Regulation and Symbiotic Role of *nirK* and *norC* Expression in *Rhizobium etli*

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Rhizobium etli CFN42 is unable to use nitrate for respiration and lacks nitrate reductase activity as well as the *nap* or *nar* genes encoding respiratory nitrate reductase. However, genes encoding proteins closely related to denitrification enzymes, the *norCBQD* gene cluster and a novel *nirKnirVnnrRnnrU* operon are located on pCFN42f. In this study, we carried out a genetic and functional characterization of the reductases encoded by the *R. etli nirK* and *norCB* genes. By gene fusion expression analysis in free-living conditions, we determined that *R. etli* regulates its response to nitric oxide through NnrR via the microaerobic expression mediated by FixKf. Interestingly, expression of the *norC* and *nirK* genes displays a different level of dependence for NnrR. A null mutation in *nnrR* causes a drastic drop in the expression of *norC*, while *nirK* still exhibits significant expression. A thorough analysis of the *nirK* regulatory region revealed that this gene is under both positive and negative regulation. Functional analysis carried out in this work demonstrated that reduction of nitrite and nitric oxide in *R. etli* requires the reductase activities encoded by the *norCBQD* and *nirK* genes. Levels of nitrosylhemoglobin complexes in bean plants exposed to nitrate are increased in a *norC* mutant but decreased in a *nirK* mutant. The nitrate-induced decline in nitrogenase-specific activity observed in both the wild type and the *norC* mutant was not detected in the *nirK* mutant. This data indicate that bacterial nitrite reductase is an important contributor to the formation of NO in bean nodules in response to nitrate.

The nitrogen cycle includes a series of both reductive and oxidative reactions in which prokaryotes have an important role. Dinitrogen (N₂) is incorporated into the biosphere by biological or chemical fixation to generate ammonium and returns to the atmosphere by denitrification. The biological denitrification process consists of a sequential reduction of nitrate or nitrite to nitrogen gas when oxygen concentrations are limited. Reduction of nitrogen oxides is coupled with energy conservation and permits cell growth under anaerobic conditions. The denitrification process depends on four nitrogen oxide reductases: respiratory or periplasmic nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, encoded by *narGHJI* (or *napEDABC*), *nirK* (or *nirS*), *norCBQD*, and *nosRZDYFLX* genes, respectively (van Spanning et al. 2005, 2007; Zumft and Körner 1997).

In general, expression of denitrification genes requires a low oxygen concentration, the presence of a nitrogen oxide, and control by transcriptional regulators belonging to the CRP/FNR family (Baek and Shapleigh 2005; Bedmar et al. 2005; Hendriks et al. 2000; Mesa et al. 2003; Tosques et al. 1997; Wang et al. 1999; Zumft and Körner 1997; Zumft 2002). FNR is a protein that contains an O₂-sensitive [4Fe-4S]²⁺ cluster that directly senses oxygen. In *Escherichia coli*, it functions as a global regulator controlling the expression of up to 100 genes in response to oxygen limitation, and its active DNA-binding form is a homodimer containing two [4Fe-4S]²⁺ clusters (Kang et al. 2005; Kiley and Beinert 2003). The protein binds through a helix-turn-helix motif to the promoter region of target genes, repressing the expression of genes for aerobic respiration and inducing genes involved in alternative forms of anaerobic respiration.

The consensus DNA binding site for *E. coli* FNR is a symmetrical motif (TTGAT-N4-ATCAA) called the FNR box or anaerobox (Kiley and Beinert 1999, 2003; Körner et al. 2003). FNR homologs have been identified in a wide number of bacteria. Some of these proteins conserve an N-terminal cysteine-rich motif, which binds the Fe-S cluster, while others, like the members of the FixK group, lack it. Based on genome analysis, the number of members of the CRP/FNR family now includes 369 members distributed in 14 phylogenetic branches (Körner et al. 2003). The FnrN group of regulators includes putative proteins with a slight modification of the conserved cysteine motif of FNR, allowing the assembly of a [4Fe-4S]²⁺ cluster and thus redox sensing. On the other hand, regulators belonging to the FixK branch are structurally homologous to FnrN but lack the N-terminal cysteine-rich motif. A characteristic frequently found in sequenced bacterial genomes is the existence of multiple members of the CRP/FNR regulators in the same host (Körner et al. 2003).

The reduction of nitrite (NO₂⁻) to nitric oxide (NO), the central reaction in denitrification, is catalyzed by nitrite reductase. There are two types of nitrite reductases with different prosthetic groups. The Cu-containing nitrite reductase (CuNir, encoded by *nirK*) uses copper as a redox active metal, and is a trimeric protein containing two types of copper centers. The other, a dimeric *cd*₁-type nitrite reductase (cytochrome *cd*₁), utilizes heme-bound iron and is encoded by the *nirS* gene. Phylogenetic distribution of NirK and NirS reductases appears to be random; however, even when both types of enzymes can be found in a single genus, they cannot coexist in a single bacteria (Shapleigh 2006; Zumft and Körner 1997).

Due to its toxicity, NO has to be reduced to nitrous oxide immediately. A membrane protein complex formed by two sub-

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units encoded by the *norC* and *norB* genes catalyzes the reduction of two molecules of NO to nitrous oxide (N₂O) and water (cNOR). NorC is a *c*-type cytochrome, while NorB contains two *b*-type hemes. In general, *norCB* genes are clustered with the *norQ* and *norD* genes. Therefore, to remove the NO, reactions for reduction of nitrite and nitric oxide must be regulated in a concerted way (Hendriks et al. 2000; Philippot 2002; Zumft 2005). In addition to cNOR, another two different bacterial NOR have been characterized (qNOR and qCu_ANOR) (de Vries et al. 2007; Zumft 2005).

In plants, NO has been proposed as a signaling molecule involved in diverse physiological processes, such as defense response, growth, and development (Besson-Bard et al. 2008). Nitric oxide in plants can be produced from nitrite via nonenzymatic or enzymatic pathways. Several enzymatic pathways have been proposed for the generation of NO in plants. One is a nitrate-dependent pathway that is catalyzed mainly by nitrate reductase. Additionally, two other enzymatic pathways that use L-Arg or polyamines as substrates have been reported. The corresponding enzymes in the last two pathways remain as yet unidentified, and the hypothesis that plants do possess a nitric oxide synthase (NOS)-like enzyme is highly controversial (Besson-Bard et al. 2008). The sources of NO in nodules are not clear; nodular NO may be a product of bacterial denitrifying metabolism (Meakin et al. 2007; Sánchez et al. 2010) or may also be produced in plant root tissue from arginine via an unidentified NOS-like enzyme (Baudouin et al. 2006). In symbiosis, NO is produced in early stages of the process and in the mature nodule. For example, in *Lotus japonicus*, a rapid and transient NO production is observed when roots are inoculated with *Mesorhizobium loti* (Shimoda et al. 2005). In *Medicago* spp., NO is involved in nodule formation and functioning (Ferrarini et al. 2008; Pii et al. 2007). In addition, modulation of NO levels are involved in the establishment of the symbiosis (Nagata et al. 2008). NO also interfere with nitrogen fixation in soybean (Trinchant and Rigaud 1982), *Alnus firma* (Sasakura et al. 2006), and *Lotus japonicus* nodules (Kato et al. 2010; Shimoda et al. 2009). NO affect nitrogenase activity or can bind to leghemoglobin to form nitrosylleghemoglobin (Lb-NO) complexes in soybean nodules (Mathieu et al. 1998; Meakin et al. 2007; Sánchez et al. 2010).

Denitrification capability is widespread in prokaryotes, including nitrogen-fixing bacteria. Among rhizobia, only *Bradyrhizobium japonicum*, the microsymbiont of soybean, has been shown to reduce NO₃⁻ simultaneously to NH₄⁺ and N₂ when cultured microaerobically with nitrate as terminal electron acceptor and sole source of nitrogen. Denitrification in *B. japonicum* depends on the consecutive action of the *napEDABC*, *nirK*, *norCBQD*, and the *nosRZDYFLX* genes (Bedmar et al. 2005). Other rhizobia, like *Sinorhizobium meliloti*, are only partial denitrifiers that produce N₂O as an end product, even when they contain the complete set of genes for microaerobic nitrate reduction (Becker et al. 2004; Holloway et al. 1996). The extreme situation is represented by *Rhizobium sllae* (formerly *Rhizobium hedyari*), which contains only the Cu-containing nitrite reductase (Toffanin et al. 1996).

Rhizobium etli CFN42 fixes nitrogen in association with bean plants, and its genome contains a chromosome and six large plasmids (pCFN42a to pCFN42f) whose sizes range from 184.4 to 642.5 kb (González et al. 2006). In *R. etli* CFN42, some important regulatory elements present on the pSym in other *Rhizobium* species are not encoded on this replicon. A *fix* region containing copies of the *fixK*, *fixNOQP*, *fixGHIS-stoR* genes and the *fixL* gene, which controls the expression of *fixKf*, is located on pCFN42f (Girard et al. 2000; Granados-Baeza et al. 2007). Downstream of the pCFN42f *fix* region, there is a gene cluster related with the denitrification

process that includes genes encoding for the Cu-containing nitrite reductase (*nirK*), a cytochrome-*c* type nitric oxide reductase (*norCBQD*), and a pseudoazurin (*azuPf*) (Girard et al. 2000; González et al. 2006). An additional gene, *nnrR*, is also present in the *nirK-norC* region in *R. etli*. In *B. japonicum* NnrR was described as a transcriptional regulator for *nirK* and *norC* genes in the presence of N oxides (Mesa et al. 2003).

Previously, we reported that *R. etli* shows nitrite reductase activity after incubation of the cells under anaerobic conditions. In this condition, a *R. etli nirK* mutant exhibits a poor nitrite reductase activity and nitrite uptake. In addition, *R. etli* is not able to use nitrate for respiration and does not present nitrate reductase activity. These results indicate that *R. etli* CFN42 is a nondenitrifying bacteria (Bueno et al. 2005). Therefore, the presence of NirK and NorC coding regions in an otherwise nondenitrifying bacterium can be explained by proposing that these enzymes have an N oxide-detoxifying role in *R. etli*, thus preventing accumulation of NO inside the cell. To gain insight into how *R. etli* responds to reactive nitrogen species, we carried out a genetic and functional characterization of the reductases encoded by *R. etli nirK* and *norC* genes. Also, we analyzed the participation of different regulatory proteins in the control of the expression of these genes. Nitrogenase-specific activity and the proportion of Lb-NO complexes formed in bean nodules demonstrate that *norC* has an important role in the microsymbiont in protection from NO and indicates that bacterial nitrite reductase is an important contributor to the formation of NO in nodules in response to nitrate.

RESULTS

Structural organization of *R. etli nirK* and *norC* region.

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*), and pseudoazurin (*azuPf*). 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. Similar to denitrifiers, the *R. etli nor* gene cluster consists of *norCBQD*. An additional gene, annotated as *norE*, that codifies for an accessory protein of the nitric oxide reductase in *S. meliloti* (SMA1279) was found upstream of the *norC* operon. The *R. etli nirK* gene is transcribed in the opposite direction of open reading frame (ORF) RHE_PF00524, which encoded for a hypothetical protein similar to NnrS from *Rhizobium* sp. strain NGR234 (NGR_c09940) (Fig. 1).

In general, the *nirK* gene is clustered with *nirV*, an ORF that encodes for a putative periplasmic nitrite reductase. In *R. etli* CFN42, the structural arrangement of the *nirK-nirV* region suggests that these genes are part of the same operon. The predicted translational start of the *nirV* coding region is located 82 bp downstream of the *nirK* stop codon. Two additional ORF are located in the proximity of *nirK* and *nirV* in *R. etli*, *nnrR*, which encodes NnrR, the FNR-type transcriptional regulator of denitrification genes, and RHE_PF00528, similar to denitrification regulator protein NnrU. The translational start of *nnrR* is located just 38 bp downstream of the *nirV* coding region, while the intergenic region between *nnrR* and *nnrU* coding regions comprises only 13 bp (González et al. 2006; Kazusa DNA Research Institute Rhizobase).

To determine if *nirK*, *nirV*, *nnrR*, and *nnrU* are arranged as an operon, we evaluated microaerobic expression of these genes by reverse transcription-polymerase chain reaction (RT-PCR). An insertional mutation within the *nirK* coding region

abolished the expression of *nnrR*, indicating that a mutation in this gene exerts a polar effect on the expression of *nnrR* (data not shown). In addition, the synthesis of different cDNA products corresponding to *nirK-nirV*, *nirV-nnrR*, or *nirV-nnrU* in the wild-type strain demonstrates that, in *R. etli*, *nirK*, *nirV*, *nnrR*, and *nnrU* constitute an operon.

To predict the location of the promoter for the *R. etli* *norC* and *nirK* operons, the transcriptional start sites of each transcriptional unit were identified in assays using the rapid amplification of cDNA 5' ends (RACE). Total RNA was purified from wild-type cells cultured under microaerobic conditions. Our results show that the *R. etli* *norC* transcriptional start site is a G located 60 nucleotides upstream of the putative translational start codon. Analysis of the DNA region upstream of the *norC* gene revealed the presence of a purine-rich Shine-Dalgarno-like sequence (AAGGGA) six bases in front of the *norC* ATG codon. We also identified a sequence similar to the consensus for the FNR-DNA binding site (5'-TTGCG-N4-AACAA-3') (Fig. 2A) centered at position -43.5 bp from the *norC* transcriptional start site.

For *R. etli* *nirK*, the transcriptional start site was defined as an A located 43 bp upstream of the putative translation start. Similar to *norC*, a possible Shine-Dalgarno sequence (AGGA GA) was located six bases upstream from the putative translation start of *nirK*. Three sequences with high similarity to FNR-binding sites (TTGCG-N4-GTCAA, TTGTG-N4-CACAA, and TTGAG-N4-CGCAA) were identified in the *nnrS-nirK* intergenic region. They were named according to their relative distance from the *nirK* transcription start site as anaerobox 1, 2, or 3 (ANB1, ANB2, or ANB3, respectively). The ANB1 located is at -42.5, ANB2 at -65.5 and ANB3 at -96.5 bp (Fig. 2B). Interestingly, overlapping with the ANB2 there is a sequence similar to the NifA-binding site (5'-TGT-N10-ACA) (upstream activator sequence) without any putative -12/-24 type promoter in the region.

Oxygen as a major signal

for *R. etli* *nirK* and *norC* gene expression.

In *B. japonicum*, the expression of *nir* and *nor* genes in response to low oxygen concentration and NO (or a chemically

related species) is under the control of FixK and NnrR, respectively, which are two regulatory proteins belonging to the CRP/FNR family (Mesa et al. 2003). Presence of putative class II promoters in the *R. etli* *norC* and *nirK* regulatory regions suggests that microaerobic conditions are a prerequisite for their expression.

To evaluate the oxygen concentration response of these genes in *R. etli*, the regulatory regions of *norC* and *nirK* were fused to the promoterless *uidA* reporter gene in plasmid pBBMCS53 (Girard et al. 2000). A 401-bp PCR product that contains the *norC* upstream region was cloned to construct plasmid pNIC-01 (*norC::uidA*) (Fig. 2A). A 696-bp PCR product containing the *nnr-nirK* complete intergenic region, including all anaeroboxes, was cloned in both directions, generating plasmids pNIC-02 (*nnrS::uidA*) and plasmid pNIC-03 (*nirK::uidA*) (Fig. 2B). These plasmids were introduced separately into the *R. etli* wild-type strain. As shown in Table 1, only marginal expression of the *norC* and *nirK* genes was observed under aerobic conditions. A low-oxygen concentration (1%) induced *norC* expression nearly 50-fold and *nirK* expression 10-fold. Addition of 100 μ M sodium nitroprussiate (SNP, a NO-generating agent) or 1.0 mM nitrite to microaerobic cultures increased *norC* and *nirK* microaerobic expression about twofold. In contrast, expression of *nnrS* was not detected in aerobic and low-oxygen concentration conditions even in the presence of an N oxide.

To study the participation of FixKf and NnrR as regulators of the expression of the *R. etli* *norC* and *nirK* genes, expression patterns of plasmids pNIC-01 and pNIC-03 in the wild-type and in the *fixKf:: Ω Km* (CFNX637) and *nnrR::loxSp* (CFNX700) mutant backgrounds were analyzed. The microaerobic expression of *norC* and *nirK* is fully dependent on a functional FixKf protein (Fig. 3); in contrast, a differential dependence on NnrR for expression of these genes was observed. While expression of *norC* is almost abated in an *nnrR* mutant background (Fig. 3A), the *nirK* promoter shows only a marginal dependence of NnrR for expression in low-oxygen levels (Fig. 3B). These results indicate that, in addition to FixKf, the presence of NnrR is indispensable for *norC* expression. A full expression of *nirK* was observed in microaerobic cultures in

Fig. 1. Genomic context of the denitrification genes in *Rhizobium etli* CFN42. The region shown is located from bases 563,249 to 594,052 in plasmid f. Functional categories are indicated according to the annotation in databases: nitrogen fixation (white arrows), denitrification (gray arrows), FNR-type regulators (striped arrows), electron transfer (dotted arrows), hypothetical proteins (black arrows), and putative FNR-binding sites (black squares).

Table 1. Expression of *norC::uidA* and *nirK::uidA* gene fusions in *Rhizobium etli* wild type under free-living conditions

Strain	Fusion genotype	β -glucuronidase specific activity ($\pm$ SD) ^a		
		Aerobic	1% O ₂	1% O ₂ + N oxide
CE3/pNIC-01	<i>norC::uidA</i>	8.4 $\pm$ 0.51	410 $\pm$ 45	741 $\pm$ 49 ^b
CE3/pNIC-02	<i>nnrS::uidA</i>	ND ^c	ND ^c	ND ^c
CE3/pNIC-03	<i>nirK::uidA</i>	108.0 $\pm$ 11	1,078 $\pm$ 61	2,130 $\pm$ 120 ^d

^a β -glucuronidase-specific activity ($\pm$ standard deviation [SD]). In nanomoles per min per milligram of protein; means from at least two independent determinations.

^b After 10 h at 30°C, 100 μ M sodium nitroprussiate was added to microaerobic cultures and they were incubated 2 h more.

^c ND = not detected.

^d After 10 h at 30°C, 1.0 mM NaNO₂ was added to microaerobic cultures and they were incubated 2 h more.

the presence of nitrite and FixKf and NnrR regulators. Complementation of CFNX637 (*fixKf::ΩKm*) and CFNX700 (*nnrR::loxSp*) mutant strains with plasmids *pfixKf* (pGHN-06) and *pnnrR* (pGHN-05), containing the wild-type *fixKf* and

nnrR genes, respectively, under the control of the *lacZ* promoter, restored the maximal *nirK* microaerobic expression observed when nitrite is present in the culture (Fig. 3B). Thus, our results clearly show that the induction of *nirK* and *norC* in response to N oxides is dependent on NnrR, which expression is microaerobically mediated by FixKf.

To define the key signal for activation of the *R. etli* NnrR transcriptional regulator, we compared the *nirK* microaerobic expression in different backgrounds in the presence of nitrite or SNP (Table 2). As observed previously, microaerobic expression of the *nirK* gene in the wild-type strain increased roughly two times in the presence of nitrite (from $1,078 \pm 61$ to $2,130 \pm 120$ [Table 2]). In contrast, in a *nirK* mutant (*nirK::loxP*, CFNX702), a strain unable to reduce NO_2^- to NO, the level of induction was significantly lower (from $1,009 \pm 75$ to $1,288 \pm 41$ [Table 2]). This suggests that reduction of NO_2^- to NO is crucial for induction of *nirK* microaerobic expression. In the presence of SNP, we observed the highest level of *nirK* expression in both wild-type and *nirK* mutant (Table 2). Microaerobic expression in a NnrR mutant was not affected by the presence of NO_2^- or NO (Table 2). This result indicates, in accordance with Figure 3, that a functional NnrR regulator is a requisite for the response of these genes to the presence of nitrogen oxides. Further evidence to support this interpretation

Fig. 2. Regulatory regions of *Rhizobium etli* *norC* and *nirK* genes. **A**, Sequence and schematic representation of the *norC* promoter region and construction of the *norC-gus* transcriptional fusion in plasmid pBBMCS53. **B**, Sequence and schematic representation of the *nirK* promoter region and fragments cloned in plasmid pBBMCS53 to generate *nnrS-gus* and *nirK-gus* transcriptional fusions. Putative FNR-binding sites are indicated as open boxes labeled ANB; NifA-UAS is underlined and the possible Shine-Dalgarno sequences are double underlined. The transcriptional start sites are indicated as +1 and by bent arrows. Relative positions of the regulatory elements identified and the size of fragments cloned in plasmid pBBMCS53 are indicated.

Fig. 3. Microaerobic expression of **A**, *norC* and **B**, *nirK* genes in different *Rhizobium etli* strains. Cells were grown microaerobically for 10 h (gray bars) and the expression was induced (white bars) by incubating 2 h more with sodium nitroprussiate to a final concentration of 100 μM (A) or with nitrite at 1 mM (B). β-glucuronidase-specific activity is expressed as nanomoles per min per milligram of protein. Data are the mean of two replicas from three independent experiments.

Table 2. Nitric oxide as an environmental signal for the induction of the *nirK* microaerobic expression mediated by NnrR

Strain	Fusion genotype	β-glucuronidase specific activity (± SD) ^a		
		1% O ₂	1% O ₂ + NaNO ₂ ^b	1% O ₂ + SNP ^c
CE3/pNIC-03	<i>nirK::uidA</i>	1,078 ± 61	2,130 ± 120	2,445 ± 131
NirK/pNIC-03	<i>nirK::uidA</i>	1,009 ± 75	1,288 ± 41	1,839 ± 136
NnrR/pNIC-03	<i>nirK::uidA</i>	894 ± 88	895 ± 26	936 ± 12

^a β-glucuronidase-specific activity (± standard deviation [SD]). In nanomoles per min per milligram of protein; means from at least two independent determinations.

^b After 10 h at 30°C, 1.0 mM NaNO₂ was added to microaerobic cultures and they were incubated 2 h more.

^c After 10 h at 30°C, 100 μM sodium nitroprussiate (SNP) was added to microaerobic cultures and they were incubated 2 h more.

was obtained by analyzing the microaerobic expression of *nirK* in a wild-type background with NaNO₂ or SNP plus a NO scavenger (c-PTIO). In these experiments, the microaerobic expression of *nirK* was not affected by the presence of NaNO₂ or SNP when 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide) (cPTIO) was added to the cultures (data not shown). These results clearly demonstrate that NO is the signal required for the induction of the microaerobic expression of *nirK* mediated by NnrR.

Functional analysis of the putative Fnr-binding sites in the expression of *nirK*.

As noted above, we identified three sequences with high similarity to FNR-binding sites in the *nnrS-nirK* intergenic region. To analyze the functionality and participation of each putative anaerobox in the expression of the *nirK* operon, DNA fragments containing deletions of the *nirK* upstream sequence were synthesized by PCR and were fused with a promoterless β -glucuronidase gene. Plasmids containing transcriptional fusions were introduced separately into different *R. etli* CFN42 derivatives and their transcriptional regulation was studied under microaerobic conditions in comparison with the expression pattern observed with plasmid pNIC-03 (which contains the complete *nnrS-nirK* intergenic region [Fig. 2B]). A 360-bp-long fragment in plasmid pNIC-04 (*nirK::uidA* ANB1 and ANB2) carries ANB1 and ANB2, while pNIC-05 (*nirK::uidA* ANB1) only contains ANB1 in a 329-bp-long fragment. The level of *nirK* microaerobic expression observed in the wild-type strain or in the different mutant backgrounds remained unaffected when ANB3 was deleted, indicating that this sequence is not required for *nirK* expression (Table 3, compare lines 1 and 2, 4 and 5, 7 and 8, and 10 and 11). The absence of ANB2-induced *nirK* expression (Table 3, lines 2 and 3, 5 and 6, 8 and 9, and 11 and 12) suggests that the regulatory region present in pNIC-05 is sufficient for *nirK* expression and is probably the binding site for either FixKf, NnrR, or both. The increased microaerobic expression of *nirK* observed when ANB2 is deleted may be due to elimi-

nation of multiple FNR sites or ANB2 may be a site recognized by a repressor protein.

Since a potential NifA-binding site overlaps with ANB2, we analyzed *nirK* expression in a *R. etli nifA* mutant background (CFNX247). Interestingly, mutation of the *nifA* gene causes an overexpression of *nirK* even in the presence of the putative NifA-binding site (Table 3, lines 10 to 12), indicating that microaerobic expression of *nirK* in *R. etli* is subject to both positive and negative regulation, entailing the participation of two Fnr-type regulators (FixKf and NnrR) and a NifA dependent regulator. However, expression of *nirK* was also augmented 20% in the NifA mutant in the absence of ANB2 (Table 3, compare lines 11 and 12). Thus, the participation of several factors governs the expression of *nirK* in *R. etli* CFN42.

To define more exactly the importance that each anaerobox has as a regulatory site for the expression of *nirK*, site-specific mutations were introduced in ANB1 (from TTGCG-N4-GTC AA to ATAACG-N4-GTCAA) and ANB2 (from TTGTG-N4-CACAA to TTGTG-N4-GATAT). In each case, changes were designed to interrupt the complementarity in the palindromic regions; moreover, since ANB1 overlaps with the -35 regulatory sequence, changes introduced in this anaerobox were chosen to avoid affecting this region. DNA fragments containing specific mutations were synthesized by PCR and were fused with a promoterless β -glucuronidase gene. The plasmids constructed (pNIC-06 and pNIC-07) were introduced separately into different *R. etli* CFN42 derivatives and their transcriptional regulation was studied under microaerobic conditions in comparison with the expression pattern observed with plasmid pNIC-03, which contains the complete *nnrS-nirK* intergenic region (Fig. 2B). The microaerobic expression of *nirK* is drastically affected when ANB1 is mutated (Table 4). The very low expression levels observed confirm that this region is an essential target site for a positive transcriptional regulator that controls *nirK* microaerobic expression. Interestingly, when microaerobic expression of *nirK* is under the control of a regulatory region carrying specific mutations in ANB2 (plasmid pNIC-07), the level of expression is only half that

Table 3. Functional analysis of the *nirK* regulatory region

Strain	Strain genotype	Fusion genotype	β -glucuronidase specific activity ^a
CE3/pNIC-03	Wild-type	<i>nirK::uidA</i> ANB1,2,3	1,078 $\pm$ 61
CE3/pNIC-04	Wild-type	<i>nirK::uidA</i> ANB1,2	1,037 $\pm$ 52
CE3/pNIC-05	Wild-type	<i>nirK::uidA</i> ANB1	1,920 $\pm$ 64
CFNX637/pNIC-03	<i>fixKf::</i> Ω Km	<i>nirK::uidA</i> ANB1,2,3	35 $\pm$ 12
CFNX637/pNIC-04	<i>fixKf::</i> Ω Km	<i>nirK::uidA</i> ANB1,2	51 $\pm$ 23
CFNX637/pNIC-05	<i>fixKf::</i> Ω Km	<i>nirK::uidA</i> ANB1	59 $\pm$ 7
CFNX700/pNIC-03	<i>nnrR::loxSp</i>	<i>nirK::uidA</i> ANB1,2,3	886 $\pm$ 59
CFNX700/pNIC-04	<i>nnrR::loxSp</i>	<i>nirK::uidA</i> ANB1,2	867 $\pm$ 63
CFNX700/pNIC-05	<i>nnrR::loxSp</i>	<i>nirK::uidA</i> ANB1	2,064 $\pm$ 22
CFNX247/pNIC-03	<i>nifA_Δ::</i> Ω Sp	<i>nirK::uidA</i> ANB1,2,3	1,834 $\pm$ 73
CFNX247/pNIC-04	<i>nifA_Δ::</i> Ω Sp	<i>nirK::uidA</i> ANB1,2	1,859 $\pm$ 108
CFNX247/pNIC-05	<i>nifA_Δ::</i> Ω Sp	<i>nirK::uidA</i> ANB1	2,372 $\pm$ 53

^a β -glucuronidase-specific activity ($\pm$ standard deviation). In nanomoles per min per milligram of protein; means from at least three independent determinations.

Table 4. Mutational analysis of the FNR sites in the *nirK* regulatory region

Strain	Fusion genotype	ANB ^a	Expression level ^b	
			Aerobic	Microaerobic
CE3/pNIC-03	<i>nirK::uidA</i>	1,2,3	108 $\pm$ 11	1,078 $\pm$ 61
CE3/pNIC-06	<i>nirK::uidA</i>	1m ^c , 2, 3	12 $\pm$ 4	38 $\pm$ 3
CE3/pNIC-07	<i>nirK::uidA</i>	1,2m ^d , 3	67 $\pm$ 5	510 $\pm$ 10

^a FNR-binding site present in the transcriptional fusion.

^b β -glucuronidase-specific activity ($\pm$ standard deviation). In nanomoles of NO per h per milligram of protein; means from at least three independent determinations.

^c Mutations in ANB1: from TTGCG-N4-GTCAA to ATAACG-N4-GTCAA.

^d Mutations in ANB2: from TTGTG-N4-CACAA to TTGTG-N4-GATAT.

found when *nirK* expression is driven by a wild-type regulatory region (Table 4). Therefore, we hypothesize that ANB2 is also a target sequence for a positive regulator necessary for attaining the highest microaerobic expression of *nirK*.

R. etli nirK and *norC* encode functional reductases.

Previously, we reported that, in *R. etli* CE3, the *nirK* gene encodes a functional nitrite reductase. In contrast to the *R. etli* wild-type strain, a *nirK::ΩKm* mutant strain (CFNX699) lost the methyl viologen (MV)-dependent Nir activity and the capacity to consume nitrite from the medium in anaerobic cultures (Bueno et al. 2005). In this work, we demonstrate that disruption of *nirK* with a Ω Km interposon exerts a polar effect on the downstream genes in the operon, including the gene encoding the NnrR transcriptional regulator. To avoid polarity effects on *nnrR*, a new *nirK::loxPsp* allele was generated. From this, an in-phase knock-out allele was derived (*nirK::loxP*) by Cre-mediated excision of the spectinomycin (Sp) cassette (strain CFNX702, discussed below). To evaluate the functionality of the reductase encoded by the *R. etli nirK* gene, nitrite reductase activity was determined in cell extracts from the wild-type and the *nirK::loxP* (CFNX702), *fixKf::ΩKm* (CFNX637), and *nnrR::loxPsp* (CFNX700) mutant strains grown microaerobically (Fig. 4). Nir activity was only detected in the wild-type strain.

Since specific mutations in the *nirK* regulatory sites (ANB1 and ANB2) affect gene expression, we performed a complementation analysis in the *nirK* mutant to ascertain if these mutations also have an effect on the Nir activity. To that end, three different plasmids were constructed: i) *pnirK*-RRWt (pGHN-01), in which the expression of *nirK* is under the control of the wild-type regulatory region; ii) *pnirK*-ANB2m (pGHN-03), in which the *nirK* regulatory region carried site-specific mutations in ANB2; and iii) *pnirK*-ANB1m (pGHN-02), in which the ANB1 was mutated. Wild-type Nir activity was restored upon introduction of plasmids pGHN-01 and pGHN-03 in the *nirK* mutant (Fig. 4). In contrast, plasmid pGHN-02, with ANB1 mutated, was unable to restore this activity. These data are consistent with a possible promoter located in ANB1 that is necessary for full expression of nitrite reductase activity.

As mentioned, a mutation in *nnrR* provokes a reduction of just 50% in *nirK* expression (Fig. 3B). Interestingly, Nir enzymatic activity was not detected in this background (Fig. 4), suggesting that NnrR is controlling other genes needed for Nir

activity. If this was the case, no Nir enzymatic activity should be seen in a *nnrR* mutant, even under conditions that restore full *nirK* expression. To substantiate this, two constitutive expression plasmids were generated, *pnirK* and *pnnrR* (pGHN-04 and pGHN-05, respectively), putting the *nirK* and the *nnrR* coding regions under the control of the *lacZ* promoter. Introduction of pGHN-04 in the *nnrR* mutant background failed to restore Nir activity, while wild-type activity was recovered in the *nnrR* mutant complemented with pGHN-05 (Fig. 4). These data support our interpretation that additional genes, controlled by *nnrR*, are required for full expression of Nir enzymatic activity.

R. etli responds to nitric oxide by the expression of *nirK* and *norC* genes.

The in vivo relevance of the functions encoded by the *nirK* and *norC* genes can be further ascertained by exploring the response of the corresponding mutants to both nitrite and to nitric oxide generators, such as SNP. Although none of the mutants described here affect growth under microaerobic conditions (Fig. 5A), the presence of 1 mM NaNO₂ drastically affects this capacity, both in the wild-type strain and in the *norC* mutant and, to a lesser extent, in the *nnrR* mutant. Both the *nirK* and the *nnrR* mutants displayed a marked inhibition of growth in the presence of nitrite upon complementation with the corresponding genes, indicating that nitrite sensitivity in the wild-type strain is due to the presence of NirK and NnrR proteins (Fig. 5B). This sensitivity is most likely due to the role of both proteins, either directly or indirectly, to produce nitric oxide. To evaluate this possibility, levels of NO production and consumption were evaluated in the wild type and in the *nirK* mutant with a nitric oxide electrode (discussed below). As shown in Table 5, high levels of NO production were seen in the wild type, while in the *nirK* mutant, levels of this metabolite were almost negligible.

Significant levels of NO consumption were detected in the wild type. This activity is due to the product of *norC*, since levels of consumption were virtually undetectable in the corresponding mutant. Moreover, the *norC* mutant showed levels of NO production twice as high as that seen in the wild type. These data allow us to predict that the *norC* mutant should be hypersensitive to the effects of a nitric oxide generator such as SNP and to nitrite as well. As expected, the presence of 100 μ M SNP in the culture severely affects the microaerobic growth of the *norC* (CFNX701) and *nnrR* (CFNX700) mutants, while the growth of the wild type was relatively resistant to SNP addition (Fig. 5C). Confirmation of the in vivo role of NorC in detoxifying the NO produced from nitrite was achieved by growth tests under microaerobic conditions of both the wild type and the *norC* mutant in the presence of sublethal concentrations of NaNO₂ (30 μ M). The wild-type strain was still able to grow microaerobically in the presence of 30 μ M NaNO₂, while the *norC* mutant failed to grow in this condition (Fig. 5D). These data clearly indicate that the product of *norC* is required in vivo to detoxify NO and that the balance between the activities of NirK and NorC determine the level of tolerance to NO.

Symbiotic expression of the *nirK* and *norC* genes.

Ex planta, expression of both *nirK* and *norC* genes require low-oxygen conditions. This expression is further enhanced by the presence of a nitrogen oxide. To test if these genes are also expressed in nodules, common bean plants (*Phaseolus vulgaris*) were inoculated with either the wild-type, *nirK*, or *norC* mutant strains and were watered with or without nitrate.

The expression of *nirK* and *norC* genes in nodule was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). RNA was purified from nodules of bean plants that were inoculated with *R. etli* wild-type strain. The presence of a cDNA

Fig. 4. Methyl viologen-dependent nitrite reductase activity in free-living conditions of both the *nirK* or *nnrR* mutant derivatives carrying plasmids that express *nirK* or *nnrR* controlled by different promoters, i.e., wild-type promoters (white bars), mutated promoters (gray bars), and constitutive promoters (black bars). Nir activity is expressed as nanomoles NO₂⁻ consumed per h per milligram of protein. Data show the mean of three independent experiments assayed three times. Error bars are indicated.

product corresponding to *nirK* and *norC* confirm that these genes are expressed in bean nodules (data not shown). Measurements of Nir activity in cell extracts from bean nodules revealed that the nitrite reductase activity in the wild-type nodules was about 4.8 times higher than that observed in extracts from nodules formed by the *nirK* mutant (95 ± 6.3 vs. 19.5 ± 0.7 nmol NO_2^- consumed per milligram of protein per h, respectively). Only marginal increases in Nir activity were observed in plants treated with 4 mM KNO_3 for 12 days before harvesting. Thus, in *R. etli* the symbiotic condition per se is sufficient to achieve a maximal activity of the reductase encoded by the *nirK* gene.

To evaluate the potential contribution of the bacterial micro-symbiont to NO production in bean plants, the proportion of Lb-NO complexes in nodules was quantified by UV-visible absorbance spectroscopy. To this end, bean plants were inoculated with wild type, *nirK*, or *norC* mutant strains and were watered with a solution containing nitrate at 14 days postinoculation (dpi). The proportion of Lb-NO complexes formed in nodules from plants cultivated without KNO_3 was approximately 15 to 20%, irrespective of the strain used for inoculation (Table 6). However, Lb-NO formation increased to 64% with the application of nitrate in wild-type nodules and up to

95% in plants inoculated with the *norC* mutant. The marked increase in Lb-NO formation in the *norC* mutant may be due to impaired elimination of NO in these nodules, indicating an important role of the microsymbiont in protection from NO. Moreover, null mutations in the *nirK* gene prevent nitrate-induced increase in the formation of Lb-NO complexes, which remained at low levels (15 to 20%). This indicates that bacterial nitrite reductase is an important contributor to the formation of NO in nodules.

None of the mutants employed affected nitrogenase-specific activity in the absence of nitrate (Table 6). Nitrate induces a threefold decline in nitrogenase-specific activity for both the wild type and the *norC* mutant; in contrast, the nitrogenase-specific activity in nodules induced by the *nirK* mutant did not show a nitrate-induced decline (Table 6). This result may be explained by the reduced amount of Lb-NO present in these nodules, as compared with the wild type and the *norC* mutant.

DISCUSSION

In this work, we demonstrate that, in *R. etli* CFN42, the *nirK* and *norCBQD* genes located on plasmid pCFN42f encode a

Fig. 5. Effect of nitrogen oxides on the microaerobic growth of *Rhizobium etli*. **A**, **B**, and **C**, Cells were cultivated in minimal medium at 30°C for 10 h or **D**, 32 h under **A**, microaerobic conditions or **B**, microaerobic conditions plus 1.0 mM NaNO_2 , **C**, plus 100 μM sodium nitroprussiate (SNP), or **D**, plus 30 μM NaNO_2 . Squares indicate growth of wild-type strain under microaerobic conditions (■), plus NaNO_2 (□), or plus SNP (▣); triangles indicate growth of *nirK* mutant strain CFNX702 under microaerobic conditions (▲), plus NaNO_2 (△), or plus SNP (▴). Growth of complemented *nirK* mutant strain CFNX702 with plasmid pGHN-04 under microaerobic conditions with NaNO_2 is indicated by --▲--. Circles indicate growth of *norC* mutant strain CFNX701 under microaerobic conditions (●), plus NaNO_2 (○), or plus SNP (◐). Diamonds indicate growth of *mnrR* mutant strain CFNX700 under microaerobic conditions (◆), plus NaNO_2 (◇), or plus SNP (◈). Growth of complemented *mnrR* mutant strain CFNX700 with plasmid pGHN-05 under microaerobic conditions with NaNO_2 is indicated by --◆--.

Table 5. Nitric oxide production and consumption in *Rhizobium etli*

Strain	Genotype	NO production ^a	NO consumption ^b
CE3	Wild-type	219.0 ± 15	42 ± 4
CFNX702	<i>nirK::loxP</i>	3.0 ± 0.1	Not measured
CFNX701	<i>norC::loxPSP</i>	557.2 ± 43	Not detected

^a NO produced in free-living cells after the addition of 30 μl of 50 mM NaNO_2 , expressed in nanomoles of NO per h per milligram of protein. Means from at least two independent determinations.

^b NO consumed in free-living cells after the addition of 100 μl of a saturated NO solution (1.91 mM), expressed in nanomoles of NO per h per milligram of protein. Means from at least two independent determinations.

functional Cu-containing nitrite reductase and a cytochrome *c*-type nitric oxide reductase, respectively. Previously, we demonstrated that *R. etli* fails to grow anaerobically with KNO₃ and lacks nitrate reductase activity (Bueno et al. 2005). In addition, we confirm by genome analysis that *R. etli* lacks the nitrate and the nitrous oxide reductases. As a consequence of the low efficiency of energy coupling using nitrite reduction, *R. etli* CFN42 displays a poor anaerobic growth at the expense of nitrite. Therefore, we propose that, in *R. etli* CFN42, the accumulation of toxic levels of nitric oxide is prevented by the NO reductase, acting in concert with the NO₂ reductase.

FixK and NnrR, two members of the CRP/FNR family, have been described as regulatory proteins for the expression of genes encoding reductases involved in denitrification. FixK is needed for activation of these genes under oxygen-limited conditions, while the response to the presence of N oxide depends of NnrR (Bedmar et al. 2005; Hartsock and Shapleigh 2010; Mesa et al. 2003; Robles et al. 2006). Recently, the participation of additional regulatory proteins for expression of these genes was reported. Among them, NifA is required in *B. japonicum* for a full expression of *nirK*, *norC*, and *napE* genes (Bueno et al. 2010), while *Agrobacterium tumefaciens* employs ActR and FnrR to control *nirK* and *norC* expression (Baek et al. 2008).

The novel operon arrangement reported here for *nirK* and one of its transcriptional regulators (*nnrR*) differentiates the way that *R. etli* regulates these genes. In the *norC* and *nirK* regulatory regions, we identified class II promoters in which an FNR regulator activates expression in response to low-oxygen concentrations (Wing et al. 1995). Expression analysis clearly shows that a functional FixKf protein is entirely required to activate, under low-oxygen conditions, *nirK* and *norC* promoters, while response to N oxides under microaerobiosis is mediated by NnrR. A differential dependence for NnrR was observed. Full expression of *norC* in response to N oxides under microaerobiosis requires the presence of FixK and NnrR. In contrast and similar to that described in *B. japonicum* (Mesa et al. 2003), microaerobic expression of *nirK* is still observed in an *nnrR* mutant background. Since *nirK* and *nnrR* are part of a single operon, this has the important consequence that NnrR controls its own N oxide-dependent expression. The signal required for the induction of the microaerobic expression of *nirK* mediated by NnrR is the production of NO. This regulatory arrangement in *R. etli* ensures that expression of *nirK* and *norC* is coupled to facilitate NO detoxification.

Three putative FNR sites were found in the *nirK* regulatory region. The observation that a *nirK* regulatory region with only ANB1 is highly expressed supports the idea that, in *R. etli*, the presence of more than one anaerobox has a repressive effect, as reported in other systems (Green et al. 2001). In addition, both ANB1 and ANB2 are target sites for other positive regulators, since a low expression level was observed when point mutations were introduced. Therefore, in *R. etli* CFN42, activation of *nirK* depends on FixKf, low oxygen conditions and

the FNR site ANB1. For maximum *nirK* expression, *nnrR* needs to be activated by NnrR when NO is present. Under these conditions, NnrR also activates *norC* to prevent NO accumulation. The removal of FixKf results in the loss of *nirK* expression, whereas in a *nnrR* mutant, levels of *nirK* are just halved. Interestingly, this mutant lacks any nitrite reductase activity. The dependence on NnrR for complete enzymatic activity may imply that this protein regulates some additional elements necessary for the nitrite reductase activity.

Additional members of the NnrR regulon in *R. etli* may be *cysG* and *azuPf*. It was reported that in *R. etli* CE3, the siroheme cofactor (product of the CysG activity) is required for nitrite reductase activity (Tate et al. 1997). The presence of a putative FNR-binding site in the *R. etli* *cysG* regulatory region (L. Girard, unpublished data) opens the possibility that this gene is also a NnrR target gene. Preliminary data from our laboratory suggest that the *azuPf* gene, encoding a blue copper pseudoazurin (a possible electron donor for the nitrite reductase), is also part of the NnrR regulon.

The structuring of regulatory circuits for controlling *nirK* and *norC* shows similarities between rhizobiales but also important differences. Common to all systems is the requirement of *nnrR*. A remarkable difference between *Agrobacterium* spp. and both *R. etli* and *B. japonicum* is the role of FixK as a regulator for *nirK* expression. Unlike *R. etli* and *B. japonicum*, FixK is not needed in *A. tumefaciens* for control of *nirK*, which is controlled by FnrN and ActR instead (Baek et al. 2008). Experiments to determine the involvement of ActR as an additional regulator of *nirK* in *R. etli* are considered for the future.

At very low oxygen concentrations, denitrification genes in *B. japonicum* need NifA for maximal expression (Bueno et al. 2010). In contrast, this regulator in *R. etli* has a negative effect on the expression of the *nirK* operon. In both cases, NifA appears to modulate by a factor of two the level of expression of *nirK*. It has been reported that the presence of both nitrite and a functional nitrite reductase affects oxygen respiration when nondenitrifying bacteria are growing in low-oxygen conditions (Toffanin et al. 1996). Furthermore, the binding of nitric oxide to cytochrome *c* oxidase can inhibit its activity (Brown and Cooper 1994). Therefore, we hypothesize that modulation of the *nirK* operon by NifA can be used in nondenitrifying bacteria, such as *R. etli*, to protect its symbiotic cytochrome oxidase operating during nitrogen fixation.

Nitric oxide participates in diverse plant processes as a signaling molecule. Several studies have shown the production of NO during different stages of the legume-rhizobia symbiosis (Baudouin et al. 2006; Cueto et al. 1996; Sánchez et al. 2010; Shimoda et al. 2005). Nevertheless, the exact source of NO in nodules is still controversial, with proposals that nodular NO comes as a product of bacterial denitrifying metabolism (Meakin et al. 2007; Sánchez et al. 2010) or may also be produced in plant root tissue from arginine via an unidentified NOS-like enzyme (Baudouin et al. 2006). In addition, in plant

Table 6. Nitrogenase activity and proportion of nitrosylhemoglobin (Lb-NO) in bean nodules formed by *Rhizobium etli* wild-type, *nirK*, and *norC* strains

Strain	Genotype	-KNO ₃		Lb-NO ^b	+KNO ₃	
		14 dpi ^a	25 dpi ^a		25 dpi ^a	Lb-NO ^b
CE3	Wild-type	38 ± 11	23 ± 8*	20 ± 3	13 ± 3*	66 ± 3
CFNX702	<i>nirK::loxP</i>	30 ± 13	25 ± 5	18 ± 3	31 ± 11	20 ± 2
CFNX701	<i>norC::loxPSP</i>	35 ± 8	31 ± 8*	15 ± 2	13 ± 5*	90 ± 5

^a Nitrogenase-specific activity determined at 14 and 25 days postinoculation (dpi), expressed in micromole ethylene per gram of nodule per h. Data are the average of nine control plants (-KNO₃) or nine plants watered with 4 mM KNO₃ for 12 days (+KNO₃). Asterisks (*) indicate that the means of the samples are different from the control plants at the 0.05 level ($P = 0.05$, Student's *t*-test).

^b Percentage of Lb-NO complexes was determined by UV-Vis spectra. Nodules were isolated at 25 dpi from control plants or plants watered with KNO₃ for 12 days. Data are the average of two independent assays.

cells, NO can be produced from nitrate via both nonenzymatic and enzymatic pathways, i.e., those catalyzed by nitrate reductase (Meyer et al. 2005) or by the plasma membrane-bound nitrite NO reductase (Stöhr et al. 2001). In the root nodules, as a result of the reducing conditions needed for nitrogen fixation, formation of the Lb-NO complex is believed to occur as a result of the reduction of exogenous nitrate. In this condition, NO binding may inhibit the oxygen-carrying activity of the leghemoglobin. It has also been observed that, as a result of the exposition of root nodules to high concentrations of nitrate, results in an increased yield of Lb-NO with a concomitant decrease in acetylene (ethylene) reducing activity. Thus, formation of Lb-NO as a result of nitrate reduction appears to have deleterious effects on the functional activity of root nodules (Mathieu et al. 1998; Sánchez et al. 2010).

In this study, we found that levels of Lb-NO in bean plants exposed to nitrate are increased in a *norC* mutant but decreased in a *nirK* mutant. These results are consistent with production of NO from the bacterial nitrite reductase and its elimination by the bacterial nitric oxide reductase in bean nodules. Important as the bacterial contribution to NO metabolism seems to be, it should be pointed out that it is not the only route to NO production. Even in a *nirK* mutant, low levels of Lb-NO were seen, indicating that other routes of unknown origin are participating in NO production. In contrast to our observations, in soybean nodules formed by *nirK* and *norC* mutants, the maximal formation of Lb-NO complexes require a physiological stress condition such as flooding in addition to nitrate. In this condition, nitrogenase activity in *nirK* nodules is not affected (Sánchez et al. 2010).

Interestingly, in bean nodules, the nitrate-induced decline in nitrogenase-specific activity observed in both the wild type and the *norC* mutant was not detected in the *nirK* mutant. This can be explained by the high levels of Lb-NO observed in the former two strains and the reduced levels of Lb-NO in the latter strain. Thus, these results reveal the commitment of these reductases to safeguard nitrogenase activity in *R. etli* from toxic levels of nitrogen reactive species.

Thus, results from our present work clearly demonstrate that, in *R. etli* CFN42, the product of *norC* is required in vivo to detoxify NO and that the balance between the activities of NirK and NorC determine the level of tolerance to NO. The particular genomic arrangement in *R. etli* of *nirK* and *nnrR* genes in a single operon suggests that NnrR controls its own expression in response to NO. Nitrite reductase activity and *norC* expression are totally dependent on NnrR. Therefore, gene expression in response to NO is tightly regulated in this bacterium to prevent deleterious effects by the expression of the nitrite reductase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used are listed in Table 7. *Rhizobium* strains were grown at 30°C in PY rich medium (Noel et al. 1984) or in Y minimal medium (MMY) with succinate (10 mM) and ammonium chloride (10 mM) as carbon and nitrogen sources, respectively (Bravo and Mora 1988). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. Antibiotics were added at the following concentrations: carbenicillin, 100 µg ml⁻¹ (*E. coli*); streptomycin (Sm), 200 µg ml⁻¹ (*R. etli*); gentamicin (Gm), 15 µg ml⁻¹ (*E. coli* and *R. etli*); kanamycin (Km) 30 µg ml⁻¹ (for both *E. coli* and *R. etli*); nalidixic acid 20 µg ml⁻¹ (*R. etli*); and spectinomycin (Sp), 100 µg ml⁻¹ (*E. coli* or *R. etli*). When required, sucrose was added at 12.5% (wt/vol). For detection of β-galactosidase and β-glucuronidase activity, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, respectively, were used in plates at a concentration of 20 µg ml⁻¹.

For growth under microaerobic conditions, cultures were grown to mid-exponential phase in PY medium. Cells were collected by centrifugation, were washed with sterile MMY, and were concentrated 100-fold. MMY containing 10 mM of succinate and 10 mM of ammonium chloride as carbon and nitrogen sources (20 ml) were inoculated with these suspensions at an initial optical density at 540 nm of 0.05. Cultures were incubated in 150-ml bottles closed with an airtight stopper and were flushed with several volumes of an oxygen and argon (1:99, vol/vol) mixture. Cultures were grown with shaking (200 rpm) for 10 h at 30°C (Granados-Baeza et al. 2007). When needed, cells were cultivated with 10 mM KNO₃, 30 µM or 1.0 mM NaNO₂, 100 µM SNP or 1 mM cPTIO (Invitrogen, Carlsbad, CA, U.S.A.); induction experiments were carried out by adding the corresponding N oxide to microaerobic cultures and incubating for 2 h more.

Microbiological and recombinant DNA methods.

Total and plasmid DNA isolation, digestion with restriction enzymes, cloning, agarose gel electrophoresis, and *E. coli* transformation were performed using standard protocols (Sambrook et al. 1989). Enzymes used for DNA restriction and modification were purchased from either Invitrogen or Fermentas (Vilnius, Lithuania) and were used according to the instructions of the manufacturer.

Conjugative transfer of plasmids from *E. coli* to *Rhizobium* spp. was done routinely by triparental crosses, using either pRK2013 or pRK2073 as helpers. For determination of plasmid profiles, a modified Eckhardt procedure was used (Hynes and McGregor 1990).

For Southern hybridizations, DNA was digested with appropriate restriction enzymes, electrophoresed in 1% (wt/vol) agarose gels, and blotted onto nylon (Hybond N+). Hybridization was carried out under high stringency conditions using Rapid-hyb buffer (Amersham, Bucks, U.K.). Specific probes were normally obtained by PCR and were labeled with α³²P-CTP by random priming, using Amersham's Rediprime system.

Enzymatic assays.

Nitrate reductase and nitrite reductase activities were determined by a colorimetric MV-dependent assay (Bueno et al. 2005; Delgado et al. 2003; Mesa et al. 2003). To prepare free-living cell suspensions, 20 ml of microaerobic cultures were spun down at 8,000 rpm, 4°C for 10 min, were washed in 50 mM Tris-HCl buffer until no nitrite was detected, and were resuspended in 1.2 ml of the same buffer. For determinations of Nir activity, aliquots of 200 µl of cell suspension (1 to 3 mg of protein) were added to a reaction mixture containing 20 mM Tris-HCl buffer (pH 7.5), 0.2 mM KNO₂, and 0.4 mM MV. The reaction was started by the addition of 50 µl of freshly prepared sodium dithionite solution (30 mg/ml) in 300 mM NaHCO₃. After incubation for 20 min at 30°C, the reaction was stopped by shaking, until the samples had lost their blue color. Control reactions were run at the same time, but the sodium dithionite was oxidized at the start of the reaction. Nitrate reductase reaction mixture contained 20 mM Tris-HCl buffer (pH 7.5), 20 mM KNO₃, 0.4 mM of MV, and 200 µl of cell suspension (1 to 3 mg of protein). The reaction was started by addition of 50 µl of freshly prepared sodium dithionite solution (8 mg ml⁻¹) in Tris-HCl buffer. After incubation for 30 min at 30°C, the reaction was stopped by vigorous shaking until the samples had lost their blue color. Nitrite was estimated by the diazotation reaction, adding equal amounts of sulphanilamide, naphthylethylene diamine dihydrochloride, and cell suspension. This mixture was then incubated at room

temperature for 20 min, measuring the absorbance at 450 nm of each sample. The amount of nitrite was determined from a standard curve of known nitrite amounts.

Quantitative β -glucuronidase activity was measured from MMY cultures of *R. etli* strains harboring transcriptional fusions as described previously (Granados-Baeza et al. 2007). After 10 h of growth, gene expression was induced using either 10 mM NaNO₃, 1.0 mM NaNO₂, or 100 μ M SNP. Quantitative β -glucuronidase activity was performed 2 h after induction on 1.0-ml culture samples, using as substrate 1.25 mM PNPG (4-nitrophenyl β -D-glucuronide) (Girard et al. 2000). Protein concentration was determined using the Bradford reagent or by Lowry assay over a second set of 1.0-ml samples. Specific activities are reported as nanomoles of product per minute per milligram of protein.

PCR amplification.

Specific PCR primers were designed using the Oligo 6.0 software and were purchased from Unidad de Síntesis Química IBT-UNAM (Cuernavaca, Mexico). PCR amplifications were done in a Mastercycler 5330 (Eppendorf, Hamburg, Germany). Accuprime high fidelity *Taq* DNA polymerase (Invitrogen) was used in PCR reactions with a cycling regime that includes a denaturing step at 94°C for 1 min, followed by 30 cycles of

94°C for 30 s, 45 to 60°C (according the primers' melting temperatures) for 30 s, and 72°C for 90 s. A final elongation step was made at 72°C for 5 to 10 min, according to the length of the DNA fragment to be amplified.

RT-PCR analysis.

Total RNA was purified from both wild type and *nirK* mutant strains (*nirK*:: Ω Km, strain CFNX699 or *nirK*::*loxP*, strain CFNX702) grown under microaerobic conditions using the High Pure RNA isolation kit (Roche, Branchburg, NJ, U.S.A.). Single-stranded cDNA synthesis was performed using the RevertAid H Minus first-strand cDNA synthesis kit (Fermentas). Specific primers for genes forming the *nirK* operon analysis were designed and used to obtain RT-PCR products. The PCR products were analyzed by electrophoresis in agarose gels.

5' RACE.

Transcriptional start sites for the *nirK* and *norC* operons were identified in assays using the 5' RACE system, version 2.0 (Invitrogen). scDNA was synthesized from 2 μ g of total RNA purified from microaerobic cultures of the wild-type strain derivatives carrying *nirK* and *norC* transcriptional fusions in plasmid pBBMCS53 (pNIC-01 and pNIC-03, respectively), using complementary primer gusLw (5'-ACAGGACGTAACA

Table 7. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Reference
<i>Rhizobium etli</i>		
CFN42	Wild type (type strain)	Quinto et al. 1982
CE3	Sm ^r derivative of CFN42	Noel et al. 1984
CFNX247	CE3 derivative, <i>nifAD</i> :: Ω Sp-Sm	Girard et al. 1996
CFNX637	CE3 derivative, <i>fixKf</i> :: Ω Km	Girard et al. 2000
CFNX699	CE3 derivative, <i>nirK</i> :: Ω Km	Bueno et al. 2005
CFNX700	CE3 derivative, <i>nnrR</i> :: <i>loxP</i>	This work
CFNX701	CE3 derivative, <i>norC</i> :: <i>loxP</i> Sp	This work
CFNX702	CE3 derivative, <i>nirK</i> :: <i>loxP</i>	This work
<i>Escherichia coli</i>		
DH5 α	<i>supE44lacU169(φ80lacZΔM15) hsdR17recA1endA1gyrA96 thi-1relA1</i>	Sambrook et al. 1989
HB101	<i>supE44 hsdS20 (r-gm-b) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Sambrook et al. 1989
BW21038	DE3 <i>lacX74 uidA (ΔMlu1)::pir⁺ miniTn5 Lac4 (Cm^r lacI^q lacZ ΔM15) recA1</i>	Metcalfe et al. 1996
Plasmids		
pSK ⁺	Cloning vector, Ap ^r	Stratagene
pCR2.1 TOPO	Cloning vector for polymerase chain reaction products, Ap ^r Km ^r	Invitrogen
pJQ200mp18	Suicide cloning vector, Gm ^r	Quandt and Hynes 1993
pK [*] <i>mobsacB</i>	Suicide cloning vector, Km ^r	Schäfer et al. 1994
pBBR1MCS5	Broad-host-range cloning vector, Gm ^r	Kovach et al. 1995
pBBMCS53	Δ <i>lacZ</i> pBBR1MCS-5 derivative, carrying the promoterless <i>uidA</i> gene, Gm ^r	Girard et al. 2000
pRK2073	Conjugation helper plasmid, Sm/Sp ^r	Better and Helinski 1983
pRK2013	Conjugation helper plasmid, Km ^r	Figurski and Helinski 1979
pRK415	Broad-host-range plasmid, Tc ^r	Keen et al. 1988
pTR102	Broad-host-range plasmid, Tc ^r	Weinstein et al. 1992
pNIC-01	pBBMCS53 derivative <i>norC</i> :: <i>uidA</i>	This work
pNIC-02	pBBMCS53 derivative <i>nnrS</i> :: <i>uidA</i>	This work
pNIC-03	pBBMCS53 derivative <i>nirK</i> :: <i>uidA</i> transcriptional fusion with 463-bp upstream NirK ATG, containing anaeroboxes 1, 2, and 3	This work
pNIC-04	pBBMCS53 derivative <i>nirK</i> :: <i>uidA</i> transcriptional fusion with 133-bp upstream NirK ATG, containing anaeroboxes 1 and 2	This work
pNIC-05	pBBMCS53 derivative <i>nirK</i> :: <i>uidA</i> transcriptional fusion with 103-bp upstream NirK ATG, containing anaerobox 1	This work
pNIC-06	pBBMCS53 derivative <i>nirK</i> :: <i>uidA</i> transcriptional fusion with specific mutations in anaerobox 1	This work
pNIC-07	pBBMCS53 derivative <i>nirK</i> :: <i>uidA</i> transcriptional fusion with specific mutations in anaerobox 2	This work
pGHN-01	pNIC-03 derivative with the complete <i>nirK</i> coding region	This work
pGHN-02	pNIC-06 derivative with the complete <i>nirK</i> coding region	This work
pGHN-03	pNIC-07 derivative with the complete <i>nirK</i> coding region	This work
pGHN-04	pTR102 derivative carrying the <i>nirK</i> gene under the <i>lacZ</i> promoter	This work
pGHN-05	pTR102 derivative carrying the <i>nnrR</i> gene under the <i>lacZ</i> promoter	This work
pGHN-06	pRK415 derivative carrying the <i>fixKf</i> gene under the <i>lacZ</i> promoter	This work
pGHN-07	pK [*] <i>mobsacB</i> derivative, <i>nnrR</i> :: <i>loxP</i> Sp	This work
pGHN-08	pK [*] <i>mobsacB</i> derivative, <i>norC</i> :: <i>loxP</i> Sp	This work
pGHN-09	pJQ200mp18 derivative, <i>nirK</i> :: <i>loxP</i> Sp	This work

^a Sm, Sp, Km, Cm, Ap, Gm, and Tc = streptomycin, spectinomycin, kanamycin, chloramphenicol, gentamicin, and tetracycline, respectively. ^r = antibiotic resistance.

TAAGGGACT-3') or *norCGSP1* (5'-CGAGGAAGTCCGCGA GAT-3'). After first-strand cDNA synthesis, the mRNA was removed by treatment with RNase and the scDNA was purified using a S.N.A.P. column. Terminal deoxynucleotidyl transferase and deoxycytidine triphosphate were used to add homopolymeric tails to the 3' ends of the cDNA. Nested PCR reactions were done using gene-specific primers *nirKGSP-2* (5'-AAGG ATCCACCTTCGGCCTTCTGCTGATG-3') (in positions 205 to 227) or *norCGSP2* (5'-AAGCTCGAGACGTGTTTCCC-3') (in positions 171 to 184) and the abridged anchor primer (AAP, GGCCACGCGTCGACTAGTACGGGIIIGGGIIIG, provided in the kit). The products were cloned into the pCR2.1 TOPO vector for sequencing.

Plasmid construction.

Specific mutagenesis of the *nnrR* gene was done by PCR amplification of the corresponding genomic region from strain CE3, using the specific primers UpDRnnrR-BamHI (5'-CGGG ATCCGACCCGCGCACCATTCTCATT-3', underlined, introduced *Bam*HI site) and LwDRnnrR-BamHI (5'-CGGGATCCG CAAACAGCAGCCACAGAACC-3', underlined, introduced *Bam*HI site). The 2,345-bp PCR product (containing the 3' end of *nirV*, the complete coding region for *nnrR*, and the 5' end of the *nnrU* gene) was cloned by T-A annealing into pCR 2.1-TOPO (Invitrogen). In this plasmid, a *nnrR::loxP*Sp deletion-substitution allele was generated by deleting a 49-bp sector (from nucleotide 233 to 282) by digestion with *Sma*I and inserting in its place a *Sma*I-*Sma*I *loxP*Sp cartridge. The fragment containing the *nnrR::loxP*Sp deletion-substitution allele was then removed by *Bam*HI digestion and was subcloned into *Bam*HI-restricted pK*mobsacB vector (Schäfer et al. 1994), giving rise to plasmid pGHN-07.

To introduce a mutation into the *norC* gene, a 1,475-bp product was obtained by PCR using total DNA from strain CE3 as template with the specific primers UpDRnorC-EcoRI (5'-CGG AATTCCATCTCATGACGACCTCTGG-3', *Eco*RI site underlined) and LwDRnorB-EcoRI (5'-CGGAATTCAGGTTGAC GGGTTGTAGAA-3', *Eco*RI site underlined). The *Eco*RI sites introduced by the primers were used to clone the fragment into the pSK+ vector (Stratagene, La Jolla, CA, U.S.A.). To construct a suicide plasmid useful for homogenotization, the *Eco*RI-*Xho*I fragment containing the *norC* allele was cloned into the *Eco*RI-*Sal*I sites in pK*mobsacB (Schäfer et al 1994). Mutagenesis of the *norC* gene was achieved by introducing into the *Bam*HI site (nucleotide 285) a *loxP*Sp interposon (Martínez-Salazar and Romero 2000), resulting in plasmid pGHN-08.

To introduce a mutation into the *nirK* gene, a 1,692-bp product was obtained by PCR using total DNA from strain CE3 as a template with primers UpnnrS-BamHI (5'-TTGGATCCTCG GAACTGCGGTGAGAAGGA-3', *Bam*HI site underlined) and LwDRnirK-BamHI (5'-ATGGATCCAGCAGCCACCTTTA GCGTAG-3', *Bam*HI site underlined). The *Bam*HI sites introduced by the primers were used to clone the fragment into the pSK⁺-1 vector (L. Girard, unpublished). Mutagenesis of the *nirK* gene was achieved by introducing into the *Eco*RI site (nucleotide 242 in the *nirK* coding region) a *loxP*Sp interposon (Martínez-Salazar and Romero 2000). To construct a suicide plasmid (pGHN-09) useful for homogenotization, the *Bam*HI fragment containing the *nirK::loxP*Sp allele, was cloned into the *Bam*HI site in pJQ200mp18 (Quandt and Hynes 1993).

To generate plasmids for expression analysis of the desired genes, the corresponding regulatory regions were cloned in the broad-host plasmid pBBRMCS53 (Girard et al. 2000) carrying a promoterless *uidA* gene. Plasmid pNIC-01, carrying a *norC::uidA* transcriptional fusion was constructed by cloning the *Eco*RI-*Xho*I 388-bp fragment synthesized by PCR with the specific primers NCUp-EcoRI (5'-TTAGAATTCCTCGGCCT

GCGT-3', *Eco*RI site underlined) and NCLw-*Xho*I (5'-AAGC TCGAGACGTGTTTCCC-3', *Xho*I site underlined). The intergenic region *nnrS-nirK* was obtained by PCR using total DNA from strain CE3 and specific primers (UpnnrS-BamHI, 5'-TT GGATCCTCGGAACTGCGGTGAGAAGGA-3' and LwnirK-BamHI 5'-AAGGATCCACCTTCGGCCTTCTGCTGATG-3', *Bam*HI sites underlined). The *Bam*HI 696-bp fragment was cloned in both directions in pBBMCS53 to obtain plasmids pNIC-02 and pNIC-03.

To construct transcriptional fusions containing deletions of the *nirK* upstream sequence, two PCR products were synthesized using primers UpnirK-gusM (5'-CAGGATCCAAGATG AGCCTTTGAACCTTG-3', *Bam*HI site is underlined) or UpnirK-gusS (5'-AAGGATCCAACAGCCAGTTTGCGCC AC-3', *Bam*HI site is underlined) with primer LwnirK-BamHI. Fragments of 360 bp (containing the 133-bp upstream NirK start codon) and 329 bp (containing the 103-bp upstream NirK start codon) were cloned separately in pBBMCS53, giving plasmids pNIC-04 (*nirK::uidA*-M) and plasmid pNIC-05 (*nirK::uidA*-S). The correct orientation of the fragments in the plasmids was verified by PCR using primer gusLw and the corresponding upper primer in each case.

Mutations of the putative FNR-binding sites (ANB1 and ANB2) in the *nirK* promoter region were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). ANB1 was modified from TTGCG-N4-GTCAA to ATAACG-N4-GTCAA (nucleotides changed are underlined). The sequence of ANB2 was modified from TTGTG-N4-CACAA to TTGTG-N4-GATAT (nucleotides underlined indicate the modifications). Both modified *nirK* regulatory regions were cloned in plasmid pBBMCS53 to obtain plasmids pNIC-06 and pNIC-07.

Four additional plasmids were constructed by cloning the complete *nirK* coding region downstream of the *nirK* regulatory regions present in plasmids pNIC-03, pNIC-06, and pNIC-07, to obtain plasmids pnirK-RRWT (pGHN-01), pnirK-ANB2m (pGHN-03), and pnirK-ANB1m (pGHN-02). Plasmids with *nirK* (pGHN-04), *nnrR* (pGHN-05), and *fixKf* (pGHN-06) genes constitutively expressed from the *lacZ* promoter were obtained by cloning the respective coding region in the conjugative plasmids pTR102 or pRK415.

R. etli mutant derivatives.

Introduction of all mutant alleles into *R. etli* was carried out by homogenotization using the *sacB* marker present in pJQ200mp18 and pK*mobsacB vectors; this marker allows an easy selection for homogenotization events by scoring for resistance to sucrose. A *R. etli* strain containing *nnrR::loxP*Sp, *norC::loxP*Sp (CFNX701), and *nirK::loxP*Sp alleles were constructed using plasmids pGHN-07, pGHN-08, and pGHN-09, respectively. Double recombinants were selected as Sm^rSp^rKm^r or Sm^rSp^rGm^r transconjugants. To verify that the desired gene replacements had occurred, both plasmid profiles as well as total DNA blots of all the derivatives were analyzed by Southern hybridization against the appropriate gene and cassette probes. Strains CFNX700 (*nnrR::loxP*) and CFNX702 (*nirK::loxP*) were obtained using the *cre-loxP* system on double recombinant strains carrying *nnrR::loxP*Sp and *nirK::loxP*Sp alleles (Martínez-Salazar and Romero 2000).

Evaluation of nitrogen-fixation capacity.

Three-day-old *Phaseolus vulgaris* cv. Negro Jamapa seedlings were inoculated with the desired strain and were grown in a greenhouse as previously described (Peralta et al. 2004). At 14 and 25 dpi, nine plants were analyzed for nitrogenase activity (by acetylene reduction) and nodule number as well as nodule and plant dry weight. Bacteria were isolated from nodules and their identities were verified by their antibiotic-resis-

tance patterns. When necessary, after 14 dpi, plants were watered with 4 mM KNO₃ for 12 days before harvesting.

Quantification of Lb-NO complexes in bean nodules.

For quantification of the percentage of Lb bound to NO within nodules, Lb extracts were prepared as reported previously (Meakin et al. 2007). Fresh nodules (0.3 g) were crushed in 4 ml of Lb extraction buffer (40 mM Na₂HPO₄·2H₂O, pH 7.4), 10 mM NaH₂PO₄·H₂O, pH 7.4, 0.02% K₃Fe(CN)₆, 0.1% NaHCO₃) and were centrifuged at 12,000 × *g* at 4°C for 20 min. The supernatant was collected and used for assays. Absorption spectra from 300 to 700 nm at 25°C were obtained from 1 ml of reduced and nitrosylated nodule extracts, using a Beckman DU 650 spectrophotometer. Absorption measurements were taken from the spectra to allow quantification of the nodular Lb-NO, following the protocol previously described by Meakin and associates (2007).

Measurement of NO production and consumption.

NO production and consumption in free-living cells was measured amperometrically using a NO electrode (Apollo 4000; World Precision Instruments, Inc., Sarasota, FL, U.S.A.) in a water-jacketed and magnetically stirred (30°C) chamber. The membrane-covered electrode was situated at the bottom of the chamber, above the stirrer. For experimental assays, the chamber was filled to 2 ml with 25 mM phosphate buffer (pH 7.4) and the cell suspension (0.2 to 0.3 mg of protein for NO production and 4 to 5 mg of protein for NO consumption). Oxygen present in the chamber was removed by incubating the cells for 5 min with glucose oxidase (to 4 U/ml), catalase (to 20 U/ml), sodium succinate (to 35 mM), and glucose (to 16 mM). To determine NO production by the cells, 30 µl of 50-mM NaNO₂ were added with a Hamilton syringe, and NO production was analyzed by adding 100 µl of a saturated NO solution (1.91 mM).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Kazusa DNA Research Institute Rhizobase database:
bacteria.kazusa.or.jp/rhizobase