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Abstract

Expression of the *Bradyrhizobium japonicum* napEDABC, nirK and norCBQD denitrification genes requires low oxygen (O₂) tension and nitrate (NO₃⁻), through a regulatory network comprised of two coordinated cascades, FixLJ-FixK2-NnrR and RegSR/NifA. To precisely understand how these signals are integrated in the FixLJ-FixK2-NnrR circuit, we analyzed β -Galactosidase activities from napE-lacZ, nirK-lacZ and norC-lacZ fusions and performed analyses of NapC and NorC levels as well as periplasmic nitrate reductase (Nap) activity, in *B. japonicum* wildtype and fixK2 and nnrR mutant backgrounds. While microoxic conditions (2% O₂ at headspace) were sufficient to induce expression of napEDABC and nirK genes and this control depends on FixK2, norCBQD expression requires, in addition to microoxia, nitric oxide gas (NO) and both FixK2 and NnrR transcription factors. Purified FixK2 protein directly interacted and activated transcription in collaboration with *B. japonicum* RNA polymerase from the napEDABC and nirK promoters, but not from the norCBQD promoter. Further, recombinant NnrR protein bound exclusively to the norCBQD promoter in an oxygen-sensitive manner. Our work suggest a disparate regulation of *B. japonicum* denitrifying genes expression with regard to their dependency to microoxia, nitrogen oxides (NO_x), and the regulatory proteins FixK2 and NnrR. In this control, expression of napEDABC and nirK genes requires microoxic conditions and directly depends on FixK2, while expression of norCBQD genes relies on NO, being NnrR the candidate which directly interacts with the norCBQD promoter.

Keywords	CRP/FNR transcription factors; FixK2-like box; NnrR; promoter; regulation; rhizobia; signal molecule
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Corresponding Author	Socorro Mesa
Corresponding Author's Institution	Consejo Superior de Investigaciones Cientificas
Order of Authors	Emilio Bueno, Eloy F. Robles, María J. Torres, Tino Krell, Eulogio J. Bedmar, María Jesús Delgado, Socorro Mesa
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Dr. Socorro MESA

Department of Soil Microbiology and Symbiotic
Systems
Estación Experimental del Zaidín
Consejo Superior de Investigaciones Científicas, CSIC
ES-18080 Granada
SPAIN

Telephone: +34 958 181600 (Ext. 201)

Fax: +34 958 129600

E-mail: socorro.mesa@eez.csic.es

To the
Editorial Office of *Nitric Oxide*

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Submission of manuscript for the special issue “Nitric Oxide in Plants” of the *Nitric Oxide* journal

Please find attached a manuscript entitled "**Disparate response to microoxia and nitrogen oxides of the *Bradyrhizobium japonicum* *napEDABC*, *nirK*, and *norCBQD* denitrification genes**" by Emilio Bueno, Eloy F. Robles, María J. Torres, Tino Krell, Eulogio J. Bedmar, María J. Delgado, and Socorro Mesa. We would be happy if this work could be considered for publication as full-length research article in the special issue “Nitric Oxide in Plants” of *Nitric Oxide*. If possible, considerer both María J. Delgado and Socorro Mesa as corresponding authors of this manuscript.

We hope you find our results interesting and suitable for publication in the *Nitric Oxide* journal and we are looking forward to your response.

With best regards,

Socorro Mesa

**Disparate response to microoxia and nitrogen oxides of the
Bradyrhizobium japonicum *napEDABC*, *nirK*, and *norCBQD*
denitrification genes**

Emilio Bueno^{a,1}, Eloy F. Robles^{a,2}, María J. Torres^a, Tino Krell^b, Eulogio J. Bedmar^a,
María J. Delgado^{a,*}, Socorro Mesa^{a,*}

^a Department of Soil Microbiology and Symbiotic Systems, and ^b Department of
Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de
Investigaciones Científicas, CSIC, E-18080 Granada, Spain

¹ Present address:

Department of Molecular Biology, Molecular Infection Medicine Sweden (MIMS),
University of Umea, Umea, Sweden

² Present address:

Division of Hemato-Oncology, Center for Applied Medical Research CIMA, University
of Navarra, IDISNA, Pamplona 31008, Spain

* To whom correspondence should be addressed:

Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del
Zaidín, CSIC, E-18080 Granada, Spain. Phone: +34 958 181600 (201/327). Fax: +34
958 129600

E-mail addresses:

emilio.bueno@umu.se (E. Bueno)

efrobles@unav.es (E.F. Robles)

mariajesus.torres@eez.csic.es (M.J. Torres)

tino.krell@eez.csic.es (T. Krell)

eulogio.bedmar@eez.csic.es (E.J. Bedmar)

mariajesus.delgado@eez.csic.es (M.J. Delgado)

socorro.mesa@eez.csic.es (S. Mesa)

ABSTRACT

Expression of the *Bradyrhizobium japonicum* *napEDABC*, *nirK* and *norCBQD* denitrification genes requires low oxygen (O₂) tension and nitrate (NO₃⁻), through a regulatory network comprised of two coordinated cascades, FixLJ-FixK₂-NnrR and RegSR/NifA. To precisely understand how these signals are integrated in the FixLJ-FixK₂-NnrR circuit, we analyzed β-Galactosidase activities from *napE-lacZ*, *nirK-lacZ* and *norC-lacZ* fusions and performed analyses of NapC and NorC levels as well as periplasmic nitrate reductase (Nap) activity, in *B. japonicum* wildtype and *fixK₂* and *nnrR* mutant backgrounds. While microoxic conditions (2% O₂ at headspace) were sufficient to induce expression of *napEDABC* and *nirK* genes and this control depends on FixK₂, *norCBQD* expression requires, in addition to microoxia, nitric oxide gas (NO) and both FixK₂ and NnrR transcription factors. Purified FixK₂ protein directly interacted and activated transcription in collaboration with *B. japonicum* RNA polymerase from the *napEDABC* and *nirK* promoters, but not from the *norCBQD* promoter. Further, recombinant NnrR protein bound exclusively to the *norCBQD* promoter in an oxygen-sensitive manner. Our work suggest a disparate regulation of *B. japonicum* denitrifying genes expression with regard to their dependency to microoxia, nitrogen oxides (NO_x), and the regulatory proteins FixK₂ and NnrR. In this control, expression of *napEDABC* and *nirK* genes requires microoxic conditions and directly depends on FixK₂, while expression of *norCBQD* genes relies on NO, being NnrR the candidate which directly interacts with the *norCBQD* promoter.

Keywords

CRP/FNR transcription factors, FixK₂-like box, NnrR, promoter, rhizobia, signal molecule

Abbreviations

BSA; bovine serum albumin; CRP/FNR, cyclic AMP receptor protein/fumarate and nitrate reductase regulator; DNR; dissimilatory nitrate respiration regulator; FPLC, fast pressure liquid chromatography; H-T-H, helix-turn-helix; IPTG, Isopropyl β-D-1-thiogalactopyranoside; ITC, isothermal titration calorimetry; IVT, *in vitro* transcription; LB medium, Luria Bertani medium; MV⁺-NR, methyl viologen-dependent nitrate reductase; Nap, periplasmic nitrate reductase; Nar, membrane-bound nitrate reductase;

66 NnrR; nitrite and nitric oxide reductase regulator; Nir, nitrite reductase; Nor, nitric
67 oxide reductase; Nos, nitrous oxide reductase; NOx, nitrogen oxides; OD₆₀₀, optical
68 density at 600 nm; PCR, polymerase chain reaction; PSY medium, Peptone-salts-yeast
69 extract medium; RNAP, RNA polymerase; SDS-PAGE, sodium dodecylsulfate
70 polyacrylamide gel electrophoresis; YEM medium, Yeast extract-mannitol medium;
71 WT, wildtype
72

73 **Highlights**

- 74 • Distinctive response of *B. japonicum* denitrification genes to microoxia and NOx
- 75 • FixK₂ activates directly transcription from *napEDABC* and *nirK* promoters
- 76 • NnrR specifically binds to the *norCBQD* promoter in an oxygen-sensitive mode

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1. Introduction

Denitrification is an alternative form of respiration in bacteria, archaea and some fungi, which consists in the dissimilatory reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to N_2 via the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O) under oxygen-limiting conditions with concomitant ATP generation. The reactions of denitrification are catalyzed by periplasmic (Nap) or membrane-bound (Nar) nitrate reductase, nitrite reductases (copper-containing NirK/cytochrome *cd*₁-containing NirS), nitric oxide reductases (cNor, qNor, or Cu_ANor) and nitrous oxide reductase (Nos) encoded by *nap/nar*, *nirK/nirS*, *nor* and *nos* genes, respectively. Reviews covering the physiology, biochemistry and molecular genetics of denitrification have been published elsewhere [1-6].

In the α -proteobacterium *Bradyrhizobium japonicum*, the nitrogen-fixing soybean endosymbiont, the four-step denitrification process depends on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes, encoding for Nap, copper-containing Nir, c-type Nor and Nos, respectively [7-10]; reviewed in Ref. [11]. In addition, associated soluble cytochromes such as cytochrome *c*₅₅₀, encoded by the *cycA* gene, are indispensable to support the electron flow during denitrification from the respiratory membrane-bound *bc*₁ complex to the NirK reductase [12].

As in other denitrifiers, maximal expression levels of the denitrification machinery in *B. japonicum*, require both, low oxygen (O_2) tension and the presence of nitrate (NO_3^-) [8, 9,10,13]. Denitrification in *B. japonicum* also depends on transcriptional regulators which are able to perceive and integrate these environmental signals into regulatory networks. In particular, the microoxic-responsive FixLJ-FixK₂ cascade, is involved in the activation of the *napEDABC*, *nirK*, and *norCBQD* genes, as suggested by the low expression levels of transcriptional fusions of each promoter region to the reporter *lacZ* gene found in both, the *fixLJ* and *fixK₂* mutant backgrounds [8,9,13]. Supporting these observations, a genome-wide transcription profiling of *B. japonicum* strains defective in *fixJ* and *fixK₂* showed that *napEDABC* and *nirK* genes are targets of both FixJ and FixK₂ [14].

A second regulatory cascade, RegSR-NifA, which responds to a tenfold lower O_2 concentration ($\leq 0.5\%$) is involved in the maximal induction of *B. japonicum* denitrification genes. Comparative transcriptome analyses of the *regR* mutant grown in anoxic denitrifying conditions together with protein-DNA binding studies carried out

with purified RegR protein showed that both *norCBQD* and *nosRZDYFLX* are probably direct targets of the response regulator RegR [15]. Further, the low O₂-responsive NifA protein is necessary for the maximal expression of *napE-lacZ*, *nirK-lacZ*, and *norC-lacZ* fusions under anoxic conditions in the presence of NO₃⁻ [16].

In the FixLJ-FixK₂ cascade, the FixK₂ protein acts as a key distributor of the microoxic signal perceived at the level of the superimposed two-component regulatory system FixLJ [14,17,18]. Hence, a moderate decrease in the O₂ concentration in the gas phase (≤5%) is sufficient to trigger ATP-dependent autophosphorylation of the deoxygenated FixL heme-based sensory kinase and subsequent transfer of the phosphoryl group to the response regulator FixJ [19,20]. Phosphorylated FixJ then induces the expression of several genes, including *fixK₂*. In turn, the FixK₂ protein activates the expression of hundreds of genes including, apart from the denitrification genes, the *fixNOQP* operon coding for the *cbb₃*-type high-affinity terminal oxidase needed for respiration under microoxia or other regulatory genes such as *rpoN_I*, *fixK_I* or *nnrR* [14]. In particular, the product of the latter, the NnrR (nitrite and nitric oxide reductase regulator) protein is also required for the maximal expression of the *nirK* and *norCBQD* promoters which occurs in denitrifying conditions [21]. By contrast, microoxic induction of the *napEDABC* or *nirK* promoters is retained in a *nnrR* mutant background [13,21], implying that the *napEDABC* or *nirK* and the *norCBQD* promoters exhibit slight differences with regard to their dependence on NnrR.

Both FixK₂ and NnrR belongs to the cyclic (cAMP) receptor protein (CRP) and the fumarate and nitrate reductase (FNR) regulator superfamily of transcription factors that mainly act as activators in a wide range of bacteria (reviewed in Ref. [22]). Despite their low sequence identity (<25%), these proteins share 4 functional domains: an N-terminal sensor domain, a β-barrel which makes contact with the RNA polymerase, a long α-helix for protein dimerization, and a DNA-binding domain with a helix-turn-helix (H-T-H) motif. In all cases studied, the active form consist of homodimeric proteins which generally bind to a 2-fold symmetric consensus DNA sequence located at distinct distances in the promoter region of regulated genes [23]. FixK₂ is a member of the FixK subgroup, proteins essential for the expression of genes required for the microoxic lifestyle in rhizobia, but differently as for the O₂-sensitive FNR proteins, lack the cysteine motif required to bind an [4Fe-4S]²⁺ cluster [22,24,25]. Instead, FixK₂ presents a single cysteine (C183) which is responsible for its oxidation-mediated posttranslational control [26]. Further, oxically purified FixK₂ does not apparently

require a cofactor for activation of targets genes in collaboration with *B. japonicum* RNA polymerase (RNAP) [27]. NnrR belongs to the NnrR clade [21,22,25], proteins that orchestrate the expression of the *nir* and *nor* gene clusters in order to keep the free concentrations of NO₂⁻ and NO below cytotoxic levels, e.g. *Rhodobacter sphaeroides* NnrR [28]. Another subgroup in the CRP/FNR family is the DNR-type proteins such as DNR from *Pseudomonas aeruginosa* [29,30], DnrD from *P. stutzeri* [31], and NNR from *Paracoccus denitrificans* [32,33]. These proteins cover similar function as the one defined for the NnrR-like proteins, however, while the DNR proteins regulate the expression of *cd₁*-type Nir encoding genes, the NnrR proteins control the expression of genes coding for copper-containing Nir enzymes. In this context, NO has been proposed as an additional key molecule that is involved in *nir* and *nor* genes regulation [1,3,34-36].

Transcription activation by CRP/FNR-type proteins require (i) direct contact between them and different parts of RNAP and (ii) binding to an imperfect palindromic DNA sequence with a consensus of AAATGTGA-N₆-TCACATTT (CRP box) or TTGAT-N₄-ATCAA (FNR box; [37,38]). The recently solved structure of FixK₂ in complex with its recognition sequence present at the promoter of the *fixNOQP* operon has allowed to determine the hallmark of FixK₂-DNA interaction and, therefore, to propose a refined consensus sequence for the FixK₂ box, TTG(A/C)-N₆-(T/G)CAA [39]. This sequence matches reasonable well with the consensus deduced from an alignment of the twelve FixK₂ box-associated promoter sequences approved by the FixK₂-dependent *in vitro* transcription (IVT) assay [14,40].

FixK₂-like boxes are present within the promoters of the *B. japonicum napEDABC* (TTGATCCAGATCAA), *nirK* (TTGTTGCAGCGCAA) and *norC* (TTGCGCCCTGACAA) genes [7-9]. Interestingly, only the *napEDABC*-associated FixK₂ box coincides with the consensus sequence. Furthermore, whether these promoters are direct targets for FixK₂ has not been yet investigated.

Despite the substantial knowledge on the external signals and cellular cues as well as regulatory proteins governing the denitrification process in *B. japonicum*, the response to different O₂ concentrations, the precise nitrogen oxides (NO_x) molecule triggering induction of each denitrifying gene, and the manner by which the FixK₂ and NnrR proteins control that process still remains unknown. The present work focused in expanding our understanding how microoxia (2% O₂), NO_x, and the regulatory proteins FixK₂ and NnrR control the expression of *B. japonicum napEDABC*, *nirK* and

norCBQD genes. Further, we also studied, for first time, the capacity of FixK₂ and NnrR proteins to directly interact and transcribe the *napEDABC*, *nirK* and *norCBQD* promoters regions by using IVT assays and isothermal titration calorimetry (ITC) experiments.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

Bacterial strains used in this work are listed in Table 1. *E. coli* cells were routinely grown in Luria Bertani (LB) medium [41] at 37°C. *E. coli* BL21 (DE3) cells for the overproduction of the FixK₂ and NnrR recombinant proteins were individually incubated at 30°C. Where needed, antibiotics were used at the following concentrations (in µg/ml): ampicillin, 200; kanamycin, 30; spectinomycin, 25; streptomycin, 25; tetracycline, 10.

Peptone-salts-yeast extract (PSY) medium [14,42] was used in routine oxic cultures of *B. japonicum*. For the β-Galactosidase activity determination, cells were grown oxically in PSY medium, collected by centrifugation (8.000 g for 10 min at 4 °C), and washed twice with Yeast extract-mannitol (YEM) medium [43]. Next, washed cells were used to inoculate, at an optical density at 600 nm (OD₆₀₀) of 0.2, 50-ml Erlenmeyer flasks containing 10 ml of YEM medium or 17-ml tubes containing 3 ml of YEM medium which were then incubated oxically or microoxically (2% O₂ in the headspace), respectively, for 24 h, and subsequently exposed for 5 h to different NO_x: 10 mM KNO₃, 1 mM NaNO₂, 100 µM NO, 2% N₂O. Given the short half-life of NO, a 5 h-period of incubation was chosen to analyze the effect of the different NO_x. Alternatively, oxic and microoxic cultures supplemented or not with 10 mM KNO₃ were incubated for 24 h after inoculation. The latter conditions were also used to prepare cells for heme-*c* proteins analysis and nitrate reductase determination, with the exception that the volume of the cultures was 200 ml of YEM medium in 1 l or 50 ml of YEM medium in 250 ml rubber stoppered bottles, respectively. In any case, in the microoxic cultures, the gas phase (2% O₂, 98% N₂) was exchanged every 8 to 16 h.

Antibiotics were added to the *B. japonicum* cultures at the following concentrations (µg/ml); chloramphenicol, 20; streptomycin, 200; kanamycin, 200; tetracycline, 100 (solid cultures), 25 (liquid cultures); spectinomycin, 200.

2.2. Plasmid construction

Plasmids used in this study are listed in Table 1. Primers sequences and their use are compiled in Table S1.

To overexpress a non-tagged NnrR protein variety, a 825-bp *NdeI*-*Bam*HI fragment from plasmid pBG0703 containing the *B. japonicum nnrR* gene was cloned into pET3a(+) (Novagen) to obtain plasmid pBG0704. The *NdeI* and *Bam*HI restriction sites were first introduced by engineering and the QuickChange method (Stratagene) in plasmids pRJ8819 and pBG0703, respectively.

Plasmids used as transcription templates were based on the plasmid pRJ9519 which contains a *B. japonicum rrn* transcriptional terminator [44]. The *napEDABC* template pRJ3322 contains a *Bam*HI-*Eco*RI restricted 297-bp fragment amplified by PCR with primers bsr7036_for_1 and bsr7036_rev_1. Plasmid pRJ8822 contains a *Bam*HI-*Eco*RI-digested 465-bp promoter fragment that was amplified by PCR using primers nirK3-for and nirK3-rev. The *norCBQD* promoter was PCR-amplified with primers norC2-for and norC2-rev, subsequently restricted with *Bam*HI and *Eco*RI enzymes, and finally cloned as a 367-bp fragment into pRJ9519, yielding plasmid pRJ8839.

The correct nucleotide sequences of all PCR-amplified fragments cloned into the corresponding plasmids were confirmed by sequencing.

2.3. β -Galactosidase activity determination

β -galactosidase activity was determined by using permeabilized cells from at least three independently grown cultures assayed in triplicate as previously described [41].

2.4. Heme-c protein analysis

Cell fractionation, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and heme-c proteins detection were performed as described earlier [7,16]. In brief, cells of *B. japonicum* were disrupted by three passages through an ice-cold French pressure cell (SLM Aminco) at about 120 MPa. Unbroken cells were removed by centrifugation (10,000 x g for 10 min at 4 °C). Membranes were prepared by further centrifugation of the supernatant at 140,000 x g for 2 h at 4 °C. The membrane pellet was washed once with 50 mM sodium-phosphate buffer (pH 7.0), resuspended in loading buffer [124 mM Tris-HCl, pH 7.0, 20% (v/v) glycerol, 5% (v/v) SDS and 50 mM 2-mercaptoethanol], incubated at room temperature for 10 min and electrophoresed

on a 12% SDS-PAGE gels at 4 °C. Proteins were transferred to a nitrocellulose filter and stained for heme-dependent peroxidase activity [45] using the chemiluminescence detection kit “SuperSignal” (Pierce). Protein concentration was estimated using the Bio-Rad assay (Bio-Rad Laboratories).

2.5. Determination of nitrate reductase activity

Methyl viologen-dependent nitrate reductase (MV⁺-NR) activity was analyzed essentially as described by Delgado et al. [7]. After 24 h of incubation under microoxic conditions in the presence and the absence of 10 mM KNO₃, cells were centrifuged and washed with 50 mM Tris/HCl buffer (pH 7.5) until no NO₂⁻ was detected at the supernatant. Then, cells were resuspended in 1 ml of the same buffer and kept on ice. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 200 μM MV, 1 mM KNO₃ and 50-100 μl of cell suspension (0.02-0.04 mg of protein). The reaction was initiated by the addition of 50 μl of freshly prepared sodium dithionite solution (30 mg/ml in 300 mM NaHCO₃) to the mixture. After incubation for 30 min at 30°C, the reaction was stopped by vigorous shaking until complete oxidation of the sodium dithionite. MV⁺-NR activity was estimated by measuring the NO₂⁻ produced from NO₃⁻ reduction by adding sulphanilamide and naphthylethylene diamine dihydrochloride [46].

2.6. Overproduction and purification of FixK₂ and NnrR proteins

Expression and purification of the functional N-terminal Histidine-tagged FixK₂ (His₆-FixK₂) protein were carried out as described in Mesa et al. [27].

Expression and purification of non-tagged NnrR was carried out as follows. *E. coli* BL21(DE3) cells carrying plasmid pBG0704 were grown in LB at 37 °C until they reached an OD₆₀₀ of 0.4. Then, cultures were incubated at 30 °C until cells reached an OD₆₀₀ of 0.6, where production of the recombinant protein was induced by the addition of 0.1 mM IPTG. After 2 h, cells were harvested (3.000 x g at 4 °C for 10 min), resuspended in 5 ml of buffer A containing 20 mM piperazine (pH 5.6) and 20 mM NaCl. The cells were disrupted by three passages through the French pressure cell, and the lysate was centrifuged at 30.000 x g at 4 °C for 60 min. The clear supernatant was loaded onto a 5-ml HiTrap MonoQ Fast Flow (Amersham) column and eluted with buffer A by fast pressure liquid chromatography (FPLC) using a LCC-500

chromatograph (Pharmacia). Protein-containing fractions were desalted and buffer exchanged by passing them through a prepacked G-25 M column (PD-10, Amersham) equilibrated with buffer B composed of 25 mM Tris-HCl (pH 7.0) and 20 mM NaCl. Proteins were loaded onto a HiTrap Heparin Fast Flow column (Amersham), and then eluted with a linear gradient of 0.02 to 1 M NaCl using FPLC. Eluted protein was analyzed by 12 % SDS-PAGE, and the gel was stained with Coomassie brilliant blue as indicated by Sambrook [47]. Eluted fractions of NnrR were pooled and kept in buffer B supplemented with 10% (wt/v) glycerol for protein storage at -70 °C. Protein concentrations were determined by using the Bio-Rad assay with bovine serum albumin (BSA) as standard. Protein concentrations used in this study refer to the dimeric state of the His₆-FixK₂ and NnrR proteins. For simplification, the prefix His₆-, will hereafter be omitted from FixK₂ protein designations.

2.7. *In vitro* transcription (IVT) activation assay

Multiple-round IVT assays were carried out as described previously [14,27,44]. Plasmids pRJ3322, pRJ8822 and pRJ8839 were used as templates to study the capacity of the FixK₂ protein to initiate transcription from the *napEDABC*, *nirK* and *norCBQD* promoters. The *fixNOQP* promoter-containing plasmid pRJ8816 [27] was applied as positive-control template. Purified FixK₂ protein was used in amounts of 1.25 or 2.5 μM.

Suitable RNA size markers were prepared *in vitro* with T3 RNA polymerase following the procedure used by Mesa et al [27]. Transcripts were visualized with a PhosphorImager and signal intensities were determined with the Bio-Rad Quantity One software (BioRad).

2.8. *Primer extension of in vitro-synthesized RNA*

The transcription start site of transcripts synthesized *in vitro* was determined with methods described previously [27,44]. Primer 9519-1 was used for primer extension with the *napEDABC* and *nirK* transcripts synthesized *in vitro* from plasmids pRJ3322 and pRJ8822, respectively. IVT assays were carried out as described above with the exception that no radiolabeled nucleoside triphosphate was used. After RNA precipitation, each reaction was resuspended in 7 μl of DEPC-treated H₂O. 2-10 μg of RNA were used for hybridization with labeled oligonucleotide (approximately 10⁵ cpm)

and 30 μ l hybridization buffer until a final volume of 40 μ l. The solution was heated at 85°C for 5 min and incubated overnight at 30°C. After ethanol precipitation, extension reactions were performed at 42 °C for 1 h. After phenol extraction followed by ethanol precipitation, extension products were loaded on 6% denaturing polyacrylamide gels adjacent to sequencing ladders generated with primer 9519-1 and plasmids pRJ3322 or pRJ8822 (approximately 200 ng each). The cDNA products were analyzed by using a PhosphorImager SF (Molecular Dynamics)

2.9. Isothermal titration calorimetry (ITC)

Three 25-bp double-stranded DNA fragments from the promoter region of each *B. japonicum* *napEDABC*, *nirK*, and *norCBQD* respectively, were used for these assays. Double strand DNA fragments were obtained by annealing stoichiometric mixtures of highly purified complementary oligonucleotides in DNA-binding buffer (10 mM Tris-HCl, pH 7.0, 250 mM NaCl, 10 mM magnesium acetate, 10 mM KCl, 5% [vol/vol] glycerol, 0.1 mM EDTA and 1 mM DTT). The mixture was then heated for 5 min at 95 °C and slowly cooled to room temperature. To verify the correctness of annealing, samples were analyzed on 4.5% (wt/vol) polyacrylamide gels. ITC was performed as described by Holdgate [48] on a VP microcalorimeter (MicroCal) equipped with a 1.437- μ l sample cell and a 300- μ l syringe, at 25 °C. Prior to analysis all samples were thoroughly dialyzed against binding buffer (25 mM Tris-HCl, pH 7.0, 100 mM NaCl). Essentially, 2 μ M DNA was placed into the ITC cell and titrated by the addition of 12.8 μ l aliquots of protein, typically at a concentration of 60 μ M. After peak integration and correction for dilution effects data were fitted by a nonlinear least-squares method (ORIGIN software; Microcal) to a function for the binding of a ligand to a macromolecule [49]. From the curve thus fitted, the reaction enthalpy (ΔH), binding constant (K_A , $K_A = 1/K_D$), and reaction stoichiometry (n) were determined. Changes in free energy (ΔG) and in entropy (ΔS) were calculated with the equation: $\Delta G = - RT \ln K_A = \Delta H - T \Delta S$, where R is the universal molar gas constant, and T the absolute temperature. For anoxic measurements, the sample cell was sparged with argon.

3. Results

3.1. The *napEDABC*, *nirK* and *norCBQD* promoters responded differently to microoxia and the presence of NOx

In order to investigate the effect of microoxia and NO_x on the expression of the *napEDABC*, *nirK* and *norCBQD* denitrifying genes, we examined their expression by using transcriptional fusions of their promoters to the *lacZ* reporter gene in cells incubated microoxically (2% O₂) in the absence or in the presence of different NO_x (NO₃⁻, NO₂⁻, NO and N₂O) for a 5 h-period.

Under microoxic conditions, the *napE-lacZ* fusion was highly induced as compared to oxic conditions (about 14-fold), but the presence of NO₃⁻ or any of its reduction products did not influence its transcription (Fig. 1A). Expression of the *nirK* gene was also induced by microoxia (about 3-fold) and the presence of either NO₃⁻, NO₂⁻ or NO increased it about 2.5-fold. However, the presence of N₂O did not induce the microoxic expression of *nirK* (Fig. 1B). In order to address the nature of the NO_x molecule that induces the expression of *nirK*, β-galactosidase activity from the *nirK-lacZ* fusion was analyzed in *napA* or *nirK* mutant strains which are unable to reduce NO₃⁻ to NO₂⁻ or NO₂⁻ to NO, respectively. As shown in Figure 1B, induction of *nirK-lacZ* fusion by NO₃⁻ was abolished in the *napA* mutant suggesting the requirement of NO₃⁻ reduction to NO₂⁻ on *nirK* gene expression. By contrary, induction of *nirK* gene expression in the presence of NO₂⁻ was retained in the *nirK* mutant confirming the implication of NO₂⁻ and not of NO, on the expression of the *nirK* gene. However, NO was also able to induce *nirK* expression to similar levels as NO₂⁻ (Fig. 1B). It might therefore be possible that in addition to NO₂⁻, NO chemically formed from NO₂⁻ that is accumulated in the *nirK* mutant [9] is also involved in *nirK* expression.

In contrast to *napEDABC* and *nirK* genes, expression of the *norC-lacZ* fusion was not induced by microoxic conditions compared to oxic conditions, however, the presence of NO₃⁻, NO₂⁻ or NO increased it by about 12-fold (Fig. 1C). Similarly to *nirK-lacZ* response, the presence of N₂O did not induce microoxic expression of the *norC-lacZ* fusion (Fig. 1C). Induction of the *norC-lacZ* expression was impaired in the *napA* and *nirK* mutants incubated in the presence of NO₃⁻ and NO₂⁻, respectively, (Fig. 1C). These results discard the implication of both NO₃⁻ and NO₂⁻ in *norC-lacZ* expression and suggest that NO might be the NO_x involved in the induction of *norCBQD* genes. The impairment of *norC* expression, in both *napA* and *nirK* mutant backgrounds was also confirmed at protein levels by heme-*c* staining assays (Fig. 1D), based on the presence of a heme-*c* group within the NorC subunit from the membrane-bound Nor. The heme-stainable band of approximately 16 kDa, which is very prominent

in membranes isolated from wild-type cells grown microoxically in the presence of nitrate, was absent in both *napA* and *nirK* mutants.

Taken together, these results demonstrate a disparate response of *B. japonicum* *napEDABC*, *nirK* and *norCBQD* to microoxia and the presence of NO_x. While microoxic conditions are sufficient to induce expression of *napEDABC* and *nirK*, the *norCBQD* genes also require a NO_x, being NO the candidate molecule which triggers their maximal expression.

3.2. Distinctive control of *FixK₂* and *NnrR* proteins on *napEDABC*, *nirK* and *norCBQD* gene expression

In *B. japonicum*, perception and transduction of the microoxic signal are mediated by two interlinked O₂-responsive regulatory cascades, the FixLJ-FixK₂-NnrR and the RegSR-NifA [18]; reviewed in Refs. [1,3,50]. A moderate decrease in the O₂ concentration in the gas phase ($\leq 5\%$) is sufficient to activate expression of FixLJ-FixK₂-dependent targets [18]. In order to investigate how FixK₂ and NnrR control the microoxic expression of *napEDABC*, *nirK*, and *norCBQD* genes, we analyzed β -Galactosidase activity from the *napE-lacZ*, *nirK-lacZ*, and *norC-lacZ* transcriptional fusions in the wildtype (WT) and $\Delta fixK_2$ and $\Delta nnrR$ strains, incubated for 24 h oxically, and microoxically (2% O₂) in the absence and the presence of 10 mM of KNO₃. The pattern of expression of the three fusions, although more pronounced, was rather similar to that found after 5 h-incubation (Fig. 2A-C, WT). Microoxic induction of both the *napEDABC* and *nirK* promoters was completely abolished in the absence of a functional *fixK₂* gene, however, this was retained in the $\Delta nnrR$ strain (Fig. 2A and B), suggesting that microoxic expression of *napEDABC* and *nirK* genes depends on FixK₂ but not on NnrR. However both FixK₂ and NnrR resulted to be essential for the maximal induction of *nirK-lacZ*, and *norC-lacZ* in response to NO₃⁻ under microoxic conditions (Fig. 2B and C).

The differential dependency of *napEDABC* and *norCBQD* expression on FixK₂ and NnrR was also confirmed at protein level by heme-staining analysis (Fig. 2D). The heme-stainable band of about 25 kDa corresponding to NapC from the Nap, was found to be quite prominent already in microoxic conditions (Fig. 2D, lane 1) and the presence of NO₃⁻, did not significantly increased its expression (Fig. 2D, lane 2). This correlated with the values of the MV⁺-NR in the WT (Fig. 2E), that were similar independently on

the presence or not of NO_3^- in the incubation medium. In line with the expression pattern of the *napE-lacZ* fusion, expression of NapC (Fig. 2D, lanes 5 and 6) as well as MV^+ -NR activity (Fig. 2E) were severely impaired in the ΔfixK_2 strain after incubation under microoxic conditions independently of the presence of NO_3^- . However, NapC was found to be expressed at comparable levels to the WT in the ΔnnrR strain, when cells of both strains were incubated microoxically (Fig. 2D, lane 3). The presence of NO_3^- in the medium slightly reduced (about 1.5-fold) the expression of the *napE-lacZ* fusion (Fig. 2A) as well as the NapC protein (Fig. 2D, comparison of lanes 2 and 4) in the ΔnnrR strain. Similarly, the values of MV^+ -NR in the ΔnnrR were slightly reduced (about 25%) compared to those observed in wild-type cells cultured under microoxic conditions with NO_3^- (Fig. 2E). This decrease is probably due to the accumulation of NO observed in the headspace of ΔnnrR cultures incubated microoxically with nitrate (2-3 μM) compared to wild-type cultures where NO was not detected (Bueno et al., unpublished).

By contrast to NapC, expression of NorC was not influenced under the same experimental settings (Fig. 2D, lanes 1 and 2). As expected, microoxia and the presence of NO_3^- were needed for the maximal expression of the 16-kDa band corresponding to NorC (Fig. 2D, lane 2) and both FixK_2 and NnrR were absolutely required (Fig. 2D, lanes 3-6). This confirmed that *napEDABC* and *norCBQD* gene expression exhibit substantial differences with regard to their dependence on microoxia and NO_3^- as well as on FixK_2 and NnrR .

3.3. *FixK₂* drives transcription from the *napEDABC* and *nirK* promoters, but not from the *norCBQD* promoter

In order to investigate whether FixK_2 could have a direct role on *napEDABC*, *nirK* and *norCBQD* activation, we monitored RNA synthesis by multiple round IVT. The three promoters were individually cloned into the template plasmid pRJ9519 [44], which carries a *rrn* terminator, yielding plasmids pRJ3322, pRJ8822 and pRJ8839, respectively. In these experiments, purified FixK_2 [27] and RNAP from *B. japonicum* [44] were used. Without FixK_2 , *B. japonicum* RNAP was unable to transcribe any of the three promoters efficiently (Fig. 3A-C, lanes 4, 7, and 10), whereas it produced a vector-encoded transcript of 107 nucleotides that served as an internal reference. As positive control, we used a plasmid harbouring the *fixNOQP* promoter (pRJ8816), that

as described previously [27], it is transcribed *in vitro* by FixK₂ producing a transcript of about 243 nucleotides (Fig. 3A, lane 3). In the presence of different concentrations of FixK₂ (1.25, and 2.5 μM), *B. japonicum* RNAP transcribed the *napEDABC* promoter efficiently producing a transcript of about 305 nucleotides (Fig. 3A, lanes 5 and 6, respectively).

We also analyzed whether FixK₂ can activate RNA synthesis from the *nirK* promoter in collaboration with *B. japonicum* RNAP. Similarly as the *napEDABC* promoter, transcription from the *nirK* promoter was activated by FixK₂ producing a weak, but theoretically expectable transcript of 240 nucleotides (Fig. 3B, lanes 8 and 9).

In contrast to the positive IVT activation mediated by FixK₂ from the *napEDABC* and *nirK* promoters, we could not detect a FixK₂-specific transcript from the *norCBQD* promoter, despite the presence of different concentrations of FixK₂ in the assay (Fig. 3C, lanes 11 and 12), although the 107 nucleotides control transcript could be observed (Fig. 3C, lanes 10-12).

To test the fidelity of the IVT results, we determined the 5' end of the transcripts individually generated by FixK₂-dependent IVT from the *napEDABC* and *nirK* promoters and compared them with that of the corresponding *in vivo* transcripts [7,16,51]. RNA synthesized *in vitro* and purified FixK₂ protein (1.25 μM) was used for primer extension with the 9519-1 primer [27] that hybridized within the backbone of the templates. The extension product was run on a sequencing gel next to a sequence ladder generated with the same oligonucleotide and plasmids pRJ3322 and pRJ8822, for the *napEDABC* and *nirK* templates, respectively. As shown in Fig. 3D, the 5' end of the *in vitro* synthesized transcript from the *napEDABC* promoter was located at 41.5 bp from the axis of symmetry of the FixK₂ binding site TTGAT-N₄-ATCAA, confirming the transcript of 305 nucleotides obtained in the IVT assay, which also corresponds to the transcriptional start site identified by Čuklina and co-workers [51]. In the case of the *nirK* promoter, the 5' end of the *in vitro* synthesized transcript (Fig. 3E) was found to be identical to the start site of the *in vivo* transcript, located at 40.5 bp from the axis of symmetry of the FixK₂ binding site TTGTT-N₄-CGCAA [16], confirming the presence of the product obtained in the IVT experiments.

3.4. *NnrR* binds to *norCBQD* but not to *napEDABC* and *nirK* promoters

IVT experiments showed that FixK₂ activates directly the transcription of *napEDABC* and *nirK* promoters in collaboration with *B. japonicum* RNAP but not of the *norCBQD* promoter. Further, *nirK* and *norCBQD* gene expression is reduced in the $\Delta nnrR$ strain grown microoxically with NO₃⁻ in comparison to the WT grown in the same conditions. Since NnrR is also a CRP/FNR-type protein, we checked the capacity of NnrR to bind to the FixK₂-like box present at *napEDABC*, *nirK* and *norCBQD* promoters by using ITC. Thus, double-stranded oligonucleotides (35 bp) containing the respective putative FixK₂-like boxes within each promoter were subjected to microcalorimetric titrations experiments with recombinant NnrR protein. Under oxic conditions, the titration profile with any of the three promoters resulted only in small, uniform and exothermic peaks which represented exclusively heats of dilution and not heats of binding (Fig. 4A). Similar results were obtained when titrations of either *napEDABC* or *nirK* promoters were repeated at anoxic conditions (Fig. 4B). However, when the *norCBQD* promoter DNA fragment was titrated in anoxic conditions, endothermic heat changes were observed (Fig. 4B). The analysis of these data with the one-binding site model (ORIGEN, Microcal) shows that the binding is driven by favourable entropy changes ($T\Delta S = 24.5 \pm 0.7$ kcal/mol) which are counterbalanced by an unfavourable enthalpy term ($\Delta H = 16 \pm 0.7$ kcal/mol) (Fig. 4C). Entropy-driven binding processes are frequently found for protein-DNA interactions and are thought to be mainly due to the displacement of DNA-bound ordered water into the bulk-solvent [52]. Furthermore, a dissociation constant (K_D) of 625 ± 80 nM, and a binding stoichiometry of 1 NnrR dimer per DNA fragment was obtained.

4. Discussion

The main focus of the present work was to contribute to the better understanding about the role of the FixK₂ and NnrR proteins in the control of *napEDABC*, *nirK* and *norCBQD* genes expression in response to microoxia and NO_x.

In *B. japonicum* as in many others denitrifiers, expression of the denitrifying machinery requires low O₂ conditions and the presence of NO₃⁻. However, in this work we found that NO₃⁻ is not required for the expression of the *napEDABC* genes in microoxic conditions. Hence, microoxia is sufficient to induce expression of a *napE-lacZ* fusion and NapC protein after 24 h incubation in the absence of NO₃⁻ (Figs. 1 and 2). In fact, when *B. japonicum* cells were incubated only for 5 h, the effect of NO₃⁻ on

napE-lacZ expression was negligible (Fig. 1). Contrary to our results, Robles and co-workers [13] observed that β -galactosidase activity from of a *napE-lacZ* fusion was 4-fold less in anoxic conditions in the absence of NO_3^- compared to those with NO_3^- . These differences could be due to the experimental conditions applied. In contrast to our conditions, where cells were incubated for a short period of time (5 or 24 h) and the gas phase (2% O_2) was replaced every 8-16 h, in Robles and co-workers work, the vials were filled-up with medium and subsequently incubated for 96 h. Therefore, in such experiments, cells might suffer of non-viable anoxia entrapment due to the O_2 limitation that is consumed rapidly by the cells.

Expression of the *nap* genes among different organisms is very variable. Similarly to that found in *B. japonicum*, expression of *nap* genes is induced microoxically, irrespective of the presence of NO_3^- in *Agrobacterium tumefaciens* [53]. In other bacteria such as the non-denitrifier *R. sphaeroides* DSM158 [54], but also in the denitrifiers *R. sphaeroides* 2.4.1 [55], *R. sphaeroides* 2.4.3 [56], *R. sphaeroides* IL106 [57], *P. pantotrophus* [58], *P. denitrificans* [59] and *Ralstonia eutropha* [60], *nap* genes are induced under oxic conditions, mostly of them independently of the presence of NO_3^- . However in these bacterial species, the Nap enzyme plays a role in cellular redox homeostasis. Since the environmental conditions at soil are rapidly fluctuating, activation of *nap* gene transcription in response to lowered O_2 concentrations in the absence of NO_3^- could be envisaged as a mechanism to provide cells with the machinery necessary to make a transiently efficient use of NO_3^- when O_2 become temporary scarce to support oxic respiration. This therefore might prevent anoxia entrapment of the cells.

FNR-type proteins such as FNR from *E. coli* and *B. subtilis*, Anr from *P. aeruginosa* and FnrP from *P. denitrificans* are able to sense O_2 at low concentrations and activate expression of genes encoding for dissimilatory nitrate reductases (Nar and Nap) [61-64]. These proteins possess an N-terminal cysteine-feature which bind $[\text{4Fe-4S}]^{2+}$ clusters. By contrary, in *B. japonicum*, the CRP/FNR-type protein FixK₂ acts as a distributor of the low- O_2 signal perceived at the level of the FixL sensor protein [14,17].

In this work, we found that microoxic induction of the *napEDABC* genes depends on FixK₂, but not on NnrR, probably due to its nitrate-independent expression. The *napEDABC* gene cluster is indeed a direct target of FixK₂, as shown in IVT transcription experiments carried out with oxically purified protein in collaboration with *B. japonicum* RNAP. However, NnrR was unable to interact with the FixK₂-like box present at the *napEDABC* promoter (Fig. 4). Therefore, the diminished expression of the

napE-lacZ fusion and the NapC protein observed in the *nnrR* mutant background under microoxic conditions in the presence of NO_3^- (Fig. 2) may be a consequence of accumulation of toxic concentrations of NO in a *nnrR* mutant strain (Bueno et al., unpublished).

Likewise to that found for *napEDABC* genes, we observed a significant microoxic induction of *nirK*, which is dependent on FixK₂ (Figs. 1 and 2). However, maximal expression of *nirK* was achieved in the presence NO_3^- which depends on NnrR (Figs. 1 and 2). Remarkably, NO_2^- and/or NO were able to induce *nirK* expression to similar levels as NO_3^- . The requirement of NO_2^- for maximal expression of genes encoding the respiratory Nir enzymes is not unprecedented, as reported for *Neisseria gonorrhoeae* AniA- and *P. stutzeri* NirS-encoding genes [65].

In many denitrifiers, expression of *nir* genes, requires low O₂ tension and the presence of a NO_x, and this control depends on DNR-type or NnrR-type regulators [66-70]. However, as we observed in *B. japonicum*, *nir* expression is induced in response to low O₂ with independency on the presence of a NO_x, in *A. tumefaciens* [66,71], *R. sphaeroides* 2.4.3 [68,72], and *P. aeruginosa* [67]. In these bacteria, in a similar manner to *B. japonicum* FixK₂, FNR-like and/or RegR-like regulators are involved in such regulation. However, while in *P. aeruginosa* depends on the FNR-type protein ANR [67], *R. sphaeroides* 2.4.3 also requires NnrR in addition to the RegR-type protein PrrA [68,72]. A more complex scenario is that found in *A. tumefaciens*, where FnrN (FNR-like), ActR (RegR-like) and NnrR proteins are required for the microoxic induction of *nirK* [66,71].

Similarly as observed for the *napEDABC* genes, *nirK* is a direct target for FixK₂, as we detected a weak, but FixK₂-specific transcript in the IVT activation assays. In order to test whether the presence of NnrR might have a positive effect of *nirK* transcription, we also carried out IVT experiments where both FixK₂ and NnrR proteins were present in the reaction. However, the same transcription efficiency of the *nirK* promoter as we found for FixK₂ alone was detected when purified NnrR was added (data not shown). Since NnrR was unable to interact with the double-stranded DNA containing the FixK₂-like box present at the *nirK* promoter region (Fig. 4), NnrR dependency of *nirK* expression (Fig. 2) might be not direct, but mediated by an additional transcriptional factor, which depends on NnrR. We have previously reported that maximal expression of the *nirK* gene depends on the O₂-sensitive protein NifA [16]. Thus, a plausible scenario could be that NifA could interact and drive transcription from the *nirK*

promoter in concert with NnrR. Unfortunately, purification of NifA seemed to be recalcitrant and we could not therefore address a putative co-requirement of NifA for NnrR-*nirK* promoter interaction.

In contrast to that found for *napEDABC* and *nirK* genes, *norC-lacZ* and NorC expression was unresponsive to microoxic conditions (Figs. 1 and 2). Hence, induction was achieved in microoxic conditions in the presence of either NO_3^- , NO_2^- or NO, being the latter the signal molecule that induce *norCBQD* expression in *B. japonicum*. The mutual dependency on NO and NnrR for the expression of *norCBQD* genes suggest a role for NnrR as an NO-responsive transcriptional activator. However, experimental evidences for such a function are still lacking. Similar dependency of *nor* gene expression on NO and NnrR-like or DNR-like proteins has been previously reported in *R. sphaeroides* [28,73], *P. denitrificans* [32,74], *P. aeruginosa* [75], and *P. stutzeri* [70]. In particular, *P. aeruginosa* DNR is able to bind NO *in vitro* through a ferrous heme cofactor group and activate *nor* gene expression *in vivo* [30,76]; reviewed in Ref. [77].

Differently as we observed for *napEDABC* and *nirK* genes, FixK₂ was unable to initiate transcription from the *norCBQD* promoter in our IVT assays. However, ITC experiments demonstrated unequivocally that one double-stranded *norC* promoter DNA molecule binds to an NnrR dimer exclusively under anoxic conditions. However, FixK₂ is also necessary for *nor* expression suggesting an indirect control of this regulator. In fact, it has been previously demonstrated the dependency of *nnrR* expression on FixK₂ [21]. In this work, we have demonstrated the FixK₂-NnrR hierarchy on *norCBQD* expression at molecular level.

Whereas CRP/FNR-type proteins do require co-regulators, oxically purified FixK₂ was shown to activate transcription *in vitro* of twelve promoters in the absence of cofactors [27]. In this work, FixK₂-mediated transcription from *napEDABC* and *nirK* promoters occurred also under oxic conditions, increasing the number of direct target for FixK₂ to fourteen in total. Indeed, when the IVT experiments were performed under anoxic conditions, we did not observe a positive effect on specific transcription of *napEDABC*, *nirK* or *norCBQD* promoters (data not shown). Therefore, it seems than anoxic conditions are neither needed nor helpful for FixK₂ activity *in vitro*.

We also analyzed transcription from *napEDABC*, *nirK* and *norCBQD* promoters by NnrR in an IVT assay, similar as the one we have established for FixK₂. In contrast to that found for FixK₂, NnrR did not transcribe any of the promoters efficiently in collaboration with the *B. japonicum* RNAP (data not shown). Neither anoxic conditions

in the induction and purification of NnrR nor the presence of NO in the anoxic reactions resulted in a specific NnrR-dependent transcript, although we could detect the control transcript used as reference. Therefore, anoxic conditions are sufficient for NnrR-DNA interaction (as it shown in the ITC experiments), but not for NnrR activation (either the NnrR protein lacks an unknown cofactor or it is not in its proper conformation to drive transcription).

The *napEDABC*, *nirK* and *norCBQD* promoters individually harbour a FixK₂-like binding site, which *a priori*, both FixK₂ and NnrR proteins could bind. However, we observed a preferential interaction of FixK₂ to *napEDABC* and *nirK* promoters, and an anoxic-specific NnrR-*norCBQD* promoter binding. The molecular determinants that define the discriminatory binding of FixK₂ or NnrR to DNA could derive from differences at the protein level or/and the DNA recognition sequence (so called FixK₂ box or NnrR box).

Amino acid residues involved in specific interaction with DNA are located in the DNA recognition helix (α F) of the H-T-H DNA binding motif of the CRP/FNR proteins. Three charged residues, R180, E181, and R185, of CRP make specific contacts with the CRP box, whereas the FNR residues E209, R213, and S212 interact with defined nucleotides of the FNR box [38]. In FixK₂, L195, E196 and R200 interact with specific positions within the FixK₂ box [39], while the consensus recognition helix for DNA binding of NnrR-like regulators includes a conserved HXXSR motif. Thus, R180 of CRP, S212 of FNR, and L196 of FixK₂ provide the discriminatory contacts between the regulators and their respective targets. However, details on the recognition mechanism underlying the interaction of *B. japonicum* NnrR-DNA still remains elusive.

By inspection of aligned FixK₂-like boxes present at the *napEDABC*, *nirK* and *norCBQD* promoters (Fig. S1), we found that 3 particular nucleotides (T5, A8, G9) are invariants in the *napEDABC*- and *nirK*- associated boxes, which differs to the FixK₂-like box at the *norCBQD* promoter (Fig. S1). Whether or not these differences may define the specificity of FixK₂ and NnrR for DNA binding to the *napEDABC*, *nirK*, and *norCBQD* promoters, opens a compelling line for future investigation, which is beyond of the scope of this paper.

Altogether, our results sharpen the regulatory control of the *B. japonicum* denitrifying *napEDABC*, *nirK* and *norCBQD* genes by the FixK₂ and NnrR transcriptional factors in response to microoxic conditions and the presence of NO_x, and suggest the existence of a disparate regulation of *napEDABC*, *nirK* and *norCBQD*

denitrifying genes. While microoxia and FixK₂ are required for the induction of *napEDABC* and *nirK* expression, *norCBQD* expression needs both microoxia and NO and at least the two regulatory proteins FixK₂ and NnrR. Further, we were able to show that FixK₂ activates directly transcription from *napEDABC* and *nirK* promoter, while NnrR specifically binds to the *norCBQD* promoter.

Statement of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper, including any financial, personal or other relationships with other organizations.

Author's contributions

EB, MJD and SM conceived and designed the study. EB, EFR, MJT and SM performed experiments. EB, EFR, MJT, TK, MJD and SM analyzed the data. EB, MJD and SM wrote the manuscript. TK and EJB critically revised the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version at <http://xxx>

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Figure captions

Fig. 1. Disparate response to microoxia and the presence of NO_x of *napEDABC*, *nirK* and *norCBQD* expression. β-Galactosidase activity from *napE-lacZ* (A), *nirK-lacZ* (B) and *norC-lacZ* (C) fusions in the parental *B. japonicum* (WT), and mutant strains *napA* and *nirK*. Oxically grown cells were collected and incubated microoxically (2% O₂) for 24 h. Next, different NO_x: 10 mM KNO₃ (dark gray bars), 1 mM NaNO₂ (dotted black bars), 100 μM NO (black bars) and 2% N₂O (striped black bars) were added or not (light gray bars) to the microoxic cultures that were incubated for another 5 h. Values are the means ± standard deviations of three biological replica assayed in triplicates. (D) Heme-stained proteins in membranes prepared from parental *B. japonicum* (WT), and *napA* and *nirK* mutant strains. Cells were incubated microoxically in YEM medium with NO₃⁻ during 24 h. The 16 kDa cytochrome corresponds to the NorC membrane-bound *c*-type cytochrome. Each lane contains about 25 μg membrane proteins.

Fig. 2. Disparate control of *napEDABC*, *nirK* and *norCBQD* expression by the regulatory proteins FixK₂ and NnrR in response to microoxia and the presence of NO₃⁻. β-Galactosidase activity (means ± standard deviations of three biological replica) from *napE-lacZ* (A), *nirK-lacZ* (B) and *norC-lacZ* fusions (C) in the parental *B. japonicum* (WT), and strains Δ*nnrR*, and Δ*fixK*₂. After collecting oxically grown cells, they were incubated oxically (white bars) or microoxically (2% O₂) in YEM medium during 24 h in the absence (light gray bars) or presence (dark gray bars) of 10 mM KNO₃. (D) Heme-stained proteins in membranes prepared from parental *B. japonicum* (WT) and strains Δ*nnrR*, and Δ*fixK*₂. Cells were incubated microoxically in YEM medium during 24 h in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of 10 mM KNO₃. Heme-stained *c*-type cytochromes identified previously, including NapC and NorC, are specified at the right margin. Apparent masses of the proteins (kDa) are shown at the left margin. Each lane contains about 25 μg membrane proteins. (E) MV⁺-NR in *B. japonicum* WT, Δ*nnrR*, and Δ*fixK*₂ strains incubated during 24 h in the same conditions as indicated in (D). Data (means±standard deviations) are expressed as nmol NO₂⁻ produced x (mg protein)⁻¹ min⁻¹, from at least two cultures which were assayed in triplicate.

Fig. 3. FixK₂-mediated IVT activation from the *napEDABC* (A), *nirK* (B) and *norCBQD* promoters (C). Supercoiled template plasmids containing either of the

indicated promoters cloned upstream of a strong transcriptional terminator were used for multiple-round IVT assays with increasing amounts of purified FixK₂ protein and RNAP from *B. japonicum*. FixK₂ concentrations were as follows: no protein (lanes 4, 7, and 10), 1.25 μM (lanes 3, 5, 8, and 11), 2.5 μM (lanes 6, 9, and 12). The pRJ8816 plasmid containing the *fixNOQP* promoter which is directly activated by FixK₂ was used as positive control (lane 3). Transcripts synthesized *in vitro* in the presence of [α -³²P]UTP were separated on a 6% denaturing polyacrylamide gel and visualized by phosphorimager analysis of the dried gel. RNA size markers (M) loaded in lanes 1 and 2 were generated as described earlier (Mesa *et al.*, 2005). The 107-nucleotide transcript present in all lanes originates from a promoter located on the plasmid vector and serves as an internal control. Shown are the results from a typical transcription experiment that was repeated at least once for each individual promoter. nt, nucleotides. (D) and (E) Primer extension experiment to determine the 5' end of the *in vitro*-synthesized FixK₂-dependent *napEDABC* (D) and *nirK* (E) transcripts. Unlabeled transcripts generated by IVT of plasmids pRJ3322 and pRJ8822 with *B. japonicum* RNAP and FixK₂ (1.25 μM) were reversed transcribed with ³²P-labeled oligonucleotide 9519-1 serving as a primer. Radioactive cDNA products were loaded onto a 6% denaturing polyacrylamide gel (lane P) along with a sequence ladder obtained with the same primer and plasmids pRJ3322 and pRJ8822 as templates, for *napEDABC* and *nirK*, respectively. The relevant nucleotide sequence is indicated at the left with the transcriptional start site emphasized by a solid arrowhead. In all experiments FixK₂ protein concentrations are referred to the dimeric state.

Fig. 4. NnrR protein interact with the *norCBQD* promoter exclusively in anoxic conditions. ITC data for the binding of *B. japonicum* NnrR protein to double-stranded DNA containing the FixK₂-like box present at *napEDABC*, *nirK* and *norCBQD* gene promoters under oxic (A) and anoxic (B) conditions. In (C) the raw data output of from *norCBQD* promoter-NnrR interaction are transformed to show the heat exchange at each injection (Kcal/mol of injectant) obtained by integration of the area of each “spike” in the raw data output, as a function of the molar ratio of the NnrR-*norCBQD* binding interaction. Data were fitted with ORIGIN using the “one set of sites” algorithm of the ORIGIN software (Microcal., Northampton, MA). Derived thermodynamic parameters are given in the inset of the panel.

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Table 1

Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant description	Source of reference
Strains		
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 gyrA96 thi-1 relA1</i>	Bethesda Research Laboratories, Inc. Gaithersburg, Md.
BL21 (DE3)	F ⁻ <i>opmT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Novagen Inc.
<i>B. japonicum</i>		
110 <i>spc4</i>	Sp ^r wild type	42
GRPA1	Sp ^c Sm ^r <i>napA</i> :: Ω	7
GRK308	Sp ^c Sm ^r <i>nirK</i> :: Ω	9
9043	Sp ^c Sm ^r <i>fixK₂</i> :: Ω	17
8678	Km ^r <i>nnrR</i> :: <i>aphII</i>	21
0602	Sp ^r Tc ^r <i>napE-lacZ</i> chromosomally integrated into 110 <i>spc4</i>	13
2498	Sp ^r Tc ^r <i>nirK-lacZ</i> chromosomally integrated into 110 <i>spc4</i>	21
2499	Sp ^r Tc ^r <i>norC-lacZ</i> chromosomally integrated into 110 <i>spc4</i>	21
1108	Sp ^r Sm ^r Tc ^r <i>napE-lacZ</i> chromosomally integrated into 9043	13
2498K2	Sp ^r Sm ^r Tc ^r <i>nirK-lacZ</i> chromosomally integrated into 9043	21
2499K2	Sp ^r Sm ^r Tc ^r <i>norC-lacZ</i> chromosomally integrated into 9043	21
0715	Sp ^r Km ^r Tc ^r <i>napE-lacZ</i> chromosomally integrated into 8678	13
2498R	Sp ^r Km ^r Tc ^r <i>nirK-lacZ</i> chromosomally integrated into 8678	21
2499R	Sp ^r Km ^r Tc ^r <i>norC-lacZ</i> chromosomally integrated into 8678	21
Plasmids		
pET-28a(+)	Km ^r , expression vector	Novagen, Inc
pET-3a(+)	Ap ^r , expression vector	Novagen, Inc
pRJ9519	Ap ^r [(pBluescript SK(+)) 308-bp <i>Bst</i> XI- <i>Kpn</i> I fragment containing the <i>B. japonicum</i> <i>rrn</i> terminator cloned into the <i>Hinc</i> II and <i>Kpn</i> I sites	44
pRJ9601	Ap ^r [pBluescript SK(+)] <i>B. japonicum</i> <i>rrn</i> promoter and <i>rrn</i> terminator on a 468-bp <i>Sac</i> I- <i>Sma</i> I fragment	44
pRJ8816	Apr (pRJ9519) <i>fixNOQP</i> promoter on a 563-bp <i>Bam</i> HI- <i>Eco</i> RI fragment	27
pRJ8817	Ap ^r (pRJ9519) <i>fixGHIS</i> promoter on a 524-bp <i>Xba</i> I- <i>Eco</i> RI fragment	27
pRJ3322	Ap ^r (pRJ9519) <i>napEDABC</i> promoter on a 297-bp <i>Bam</i> HI- <i>Eco</i> RI fragment	This work
pRJ8822	Ap ^r (pRJ9519) <i>nirK</i> promoter on a 465-bp <i>Bam</i> HI- <i>Eco</i> RI fragment	This work
pRJ8839	Ap ^r (pRJ9519) <i>norCBQD</i> promoter on a 367-bp <i>Bam</i> HI- <i>Eco</i> RI	This work
pRJ8819	Km ^r [pET28a(+)] <i>nnrR</i> on a 818-bp <i>Nde</i> I- <i>Eco</i> RI fragment cloned in frame with the vector-encoded N-terminal His ₆ tag, the <i>Nde</i> I site was introduced by engineering at the previous codon of the <i>nnrR</i> start codon	S. Mesa, unpublished
pBG0703	Km ^r (pRJ8819) <i>nnrR</i> on a 825-bp <i>Nde</i> I- <i>Bam</i> HI fragment, the <i>Bam</i> HI site was introduced at the 3'end of the insert by the QuickChange method	This work
pBG0704	Ap ^r [pET-3a(+)] <i>nnrR</i> on a 825-bp <i>Nde</i> I- <i>Bam</i> HI fragment	This work

TABLE S1. Oligonucleotide primers

Primer name	Primer sequence (5'-3' sequence)	Use
nnrRmut1(2)	C ^[7801749] AAGGAGGCC <u>CATATG</u> CCAAGG ^{[7801770](1,2)}	Forward primer for amplification of a 1.352 bp fragment containing an artificially engineered <i>NdeI</i> site in the previous codon of the predicted ATG of <i>nnrR</i> [in combination with M13for (-20)]. Plasmid pRJ8676 ⁽³⁾ was used as template
M13for (-20)	GTAAAACGACGGCCAGT	Reverse primer for amplification of a 1.352 bp fragment containing an artificially engineered <i>NdeI</i> site in the previous codon of the predicted ATG of <i>nnrR</i> [in combination with nnrRmut1(2)]. Plasmid pRJ8676 ⁽³⁾ was used as template
nnrRmut1B	G ^[7802573] CGCGAATTC ^[7802582] <u>AGGATCCG</u> TCGACAAG ^(1,2)	Forward primer used together with nnrRmut2B to create a <i>Bam</i> HI site in the 3' end of the insert of plasmid pBG0703 using the QuickChange method
nnrRmut2B	CTTGTCGAC <u>CGATCCTG</u> ^[7802582] AATTCGCGC ^{[7802573](1,2)}	Reverse primer used together with nnrRmut1B to create a <i>Bam</i> HI site in the 3' end of the insert of plasmid pBG0703 using the QuickChange method
bsr7036_for_1	CGGGATCCCG ^[7751787] CCCCATCTGGTTCACAATTC ^{[7751807](1,2)}	Forward primer used together with bsr7036_rev_1 for amplification of the <i>napEDABC</i> promoter as a 306 bp <i>Bam</i> HI- <i>Eco</i> RI fragment (template for IVT)
bsr7036_rev_1	GGAATTCC ^[7752076] AGGAAGGCGAAAATCTCCAT ^{[7752056](1,2)}	Reverse primer used together with bsr7036_for_1 for amplification of the <i>napEDABC</i> promoter as a 306 bp <i>Bam</i> HI- <i>Eco</i> RI fragment (template for IVT)
nirK3-for	CGGGATCCCG ^[7805388] TTTCTTCACCCATCTTCATGC ^{[7805409](1,2)}	Forward primer used together with nirK3-rev for amplification of the <i>nirK</i> promoter as a 475 bp <i>Bam</i> HI- <i>Eco</i> RI fragment (template for IVT)
nirK3-rev	CGGAATTCCG ^[7805844] CTCTGCGGGTGAACATCTG ^{[7805825](1,2)}	Reverse primer used together with nirK3-for for amplification of the <i>nirK</i> promoter as a 475 bp <i>Bam</i> HI- <i>Eco</i> RI fragment (template for IVT)
norC2-for	CGGGATCCCGG ^[3551430] GATCGTCTCGACCTCACAT ^{[3551449](1,2)}	Forward primer used together with norC2-rev for amplification of the <i>norC</i> promoter as a 377 bp <i>Bam</i> HI- <i>Eco</i> RI fragment (template for IVT)
norC2-rev	CGGAATTCCGA ^[3551786] GCCATTGCCAATCCTTTC ^{[3551768] (1,2)}	Reverse primer used together with norC2-for for

9519-1	ATGGATGAACTCCAAGACGGTATC	amplification of the <i>norC</i> promoter as a 377 bp <i>Bam</i> HI- <i>Eco</i> RI fragment (template for IVT)
P2 _{napE-fw}	A ^[7751905] CGGA <u>TTGATCCAGATCAA</u> CGCGT ^{[7751928](1,5)}	Primer used for primer extension of <i>in vitro</i> -synthesized <i>napADABC</i> and <i>nirK</i> transcripts ⁽⁴⁾ Forward primer used to study NnrR- <i>napEDABC</i> interaction by isothermal titration calorimetry (ITC)
P2 _{napE-rv}	A ^[7751928] CGCG <u>TTGATCTGGATCAA</u> TCCGT ^{[7751905](1,5)}	Reverse primer used to study NnrR- <i>napEDABC</i> interaction by ITC
P2 _{nirK-fw}	A ^[7805740] AAGC <u>TTGTTGCAGCGCAA</u> AACT ^{[7805763](1,5)}	Forward primer used to study NnrR- <i>nirK</i> interaction by ITC
P2 _{nirK-rv}	A ^[7805763] GTGT <u>TTGCGCTGCAACA</u> AGCTTT ^{[7805740](1,5)}	Reverse primer used to study NnrR- <i>nirK</i> interaction by ITC
P2 _{norC-fw}	G ^[3551689] CTCA <u>TTGCGCCCTGACAA</u> AGAAC ^{[3551712](1,5)}	Forward primer used to study NnrR- <i>norCBQD</i> interaction by ITC
P2 _{norC-rv}	G ^[3551712] TTCT <u>TTGTCAGGGCGCAA</u> TGAGC ^{[3551689](1,5)}	Reverse primer used to study NnrR- <i>norCBQD</i> interaction by ITC

¹⁾ Numbers in brackets at the right of the nucleotide refer to the genomic coordinates of the *B. japonicum* genome sequence (<http://genome.microbedb.jp/rhizobase/>)

²⁾ Engineered *Nde*I, *Bam*HI and *Eco*RI restriction sites are underlined. Mutations are introduced as boldface letters

³⁾ Mesa et al., 2003 [1]

⁴⁾ Mesa et al., 2005 [2]

⁵⁾ Letters in boldface and underlined show the FixK₂-like box for each denitrification gene

1

Promoters

Consensus FixK₂ box

TTGA/C- N₆ -T/GCAA

2

3 **Figure S1.** FixK₂-like boxes associated with the promoters of *B. japonicum* denitrifying
4 genes. The FixK₂ consensus binding site is shown at the bottom of the figure [3].
5 Letters inside squares show the conserved determinants common for *napEDABC*- and
6 *nirK*- compared with *norCBQD*-associated FixK₂-like boxes. The vertical dotted line
7 marks a dyad symmetry axis with respect to the most conserved nucleotides, indicated
8 by bold style and underlined. The arrows indicate the interaction of FixK₂ and NnrR
9 with the FixK₂-like boxes present at each denitrifying genes promoters.

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