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ORIGINAL PAPER

Dissecting the role of NtrC and RpoN in the expression of assimilatory nitrate and nitrite reductases in *Bradyrhizobium diazoefficiens*

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Abstract *Bradyrhizobium diazoefficiens*, a nitrogen-fixing endosymbiont of soybeans, is a model strain for studying rhizobial denitrification. This bacterium can also use nitrate as the sole nitrogen (N) source during aerobic growth by inducing an assimilatory nitrate reductase encoded by *nasC* located within the *narK-bjgb-flp-nasC* operon along with a nitrite reductase encoded by *nirA* at a different chromosomal locus. The global nitrogen two-component regulatory system NtrBC has been reported to coordinate the expression of key enzymes in nitrogen metabolism in several bacteria. In this study, we demonstrate that disruption of *ntrC* caused a growth defect in *B. diazoefficiens* cells in the presence of nitrate or nitrite as the sole N source and a decreased activity of the nitrate and nitrite reductase enzymes. Furthermore, the expression of *narK-lacZ* or *nirA-lacZ* transcriptional fusions was significantly reduced in the *ntrC* mutant after incubation under nitrate assimilation conditions. A *B. diazoefficiens rpoN*$_{1/2}$ mutant, lacking both copies of the gene encoding the alternative sigma factor σ^{54}, was also defective in aerobic growth with nitrate as the N source as well as in nitrate and nitrite reductase expression. These results demonstrate that the NtrC regulator is required for expression of the *B. diazoefficiens nasC* and *nirA* genes and that the sigma factor RpoN is also involved in this regulation.

Keywords Alternative sigma factor · *Bradyrhizobium* · Nitrate assimilation · Nitrate reductase · Nitrite reductase · Two-component-regulatory system

Abbreviations

Bjgb	*Bradyrhizobium japonicum* haemoglobin
BN3	Bergersen minimal medium-nitrate
C	Carbon
CFU	Colony formation units
Flp	Flavoprotein
MU	Miller units
MV-NiR	Methyl viologen-dependent nitrite reductase
MV-NR	Methyl viologen-dependent nitrate reductase
N	Nitrogen
NarK	Nitrate/nitrite transporter
NasC	Assimilatory nitrate reductase
NirA	Assimilatory nitrite reductase
NO	Nitric oxide

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NtrB	Two-component system kinase
NtrC	Two-component system response regulator
OD_{500}	Optical density-500 nm
PSY	Peptone–salts–yeast extract
RpoN	Alternative sigma factor
WT	Wild-type
YEM	Yeast-extract-mannitol

Introduction

Bacteria have developed diverse mechanisms to sense, respond, and adapt to changes in the environmental availability of nutrients (reviewed by Shimizu 2016). Adaptive physiological responses to these changes usually include two-component signal (TCS) transduction systems that allow bacteria to respond to diverse environmental stimuli (Stock et al. 2000). Extensive studies have been done on several TCS systems including NtrB–NtrC. This TCS is a classical regulatory system involved in the regulation of expression of genes in response to nitrogen limitation (Jiang and Ninfa 1999, 2009; Pioszak et al. 2000; Schumacher et al. 2013). NtrBC has been well characterised in enteric bacteria (Merrick and Edwards 1995; Reitzer 2003; Li and Lu 2007; van Heeswijk et al. 2013). NtrB is the sensor kinase that responds to an internal signal and autophosphorylates on a conserved histidine residue. The phosphoryl group of this histidine is then transferred to a conserved aspartate residue of the response-regulator protein NtrC within the receiver domain. Once phosphorylated, NtrC binds DNA at specific promoters and activates transcription of target genes (Weiss et al. 1992; Chen and Reitzer 1995).

PII signal-transduction proteins are recognized to coordinate the regulation of central carbon and nitrogen metabolism (Leigh and Dodsworth 2007; Forchhammer 2008). Under nitrogen-limiting conditions, the ratio of α-ketoglutarate to glutamine increases and stimulates the PII functions, thereby activating the kinase activity of NtrB which, in turn, leads to phosphorylation of NtrC. Recent studies have proposed an in vivo model in which α-ketoglutarate has a predominant regulatory role acting as a metabolic signal of nitrogen regulation (Schumacher et al. 2013). The phosphorylated NtrC activates the transcription of genes involved in nitrogen scavenging, in metabolism, and in regulation (Zimmer et al. 2000), in conjunction with a specific sigma factor (σ^{54}), the product of the *rpoN* gene (Reitzer and Magasanik 1985; Ninfa et al. 1987; Kullik et al. 1991; Merrick 1993; North et al. 1993).

In bacteria, nitrate-assimilation begins with the transport of nitrate into the cell. Then, intracellular nitrate is further reduced to nitrite by a cytoplasmic molybdenum-containing nitrate reductase followed by a sirohaem-containing nitrite reductase that reduces nitrite to ammonia (Moreno-Vivián et al. 1999; Richardson et al. 2001; Luque-Almagro et al. 2011). The genetic organization of the assimilatory nitrate-reducing systems (Nas) have been well characterised in bacteria such as *Rhodobacter capsulatus* (Cabello et al. 2004; Pino et al. 2006), *Klebsiella oxytoca* (Lin and Stewart 1998), *Azotobacter vinelandii* (Gutiérrez et al. 1995), *Bacillus subtilis* (Ogawa et al. 1995), and *Paracoccus denitrificans* (Gates et al. 2011; Luque-Almagro et al. 2013). In Gram-negative bacteria, the *nas* genes are subjected to dual control: an ammonia repression by the general nitrogen-regulatory NtrBC system and a specific nitrate or nitrite induction (Luque-Almagro et al. 2011).

Bradyrhizobium diazoefficiens is a soil Gram-negative alphaproteobacterium able to form a symbiotic association with soybean plants. Like other rhizobia species *B. diazoefficiens* can assimilate soil N sources like ammonia (i.e., NH_4^+) and nitrate in free living conditions. In rhizobia, several studies have reported the role of NtrC in the regulation of genes involved in NH_4^+ metabolism (reviewed by Patriarca et al. 2002). In contrast, very little information is available on the function of NtrC in the control of nitrate assimilation genes expression in rhizobia (Szeto et al. 1987; Martin et al. 1988). Within this context, recent DNA microarray-based transcriptional profiling has revealed a NtrC-dependent regulon operating in response to nitrogen limitation in *B. diazoefficiens* and the role of NtrC in regulating the utilization of nitrite as a sole N source (Franck et al. 2015). However, the involvement of NtrC on the control of assimilatory nitrate reduction to nitrite has not been reported so far.

In *B. diazoefficiens*, a recent genetic and biochemical analysis has given novel insights into bacterial nitrate assimilation (Cabrera et al. 2016). Unlike

related bacteria that assimilate nitrate, the genes encoding the assimilatory nitrate reductase (*nasC*) and nitrite reductase (*nirA*) are located at separate chromosomal loci. The *nasC* gene belongs to the *narK-bjgb-flp-nasC* operon, which also codes for a major facilitator superfamily–type nitrate and nitrite transporter (NarK), a bacterial hemoglobin (Bjgb) previously reported to be involved in NO detoxification (Cabrera et al. 2011; Sánchez et al. 2011), and a flavin-adenine-dinucleotide dependent NAD(P)H-oxidoreductase protein (Flp). The *nirA* gene is in a cluster with loci containing a nitrate and nitrite responsive regulator system (NasST). In *B. diazoefficiens*, the nitrate-dependent expression of the *narK-bjgb-flp-nasC* operon and the *nirA* gene requires the NasST system for transcription antitermination (Cabrera et al. 2016).

In this paper, we demonstrate that NtrC is essential for the expression of the assimilatory nitrate and nitrite reductase activities. We also report that the transcription of the *B. diazoefficiens narK-bjgb-flp-nasC* operon and the *nirA* gene in response to nitrate also requires NtrC and that the alternative sigma factor RpoN is essential for the expression of the NtrC-dependent genes involved in nitrate and nitrite assimilation.

Materials and methods

Bacterial strains, plasmids and primers

Table 1 lists the bacterial strains, plasmids and primers used in this study.

Bacterial growth conditions

Bacteria were routinely grown at 28 °C in complete yeast-extract-mannitol medium (YEM) (Vincent 1974). To test growth kinetics, a single rhizobial colony cultured in 10 ml Evans minimal medium (Evans et al. 1970) with 10 g mannitol l^{-1} as the carbon source and 20 mM $(NH_4)_2SO_4$ as the N source was grown at 28 °C on a rotary shaker at 180 rpm for a week. The culture was then diluted 1:100 in fresh Evans medium and grown again for additional 3 days under the same conditions. Next, this starter culture was diluted 1:50 in Erlenmeyer flasks containing a volume of the medium to be assayed equal to 20% of the flasks' capacity. Growth curves under different N sources were performed by modifying the original Evans formulation through the addition of 10 mM $NaNO_3$ or 1 mM $NaNO_2$ as the sole N source. Growth was monitored by measuring the optical density of the cultures at 500 nm (OD_{500}) and the number of viable colony-forming units (CFU) estimated by plate counts in solid YEM after the appropriate serial dilutions every 24 h for 15 or 17 days.

Antibiotics were added to *B. diazoefficiens* cultures at the following concentrations (mg ml^{-1}): chloramphenicol 20; spectinomycin 200; kanamycin 200; and tetracycline 100.

Escherichia coli strains were cultured in Luria–Bertani medium (Miller 1972) at 37 °C. The antibiotics used were (mg ml^{-1}): gentamycin, 10; kanamycin, 25; and tetracycline, 10.

To test for enzymatic activities, *B. diazoefficiens* strains were grown at 30 °C in peptone–salt–yeast-extract (PSY) medium supplemented with 0.1% (w/v) L-arabinose (Regensburger and Hennecke 1983). Dilutions of these cultures were then transferred to Bergersen minimal medium (Bergersen 1977) supplemented with 10 mM KNO_3 as the sole N source (i.e., BN3 medium). Since the protocols for the determination of nitrate-reductase (NR) and nitrite-reductase (NiR) activity had been optimized in Bergersen media, we first confirmed that the growth phenotype of the LP4488 mutant (see further on) was similar when determined in Evans's nitrate media (data not shown).

Construction of a *B. diazoefficiens ntrC* mutant

Cloning procedures — including DNA isolation, restriction-enzyme digestion, ligation, and transformation — were performed as described previously (Sambrook and Russell 2001). Biparental matings were effected with the *E. coli* strain S17-1 (Simon et al. 1983). Electroporation was carried out with a Gene-Pulser system (Bio-Rad, Hercules, CA) at 1.5 V, 25 μF, and 200 Ω in a 0.1 cm gap-width electroporation cuvette.

Genomic- and plasmid-DNA was isolated through the use of the Wizard Genomic DNA purification Kit (Promega) and Accuprep Plasmid MiniPrep DNA Extraction Kit (Bioneer), respectively. Custom oligonucleotide primers were supplied by Genbiotech and the polymerase-chain reaction (PCR) run with the Taq DNA polymerase from Embiotec or the Pfx

Table 1 Bacterial strains, plasmids and primers used in this study

	Genotype and phenotype	Reference
Strains		
E. coli		
DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Bethesda Research Laboratories
S17-1	Tra^+, *recA pro thi hsdR chr*::RP4-2	Simon et al. 1983
B. diazoefficiens		
USDA 110	Wild-type strain,Cm^r	US Department of Agriculture, Beltsville, MD, USA
110*spc*4	USDA 110 derivative, Spc^r	Regensburger and Hennecke, 1983
LP4488	USDA 110 Δ*ntrC,* Km^r	This work
N50-97	110*spc*4 Δ$rpoN_{1/2}$, Spc^r	Kullik et al. 1991
4009	USDA 110::*narK-lacZ*, Cm^r, Tc^r	Cabrera et al. 2016
4018	USDA 110::*nirA-lacZ,* Cm^r, Tc^r	Cabrera et al. 2016
LP4488-4009	LP4488::*narK-lacZ*, Cm^r, Tc^r	This work
LP4488-4018	LP4488::*nirA-lacZ*, Cm^r, Tc^r	This work
110*spc*4-4009	110*spc*4::*narK-lacZ*, Cm^r, Tc^r	This work
110*spc*4-4018	110*spc*4::*nirA-lacZ,* Cm^r, Tc^r	This work
N50-97-4009	N50-97::*narK-lacZ*, Cm^r, Tc^r	This work
N50-97-4018	N50-97::*nirA-lacZ*, Cm^r, Tc^r	This work
Plasmids		
pG18mob2	Rhizobial suicide plasmid	Kirchner and Tauch 2003
pMFL4488	pG18mob2::*ntrC*5́::Km::*ntrC*3́, Km^r	This work
pDB4009	pSUP3535::*narK-lacZ*, Tc^r	Cabrera et al. 2016
pDB4018	pSUP3535:: *nirA-lacZ,* Tc^r	Cabrera et al. 2016

Primers	Sequence	Reference
ntrC5′FW	AGCCGCGCAAGACCACCTTC	This work
ntrC5′RV	TGCCGGTGAGCCTGACCTCA	This work
ntrC3′-*Sph*I FW	TAGCATGCCTCTATCCGCAGGACGTGAT	This work
ntrC3′-*Hind*III RV	AAAAAGCTTGCTCCGATAGACCTGGATGT	This work
ntrB5′(checking)	GCGCTTCCCAATCCCGTGCT	This work
cheqRVntrC (checking)	ATTCCGGCTTGACTGGGATG	This work
Km FW (checking)	TGTATGGGAAGCCCGATG	Mongiardini et al. 2016
Km RV (checking)	TGCCATTCTCACCGGATT	Mongiardini et al. 2016

polymerase from invitrogen. DNA was digested with the Fast Digest (Fermentas) or Promega enzymes.

To obtain the *B. diazoefficiens ntrC*-deletion mutant (ORF blr4488; http://genome.microbedb.jp/rhizobase/), upstream (237-bp) and downstream (330-bp) DNA fragments flanking the *ntrC* locus were generated by PCR from total *B. diazoefficiens* DNA by means of the ntrC5′FW, ntrC5′RV, ntrC3′FW, and

ntrC3′RV primers (Table 1). These fragments were inserted into the rhizobial suicide plasmid pG18mob2 (Kirchner and Tauch 2003) as a *Sma*I and a *Sph*I-*Hind*III fragment. Then, the kanamycin-resistance cassette from the pUC4k plasmid (Vieira and Messing 1982) was inserted in the *BamH*I restriction site, resulting in the pMFL4488 plasmid (this work). This plasmid was introduced into *E. coli* S17-1 electrocompetent cells that served as donor in a conjugative plasmid transfer to *B. diazoefficiens* USDA 110. The transconjugants obtained were screened as kanamycin-resistant, gentamycin-sensitive and the correct recombination at the target gene checked by both PCR and genetic sequencing (Macrogen Inc, Korea). The following experiments were accordingly carried out with the clone referred to as LP4488.

Determination of nitrate- and nitrite-reductase activities

B. diazoefficiens cells were grown under aerobic conditions in PSY medium, harvested by centrifugation at 8000×*g* for 10 min at 4 °C, washed twice with BN3 medium, and inoculated at an OD_{500} of ca. 0.3 in the same minimal medium. After 48 h the cells were harvested, washed with 50 mM Tris/HCl buffer (pH 7.5) to remove excess nitrite, and then resuspended in 1 ml of the same buffer before the assay for enzymatic activity. Methyl-viologen (MV)-dependent nitrate reductase (MV-NR) and nitrite reductase (MV-NiR) activities were measured as described by Delgado and coworkers with dithionite-reduced MV as an artificial electron donor (Delgado et al. 2003). The MV-NR and MV-NiR activities are expressed as nanomol of nitrite produced (for NR) or consumed (for NiR) per mg protein^{-1} min^{-1}. For more details see Cabrera et al. (2016).

β-galactosidase activity of *narK-lacZ* and *nirA-lacZ* fusions

The transcriptional-fusion plasmids pDB4009 and pDB4018 containing *narK-lacZ* and *nirA-lacZ* fusions, respectively (Table 1), were integrated by homologous recombination into the chromosomes of the wild-type (WT) strain 110*spc*4, the *ntrC* mutant, and the $rpoN_{1/2}$ double mutant (strain N50–97; Regensburger and Hennecke 1983) to produce strains 110*spc*4-4009, 110*spc*4-4018, and LP4488-4009 plus LP4488-4018, N50-97-4009, and N50-97-4018, respectively (Table 1). The correctness of recombination was checked by PCR and by sequencing analysis of the genomic DNA isolated from each strain.

The cells were grown aerobically in PSY medium, collected by centrifugation, washed twice with nitrogen-free Bergersen medium, and finally incubated aerobically in the same medium with or without the addition of 10 mM $NaNO_3$ as the N source. After cultures having an initial OD_{500} of about 0.3 had been incubated for 48 h, the ß-galactosidase activity was assayed in triplicate on permeabilized cells from at least three independently grown cultures for each strain and condition, as previously described (Miller 1972). The absorbance data at 420 and 500 nm were read for all samples and cultures with a plate reader (SUNRISE Absorbance Reader, TECAN, Männedorf, Switzerland) and recorded by means of the software XFluor4 (TECAN). The specific activities were finally calculated in Miller units (MU).

Analytical methods

The nitrite concentration was estimated after diazotization by adding the sulfanilamide–naphthylethylenediamine-dihydrochloride reagent (Nicholas and Nason 1957) and the protein concentration measured by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with a standard curve of varying bovine-serum-albumin concentrations.

Results

Involvement of NtrC and RpoN in nitrate- and nitrite-dependent growth

The *B. diazoefficiens* USDA 110–NtrBC two-component system is encoded by the *blr4487* and *blr4488* genes belonging to the *nifR3-ntrB-ntrC*-gene cluster, respectively. The targets of the NtrC protein are usually σ^{54}-dependent, and involved in the transcription of genes related to nitrogen metabolism. *B. diazoefficiens* has two functional, highly conserved *rpoN* genes ($rpoN_1$ and $rpoN_2$) encoding for the σ^{54}-RNA-polymerase alternative factor RpoN (Kullik et al. 1991). In this work, we have constructed a *B. diazoefficiens* mutant strain (i.e., LP4488) where the *ntrC* gene (i.e., *blr4488*) has been deleted. To

investigate the role of NtrC and RpoN in nitrate assimilation, the *B. diazoefficiens* mutant *ntrC* and the double mutant $rpoN_{1/2}$ were incubated aerobically in Evans minimal medium with 10 mM $NaNO_3$ as the sole N source. Growth was determined by monitoring the OD_{500} (Fig. 1, Panel a) or the number of CFU (Fig. 1, Panel b). In contrast to the *B. diazoefficiens* USDA 110 parental strain, the *ntrC* mutant exhibited a severe defect in growth, reaching an OD_{500} of only 0.097 compared to 3.8 determined in the WT cells after 15 days of incubation (Fig. 1, Panel a). As observed for *ntrC* mutant, growth rates of the $rpoN_{1/2}$ mutant were very low compared to those observed in the *B. diazoefficiens* 110*spc*4 WT strain (Fig. 1, Panel a). The maximal CFU reached by the parental strains was around 7.2×10^{10} CFU ml^{-1} after 15 days incubation, whereas *ntrC* and $rpoN_{1/2}$ mutants reached values only around 6.5×10^{8} CFU ml^{-1} (Fig. 1, Panel b). In addition, we confirmed that the growth phenotype of the mutants in Bergersen medium was the same as that observed in Evans medium (data not shown), which observation was useful for the following studies.

To test the capacity of the *ntrC* mutant to use NH_4^+ as an N source, cells were grown to early stationary phase with nitrate (to a final OD_{500} value of 0.17). Then, 20 mM NH_4Cl was added to the USDA 110 (WT) and the *ntrC* mutant cultures. A significant increase in growth of the *ntrC* mutant cells was observed that attained a OD_{500} similar to that reached by the WT cells after 10 days of incubation in the presence of NH_4^+ (Fig. 2). These observations confirm that NtrC has a key role in nitrate, but not NH_4^+, assimilation. In order to further confirm that possibility, we also tested the capacity of the NtrC-deficient LP4488 strain to grow in mineral-salts minimum medium with 20 mM NH_4Cl (a high-nitrogen condition) or 0.1 μM NH_4Cl (a nitrogen-limiting condition) as the sole N source. In concordance with previous reports, *ntrC* mutant displayed similar growth kinetics to those of the WT strain in the presence of either concentration of NH_4Cl as the sole N source (data not shown; Martin et al. 1988).

In order to study the involvement of NtrC and RpoN in nitrite assimilation, cells from the wild-type strains USDA 110 and 110*spc4* along with the *ntrC* and $rpoN_{1/2}$ mutants were incubated in Evans minimal medium with 1 mM $NaNO_2$ as the sole N source. Figure 3, Panel a indicates that a significantly delay in growth measured as OD_{500} was observed in the *ntrC*- or the $rpoN_{1/2}$-mutant cells compared to that recorded with the WT strains. In a similar manner, the kinetics

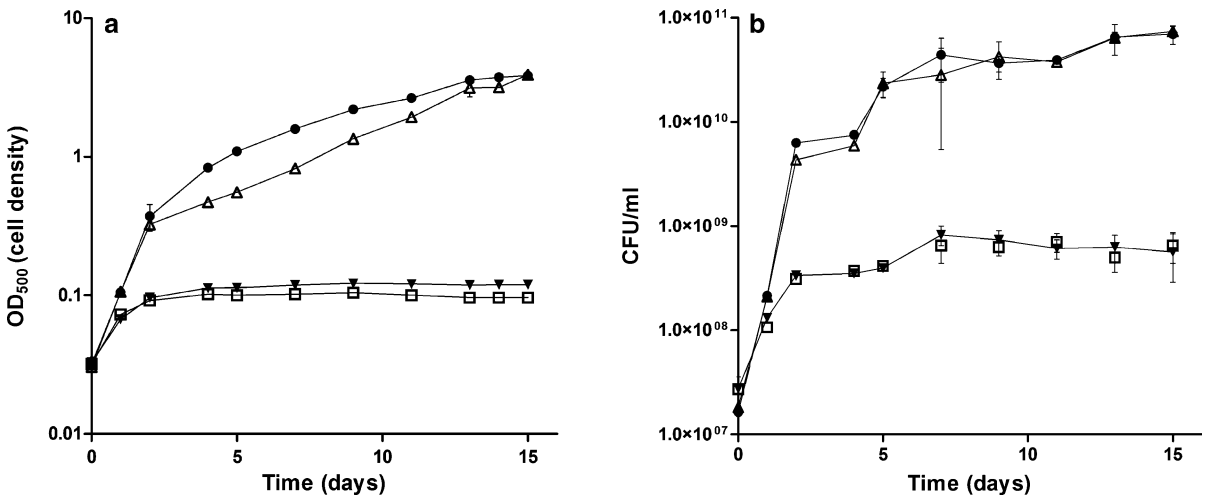

Fig. 1 Nitrate-dependent aerobic growth of wild-type *B. diazoefficiens* USDA 110 (*black circles*) and 110*spc*4 (*white upright triangles*) strains and the *ntrC* (*white squares*), and $rpoN_{1/2}$ (*black inverted triangles*) mutants in Evans minimal medium with 10 mM nitrate as N source. (Panel **a**) optical density at 500 nm of cell cultures. In the figure, the optical density of the cultures at 500 nm is plotted on the *ordinate* as a function of the time in days on the *abscissa*. (Panel **b**) viable cell counts as colony-forming units (CFU) per ml of culture. In the figure, the colony-forming units per ml of the cultures is plotted on the *ordinate* as a function of the time in days on the *abscissa*. The results presented are the means with the *error bars* representing the standard deviation from two biologic replicates assayed in triplicate. The absence of *error bars* indicates the error to be smaller than the symbol

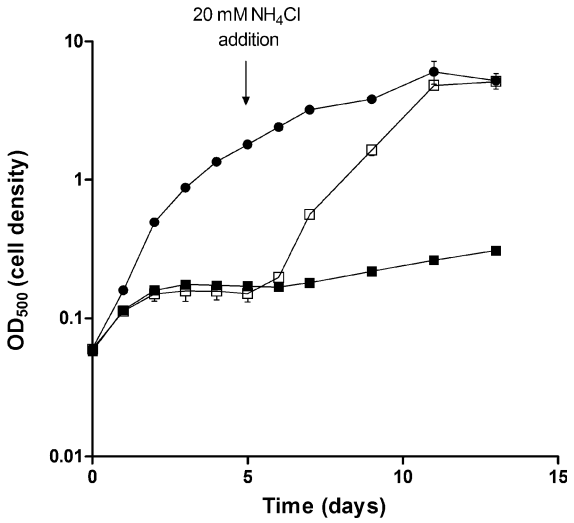

Fig. 2 Ammonium effect on the nitrate-dependent aerobic growth of the wild-type *B. diazoefficiens* USDA 110 (*black circles*) and the *ntrC* mutant (*black* and *white squares*) in Evans minimal medium with 10 mM sodium nitrate as the sole N source. The optical density at 500 nm is plotted on the *ordinate* as a function of time in days on the *abscissa* to illustrate bacterial growth. As indicated in the figure, 20 mM ammonium chloride was added to two of the four *ntrC*-mutant cultures 5 days after inoculation (*white squares*). The results presented are the means with the *error bars* representing the standard deviation from two biologic replicates assayed in triplicate. The absence of *error bars* indicates the error to be smaller than the symbol

of colony-formation counts by *ntrC* or $rpoN_{1/2}$ mutants exhibited a delay with respect to the corresponding time observed in WT cells (Fig. 3, Panel b). Moreover, mutants strains OD_{500} and CFU ml^{-1} started increasing between days 7 and 8 while their parental strains growth rose significatively at day 3 (ANOVA data analysis, $p < 0.05$; Tukey test). Nevertheless, after 10 days incubation the growth rates and extent of colony formation of both *ntrC* or $rpoN_{1/2}$ mutants were very similar to those obtained by both the USDA 110 and the 110*spc*4 wild-type strains, with no statistically significant differences between the four strains by the end of the culture period tested (Fig. 3, Panels a, b).

Nitrate- and nitrite-reductase activities are controlled by NtrC and RpoN

In this work, we also investigated whether the inability of the *ntrC* and $rpoN_{1/2}$ mutants to grow with nitrate or nitrite as the sole N source resulted from an alteration in the activity of the assimilatory nitrate and nitrite reductases, respectively. Here, MV-NR and MV-NiR activities were measured in whole cells following aerobic incubation with nitrate as the sole N source. Accordingly, and as expected, the respective NR rates observed in the *ntrC* and $rpoN_{1/2}$ mutants were about 18- and 23-fold lower than those recorded in the WT cells (ANOVA data analysis, $p < 0.01$) (Table 2). These results strongly support the inability of those mutants to grow in the presence of nitrate as the only N source (Fig. 1). The NR activity that is lost in the *ntrC* and $rpoN_{1/2}$ mutants corresponds to that of NasC since a similar phenotype had been previously observed in a *B. diazoefficiens nasC* mutant incubated under the same conditions (Cabrera et al. 2016).

NiR activity was decreased by about 5-fold in the *ntrC* and $rpoN_{1/2}$ mutants with respect to the WT strains (ANOVA data analysis, $p < 0.01$). As shown in Table 2, about 20% of the WT NiR activity, was retained in the *ntrC* and $rpoN_{1/2}$ mutants. This residual activity could explain the observed capacity of *ntrC* and $rpoN_{1/2}$ mutants to grow (Fig. 3, Panel a, b) after 10 days of incubation in a medium containing nitrite as the only N source.

These results clearly suggest that the expression of the *B. diazoefficiens* assimilatory nitrate reductase and nitrite reductase encoded by *nasC* and *nirA* respectively are controlled by NtrC and RpoN.

Role of NtrC and RpoN on the transcription of *nasC* and *nirA*

In order to evaluate the involvement of NtrC and RpoN in the expression of the *nasC* and *nirA* genes involved in the synthesis of the assimilatory NR and NiR, we used the *narK-lacZ* and *nirA-lacZ* transcriptional fusions previously constructed by Cabrera et al. (2016). The *narK-lacZ* fusion, which contains the promoter region of *narK*, the first gene of the *narK-bjgb-flp-nasC* operon containing *nasC*. Both the *narK-lacZ* and the *nirA-lacZ* transcriptional fusions were transferred to the WT strains (USDA 110 and 110*spc*4) and to the *ntrC* and $rpoN_{1/2}$ mutants. ß-galactosidase activity was monitored in the resulting strains incubated in the absence or presence of nitrate as the sole N source (Fig. 4). As previously reported (Cabrera et al. 2016), low levels of ß-galactosidase activity were observed in the *narK-lacZ* and *nirA-lacZ* fusions in USDA 110 incubated without nitrate, whereas the presence of this molecule induced the expression of the two fusions by approximately 4.4-

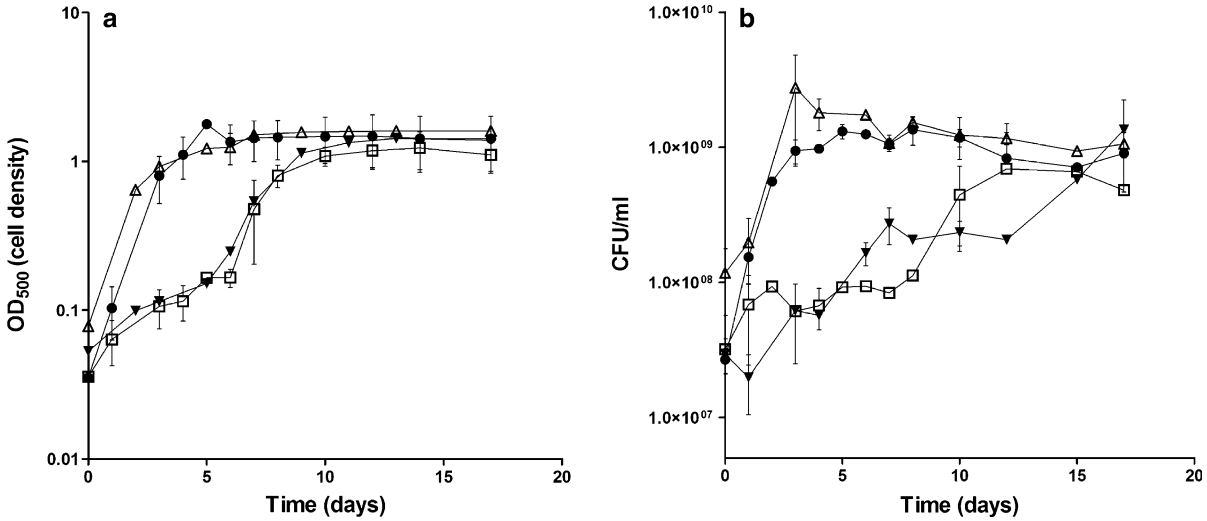

Fig. 3 Nitrite-dependent aerobic growth of the wild-type strains *B. diazoefficiens* USDA 110 (*black circles*) and 110*spc*4 (*white upright triangles*) and the mutant strains *ntrC* (*white squares*) and *rpoN*$_{1/2}$ (*black inverted triangles*) in minimal medium with 1 mM sodium nitrite as the sole N source. (Panel **a**) optical density at 500 nm of cell cultures is plotted on the *ordinate* as a function of time in days on the *abscissa*. (Panel **b**) viable cell counts as colony forming units (CFUs) per ml of culture is plotted on the *ordinate* as a function of time in days on the *abscissa*. The results presented are the means with the *error bars* representing the standard deviation from two biologic replicates assayed in triplicate. The absence of *error bars* indicates the error to be smaller than the symbol

Table 2 Methyl-viologen-dependent nitrate-reductase (MV-NR) and nitrite-reductase (MV-NiR) activities of *Bradyrhizobium diazoefficiens* USDA 110 and 110*spc*4 wild-type strains and *ntrC* and *rpoN*$_{1/2}$ mutant strains incubated aerobically for 48 h in Bergersen minimum medium with 10 mM nitrate as the nitrogen source

Strain	Relevant genotype	Activities	
		MV-NR[a]	MV-NiR[b]
USDA 110	Wild-type	8.77 ± 1.42	2.49 ± 0.40
LP4488	*ntrC*	0.49 ± 0.07	0.45 ± 0.25
110*spc*4	Wild-type	7.83 ± 0.80	2.20 ± 0.22
N50-97	*rpoN*$_{1/2}$	0.34 ± 0.07	0.42 ± 0.18

The data are expressed as the means ± the standard deviation from at least two different cultures assayed in triplicate

[a] MV-NR and [b]MV-NiR activities are expressed as nmol NO_2^- produced or consumed mg protein^{-1} min^{-1}

and 2.4-fold, respectively. Similarly, nitrate induced the expression of ß-galactosidase in the *narK-lacZ* and *nirA-lacZ* fusions in the wild-type strain 110*spc*4 by about 4.2- and 1.6-fold, respectively (Fig. 4). That the ß-galactosidase activities from the *narK*-lacZ fusions were almost undetectable in the *ntrC* and *rpoN*$_{1/2}$ mutants incubated in the presence of nitrate was notable, with those activities representing less than 1% of the WT levels (ANOVA data analysis, $p < 0.01$) (Fig. 4). This very low transcription of the *narK-bjgb-flp-nasC* operon observed in the *ntrC* and *rpoN*$_{1/2}$ mutants is consistent with the low levels of NasC activity observed in both mutants and strongly demonstrates the regulatory role of NtrC and RpoN in the transcription of the *nasC* gene. These results are in agreement with previous reports in other bacteria, where the regulation of *nasC* transcription by NtrC has already been demonstrated (Ishida et al. 2002; Ohashi et al. 2011; Romeo et al. 2012; Wang et al. 2012).

Similarly, as observed for the *narK-lacZ* fusion, a significant decrease in *nirA-lacZ* expression of about 15- and 11-fold was observed in the *ntrC* and *rpoN*$_{1/2}$ mutants, respectively, compared to the WT levels (ANOVA data analysis, $p < 0.01$) (Fig. 4). Nevertheless, about 7 and 9% of the WT ß-galactosidase activity from the *nirA-lacZ* fusion was still retained in those two mutants, respectively. These basal levels of *nirA-lacZ* expression in both mutants might explain the residual NiR activity observed in the *ntrC* and *rpoN*$_{1/2}$ mutants as well as the growth capacity recovery of those mutants after 10 days of incubation in a medium containing nitrite as the only N source.

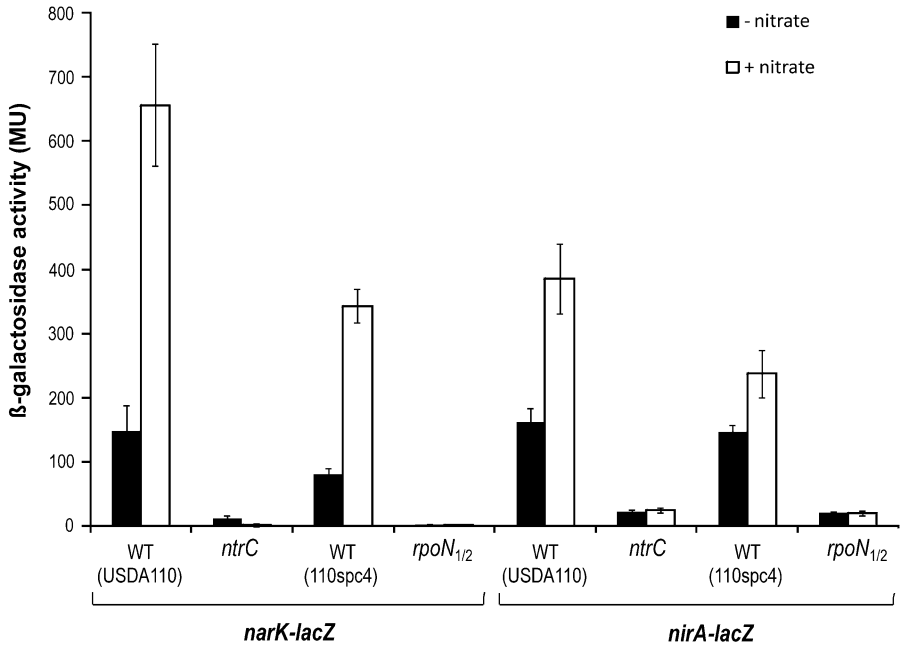

Fig. 4 ß-galactosidase activity derived from the *narK-lacZ* and *nirA-lacZ* fusions present in the WT strains *B. diazoefficiens* USDA 110 or 110*spc*4 and the *ntrC* or $rpoN_{1/2}$ mutants. Cells were cultured aerobically for 48 h in minimal medium with (*white bars*) or without (*black bars*) 10 mM nitrate as the sole N source. In the figure, the ß-galactosidase activity in Miller units (MU) is plotted on the *ordinate* for each of the strains indicated on the *abscissa*. Data are the means ± the standard error from at least three independent cultures, assayed in triplicate

Discussion

In *K. oxytoca* (Wu et al. 1999), *A. vinelandii* (Wang et al. 2012) and *Pseudomonas aeruginosa* (Li and Lu 2007; Romeo et al. 2012), NtrBC plays a role in the transcription of genes related to nitrate assimilation, but in rhizobia the main function of NtrC reported thus far implicates the transcriptional regulation of genes involved in NH_4^+ assimilation (Patriarca et al. 2002). It has been previously demonstrated the involvement of NtrC on *nirA* expression as well as the inability of a *B. diazoefficiens ntrC* mutant to grow on nitrite as sole N source (Franck et al. 2015). Our biochemical results confirm the NtrC control over *nirA* and demonstrate for the first time the involvement of NtrC as a transcriptional regulator of the *nasC* gene encoding the assimilatory nitrate reductase as well as in the ability of *B. diazoefficiens* to grow with nitrate as the sole nitrogen source. Indeed, we showed that NtrC is essential for the expression of the assimilatory nitrate and nitrite reductase activities.

The results obtained for the growth kinetics of a *B. diazoefficiens ntrC* mutant have demonstrated the previously reported role of NtrC in the nitrate-dependent growth of this bacterium (Martin et al. 1988). Consistent with these observations, the inability of another *ntrC* mutant of *Sinorhizobium meliloti* to grow on nitrate as the sole N source has also been reported (Szeto et al. 1987). A *Bradyrhizobium japonicum* $rpoN_{1/2}$ mutant was also found to be unable to use nitrate, suggesting a role of the sigma factor σ^{54} on the NtrC-dependent expression of nitrate assimilation. These results confirm previous findings where the requirement of at least one functional *rpoN* gene in nitrate assimilation by *B. japonicum* was reported (Kullik et al. 1991).

Interestingly, in this work it has also been confirmed that under nitrogen-limiting conditions (i.e., 0.1 μM NH_4Cl), the growth of the *ntrC* mutant was similar to the WT strain (data not shown). This finding suggests that NtrBC does not play a main role in NH_4^+ assimilation, perhaps because of a possible cross talk with another two-component regulatory system. In fact, downstream from the *ntrBC* genes, *B. diazoefficiens* contains the *ntrYX* loci that code for an additional two-component regulatory system, NtrYX. In support of this hypothesis, in *Azospirillum brasilense* and *Azorhizobium caulinodans* such a possible mutual interaction between the NtrYX and NtrBC has also been suggested (Pawlowski et al. 1991; Ishida et al. 2002). Furthermore, the possibility that the NtrB and NtrY in *R. capsulatus* can substitute for each other as phosphodonors for NtrC has also been proposed (Drepper et al. 2006).

With respect to nitrite-dependent growth, the *ntrC* and $rpoN_{1/2}$ mutants exhibited a strong delay in growth kinetics, but were nevertheless able to reach WT

growth rates after 10 days of incubation. This pattern is in contrast to recent studies where a *B. diazoefficiens ntrC* mutant was unable to grow with nitrite as the only N source (Franck et al. 2015). This apparent discrepancy could be explained by the different growth conditions used by Frank and colleagues from those used in this work. Whereas they used MMB minimal medium containing 2 mM nitrite as N source and 4 ml glycerol l^{-1} as the carbon source, in these experiments we used Evans minimal medium containing 1 mM nitrite and 10 g mannitol l^{-1} as those respective sources. The difference in the C/N ratio present in the two growth formulations might possibly have altered the effect of NtrC on *nirA* expression and consequently on the ability of the mutants to grow on nitrite as the sole N source.

The growth defect of *ntrC* and *rpoN*$_{1/2}$ mutants with nitrate as the N source could be explained by the significant inhibition of NR expression in those mutants. In fact, NR activity analyses showed that only 5% of WT NR activity was retained in either of the two mutants. Similarly, *narK-lacZ* expression in those mutants was nearly undetectable. With respect to NiR activity and ß-galactosidase activity from a *nirA-lacZ* fusion, a significant decrease in both activities was also observed in the *ntrC* and *rpoN*$_{1/2}$ mutants. However, a residual NiR activity (20% of WT activity) as well some basal levels of *nirA-lacZ* expression were still present in both mutants. These basal levels of *nirA* expression and NiR activity could explain how both *ntrC* and *rpoN*$_{1/2}$ mutants were able to grow on nitrite after 10 days of incubation, albeit after a significant delay.

The stronger effect of NtrC and RpoN on the NR and ß-galactosidase activity from a *narK-lacZ* fusion than on the NiR and ß-galactosidase activity from a *nirA-lacZ* fusion might explain the different growth responses of the *ntrC* and *rpoN*$_{1/2}$ mutants in media containing nitrate and nitrite as the respective sole N sources. As stated above, in contrast to the majority of bacteria where the genes encoding an assimilatory nitrate reductase or nitrite reductase are arranged in the same operon (for a review see Luque-Almagro et al. 2011), in *B. diazoefficiens* the *nasC* and *nirA* are located at separate chromosomal loci. This genetic organization may explain the slight differences observed between the expression of those genes with respect to their dependence on NtrC and RpoN. Moreover, in *A. vinelandii* and *P. denitrificans* it has been demonstrated that in addition to NtrBC, the NasST two-component system also controls nitrate assimilation, with NasT an RNA-binding protein with a positive effect over transcription and the sensor NasS a negative regulator (Wang et al. 2012; Luque-Almagro et al. 2013). In *B. diazoefficiens*, the genes coding for the NasST system are clustered with *nirA*, in contrast to other bacteria, and it was reported that this two-component system also controls nitrate assimilation in this rhizobium (Cabrera et al. 2016). Thus, it could be possible that NasST contributes to *nirA* expression, allowing a partial remaining NiR activity and the recovery of *ntrC* mutant growth in nitrite. Nevertheless, further studies must be carried out in order to elucidate how NtrBC and NasST control the expression of nitrate and nitrite reductases in *B. diazoefficiens*.

Taken together, the results reported here clearly demonstrate the fundamental role of NtrC and RpoN in the transcriptional control of the *B. diazoefficiens nasC* and *nirA* genes, those being involved in nitrate assimilation.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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