosylase ('dioxygenase') activity to $NO_3{}^-$ under micro-oxic free-living conditions or inside the nodules. For both activities, electrons from NAD(P)H might be delivered via the flavin protein encoded by *blr2808*. However, the contribution of those activities as well as the role of *blr2808* (FAD-protein) in NO detoxification needs to be demonstrated.

We have performed a comparative analysis of expression of the *blr2803–blr2809* gene cluster by looking at previous genome-wide transcription profiling of *B. japonicum* cells grown oxically, micro-oxically (0.5% O_2) or anoxically with $NO_3{}^-$ [48–50]. We found that expression of these genes is significantly induced (~2–10-fold) in cells grown anoxically in comparison with cells grown oxically or micro-oxically (Table 1). The basal expression of *blr2807* under micro-oxic conditions supports the phenotypic results showed in Figure 2, and suggests a role for this gene in NO detoxification under micro-oxic conditions. The putative involvement of *blr2807* in NO detoxification under denitrifying conditions is, at the moment, unknown.

Putative FNR-type binding sites are present in the promoter regions of the gene cluster containing *blr2807* (results not shown), indicating that low O_2 concentrations are required to induce the expression of the *blr2803–blr2809* gene cluster. Since maximal expression of this gene cluster was observed under $NO_3{}^-$-dependent anaerobic growth conditions, we might speculate that $NO_3{}^-$ or a nitrogen oxide derived from its reduction are also required to induce expression of *blr2803–blr2809* genes. The regulation of this gene cluster is currently under investigation.

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