Rhizobia are recognized to establish N₂-fixing symbiotic interactions with legume plants. *Bradyrhizobium japonicum*, the symbiont of soybeans, can denitrify and grow under free-living conditions with nitrate (NO₃⁻) or nitrite (NO₂⁻) as sole nitrogen source. Unlike related bacteria that assimilate NO₃⁻, genes encoding the assimilatory NO₃⁻ reductase (nasC) and NO₂⁻ reductase (nirA) in *B. japonicum* are located at distinct chromosomal loci. The nasC gene is located with genes encoding an ABC-type NO₃⁻ transporter, a major facilitator family NO₃⁻/NO₂⁻ transporter (NarK), flavoprotein (Fip) and single-domain haemoglobin (termed Bjgb). However, nirA clusters with genes for a NO₃⁻/NO₂⁻-responsive regulator (NasS-NasT). In the present study, we demonstrate NasC and NirA are both key for NO₃⁻ assimilation and that growth with NO₂⁻, but not NO₃⁻ requires flp, implying Flp may function as electron donor to NasC. In addition, bjgb and flp encode a nitric oxide (NO) detoxification system that functions to mitigate cytotoxic NO formed as a by-product of NO₃⁻ assimilation. Additional experiments reveal NasT is required for NO₂⁻-responsive expression of the narK-bjgb-flp-nasC transcriptional unit and the nirA gene and that NasS is also involved in the regulatory control of this novel bipartite assimilatory NO₃⁻/NO₂⁻-reductase pathway.

Key words: bacterial denitrification, bacterial haemoglobin, nitrate reduction, nitric oxide reductase, nitrite reduction.

INTRODUCTION

Fixation of atmospheric dinitrogen (N₂) by plant-associated symbiotic soil bacteria, collectively termed rhizobia, is a significant agricultural process that reduces dependence on synthetic nitrogen (N) containing fertilizers in crop production. This protects water quality and human health as well as the wider environment. In addition to N₂ fixation, the soybean endosymbiont *Bradyrhizobium japonicum* USDA110 is capable of growing anaerobically with the water-soluble nitrate (NO₃⁻) anion, as an alternative terminal electron acceptor to oxygen (O₂), which is reduced to N₂ gas by respiratory denitrification. During this process, several free N-containing intermediates are produced, including: (i) the oxoanion nitrite (NO₂⁻), (ii) the gaseous cytotoxic free-radical nitric oxide (NO) and (iii) the potent and long-lived greenhouse gas nitrogen dioxide (NO₂). In *B. japonicum*, the denitrification apparatus is encoded by the napEDABC, nirK, norCBQD and nosRZDFYLYX genes, which express the periplasmic NO₃⁻ reductase (NapABC), copper-containing NO₂⁻ reductase (NirK), cytochrome-c NO reductase (NorCB) and N₂O reductase (NosZ) enzymes, respectively [1]. This bacterium is distinguished by the ability to denitrify under both free-living and symbiotic conditions [2–4].

Several reports suggest that rhizobial denitrification is the main driver for production and release of the environmentally damaging agents NO and N₂O from alfalfa and sojbean nodules [5–8]. NO is a highly reactive and well-studied ozone-depleting agent, whereas N₂O is increasingly recognized as a powerful greenhouse gas with an estimated 300-fold higher radiative potential for global warming, molecule for molecule, compared with carbon dioxide [9–11]. Importantly, in active root nodules, NO also acts as a potent inhibitor of nitrogenase, the central enzyme of symbiotic N₂-fixation [8,12]. Under free-living denitrifying conditions, the *B. japonicum* proteins NirK and NorCB are physiologically important for the synthesis and detoxification of NO, respectively [1]. However, several studies suggest the involvement of other sinks for NO that are distinct from the recognized denitrification pathway in nodules [8,13]. For example, in related bacteria, NO may be oxidized to NO₂⁻ or reduced to N₂O by cytoplasmic detoxification enzymes. These systems include single-domain haemoglobinins (sdHbs), truncated haemoglobinins (thHbs), flavohaemoglobinins (FHbs) and flavorubredoxins (FIRd) [14–18].

Following sequencing of the *B. japonicum* USDA110 genome [19], several studies have investigated the involvement of a putative bacterial sdHb, termed Bjgb, in NO-detoxification, under free-living conditions [3,20]. This bacterial haemoglobin is encoded by the ORF blr2807 and resides within a cluster of other uncharacterized ORFs (blr2803–05) predicted to encode components of a NO₂⁻ assimilation (Nas) pathway, including: an ABC-type NO₂⁻ transport system (blr2803–05), a major facilitator superfamily (MFS)-type NO₃⁻/NO₂⁻ transporter (blr2806), an FAD-dependent NAD(P)H oxidoreductase (blr2808) and the catalytic subunit of the assimilatory NO₃⁻ reductase (blr2809), termed NasC (we note the gene for the assimilatory NO₂⁻ reductase in *B. japonicum* was previously termed nasA, but here we unify the gene nomenclature for α-proteobacteria). The genome also contains a putative
ferredoxin-dependent assimilatory NO\textsubscript{3}− reductase (NirA) that is present at blil4571–73 loci in NO\textsubscript{3}−. However, to date, a role for the proteins encoded at blr2803–09 utilizing soil bacterium Paracoccus denitrificans PD1222 [21,22]. However, to date, a role for the proteins encoded at blr2803–09 utilizing soil bacterium Paracoccus denitrificans PD1222 [21,22].

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Although the biochemical components for Nas systems may be highly modular, in related α-proteobacteria such as P. denitrificans and Rhodobacter capsulatus E1F1, genes encoding regulatory and structural elements for the NO\textsubscript{3}− assimilation pathway are typically found together [23,24]. For example, in P. denitrificans, the genes required for import and reduction of NO\textsubscript{3}− and/or NO\textsubscript{2}− are encoded by nasABGHC and the nasTS genes required for NO\textsubscript{3}−/NO\textsubscript{2}−-responsive regulatory control are found immediately upstream [25]. Here, the assimilatory NO\textsubscript{3}−/NO\textsubscript{2}− reductase apparatus includes a: NO\textsubscript{3}−/NO\textsubscript{2}− transporter (NasA), NO\textsubscript{2}− reductase (NasB), ferredoxin (NasG), NO\textsubscript{3}− transporter (NasH) and NO\textsubscript{2}− reductase (NasC). Notably, the nasG gene is highly conserved in NO\textsubscript{3}−/NO\textsubscript{2}− assimilation gene clusters, which is consistent with a key role for the NasG ferredoxin in mediating electron flux from the NAD(+) oxidizing site in NasB to the sites of NO\textsubscript{3}− and NO\textsubscript{2}− reduction in NasC and NasB respectively, in order to prevent intracellular accumulation of NO\textsubscript{2}− [25]. In P. denitrificans, the RNA-binding protein NasT has been recently shown to positively and directly regulate nas expression (i.e. nasABGHC) by interacting with the nasA leader mRNA. The NO\textsubscript{3}−/NO\textsubscript{2}−-binding sensor NasS controls NasT activity and the NasS and NasT proteins co-purify as a stable heterotetrameric regulatory complex, NasS-NasT in the absence of inducer [21]. The NasS-NasT system from B. japonicum has now been characterized by Sánchez et al. [22] and shown to regulate expression of napE and nosZ genes for the dissimilatory denitrification pathway.

The processes and enzymes for rhizobial denitrification have been well studied [1–4]; however, the biochemical apparatus for NO\textsubscript{3}−/NO\textsubscript{2}− assimilation by plant-associated rhizobia has yet to be characterized. In the present work, we demonstrate a dual role for the blr2806–09 and blil4571–73 loci for NO\textsubscript{3}−/NO\textsubscript{2}− assimilation and NO management in B. japonicum.

EXPERIMENTAL

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the present study are listed in Supplementary Table S1. Gene deletion and transcriptional reporter construction used previously established methods [26,27], with key modifications as outlined below. B. japonicum strains were grown routinely under aerobic conditions at 30°C in peptone-salts-yeast extract (PSY) medium supplemented with 0.1% (w/v) L-arabinose [28]. Growth curves for different N-sources were performed in Bergersen minimal medium [29] supplemented with 10 mM KNO\textsubscript{3} (BN3), 1 mM NaNO\textsubscript{2} (BN2) or 10 mM KNO\textsubscript{3} plus 6.5 mM l-glutamate (BGN3) as sole N-sources and incubated aerobically or anaerobically. Anaerobic conditions were reached by incubating the cells in completely filled glass serum tubes. Growth was followed by measuring attenuation (D) of cell cultures at 600 nm.

To test growth inhibition by nitrosative stress, cells were grown in Bergersen minimal medium with 6.5 mM L-glutamate (BG) as sole N-source and incubated under microaerobic conditions in serum tubes sealed with rubber septa. The headspace of these tubes was filled with a gas mixture of 2% (v/v) O\textsubscript{2} and 98% (v/v) N\textsubscript{2} and was replaced with fresh gas mixture every 24 h. Nitrosative stress was induced by adding 1 mM sodium nitroprusside (SNP) or spermine NONOate to the cultures 24 h after inoculation. To test cell survival after nitrosative stress induction, cells were grown to early stationary growth phase under the same conditions as for growth inhibition experiments (final D value at 600 nm was ∼0.5). Then, 1 mM SNP or spermine NONOate was added to the cultures (replica cultures were not subjected to nitrosative stress as controls). Cell cultures were incubated at 30°C and 0.1 ml of samples were taken periodically, serially diluted in growth medium and plated into PSY-agar. Colonies were counted after incubation for 7 days under aerobic conditions. The capacity for colony formation of control cells was considered as 100% survival.

To induce the expression of NorCB as a cellular marker for NO, B. japonicum was grown in Bergersen minimal medium where glycerol was replaced with 10 mM succinate as carbon source (BS) [30,31]. The medium was supplemented with 10 mM KNO\textsubscript{3} as sole N-source (BSN3). In these experiments, the headspace was filled with 2% (v/v) O\textsubscript{2} and 98% N\textsubscript{2} (v/v). In contrast with microaerobic growth conditions, the atmosphere for cultures was not replaced. As such, cells consumed the O\textsubscript{2} present and reached anoxic conditions after 24 h incubation.

Antibiotics were added to B. japonicum cultures at the following concentrations (μg·ml\textsuperscript{-1}): chloramphenicol 20, tetracycline 100, spectinomycin 200, streptomycin 200 and kanamycin 200. Escherichia coli strains were cultured in LB medium [32] at 37°C including tetracycline 10, spectinomycin 20, streptomycin 20, kanamycin 30 and ampicillin at 200 μg·ml\textsuperscript{-1}. E. coli S17-1 served as the donor for conjugal plasmid transfer [33].

Construction of B. japonicum narK-lacZ and nirA-lacZ transcriptional fusions

For construction of transcriptional fusion reporter plasmids, DNA fragments for the narK (520 bp) and nirA (563 bp) promoter regions were amplified using primers narK-lacZ_For/narK-lacZ_Rev and nirA-lacZ_For/nirA-lacZ_Rev, respectively (see Supplementary Table S2 for oligonucleotide sequences). Fragments were digested with PstI or PstI-EcoRI and cloned into the lacZ fusion vector pSUP3535, which is derived from pSUP202 [33] to give plasmids pDB4009 and pDB4018 respectively (Supplementary Table S1). The correct orientation of cloned inserts was verified by sequencing.

Transcriptional fusion plasmids pDB4009 and pDB4018 were integrated by homologous recombination into the chromosome of wild-type (WT) B. japonicum USDA110 and nasS and nasT mutants to produce strains 4009, 4012-4009, 4013-4009, 4018, 4012-4018 and 4013-4018 detailed in Supplementary Table S1. Correct recombination was checked by PCR analysis of genomic DNA isolated from each strain.

Growth conditions for β-galactosidase activity assay of narK-lacZ, nirA-lacZ and norC-lacZ fusions

Strains 4009, 4012-4009, 4013-4009, 4018, 4012-4018 and 4013-4018 containing the narK-lacZ or nirA-lacZ reporter-fusion constructs (Supplementary Table S1) were grown aerobically in PSY medium. Cells were harvested by centrifugation at 8000 g for 10 min, washed twice with nitrogen-free Bergersen medium and cultured aerobically in the same medium or in BN3 medium, for 48 h (until a D value of ∼0.5 at 600 nm was obtained). To measure β-galactosidase activity from the norC-lacZ fusion,
plasmid pRJ2499 (Supplementary Table S1) was integrated by homologous recombination into the chromosome of *bjgb*, *nasC*, *napA* and *nasC*, *napA* mutants to produce strains 4001-2499, 4003-2499, GRPA1-2499 and GRPA1-4003-2499 respectively (Supplementary Table S1). In order to induce expression of nor genes, cells were cultured in BSN3 medium with 2% (v/v) initial O₂ concentration.

**Construction and complementation of *B. japonicum* mutants**

Genomic and plasmid DNA isolation was carried out using the REALPURE Genomic DNA purification Kit (Real) and Qiagen Plasmid Kit (Qiagen) respectively. Custom oligonucleotide primers were supplied by Sigma, PCR was performed using the High Fidelity DNA polymerase Phusion enzyme (Thermo) and DNA digestions were carried out using Fast digest enzymes (Fermentas). All mutants constructed in the present work were made by in frame deletion of the corresponding gene using the mobilizable pK18mobsacB suicide vector that conferred kanamycin resistance and sucrose sensitivity on the host (Supplementary Table S1). To generate mutant strains, upstream and downstream regions of relevant target genes were amplified by PCR using the gene-specific primer sets detailed in Supplementary Table S2.

For the *narK* deletion mutant, upstream (834 bp) and downstream (981 bp) DNA fragments flanking *blr2806* were amplified by PCR using *blr2806_up_For*/blr2806_up_Rev and *blr2806_down_For*/blr2806_down_Rev primer pairs (Supplementary Table S2). The 981-bp fragment was inserted into pK18mobsacB as an XbaI-HindIII fragment that contained a new unique XhoI restriction site immediately downstream of the XbaI site. Subsequently, the 834-bp fragment was inserted into this plasmid as a BamHI-XhoI fragment yielding plasmid pDB4000. Double recombination of pDB4000 with the *B. japonicum* genome led to the replacement of the WT *blr2806* gene encoding a 459 amino acid (aa) protein for an in frame truncated version encoding 38 aa.

To generate *bjgb* and *nasT* deletion mutants, upstream and downstream regions flanking *blr2807* (824 and 884 bp fragments) and *bll4573* (696 and 736 bp fragments) were amplified by PCR using gene-specific primer pairs, i.e. *blr2807_up_For*/blr2807_up_Rev and *blr2807_down_For*/blr2807_down_Rev for *blr2807* and *bll4573_up_For*/bll4573_up_Rev and *bll4573_down_Rev*/bll4573_down_Rev for *bll4573* (Supplementary Table S2). The PCR products containing the upstream regions of *blr2807* and *bll4573* were inserted separately into pK18mobsacB as EcoRI-XbaI fragments and subsequently the downstream PCR products were inserted into the respective plasmids as XbaI-HindIII DNA fragments yielding plasmids pDB4001 and pDB4013 (Supplementary Table S1). Double recombination of pDB4001 and pDB4013 with the *B. japonicum* genome led to the replacement of the upstream region of the *blr2807* gene encoding a 142 aa protein and the *bll4573* gene encoding a 196 aa protein with in-frame truncated versions of 33- and 27-aa for *blr2087* or *bll4573*, respectively.

To generate the *flp* and *napC* mutants, upstream and downstream regions of *blr2808* (983 and 856 bp) and *blr2089* (829 and 840 bp) were amplified by PCR using gene-specific primer pairs *blr2808_up_For*/blr2808_up_Rev and *blr2808_down_For*/blr2808_down_Rev for *blr2808* and *blr2089_up_For*/blr2089_up_Rev and *blr2089_down_For*/blr2089_down_Rev for *blr2089* (Supplementary Table S2). The PCR product containing the upstream regions of *blr2808* and *blr2089* were cloned into separate pK18mobsacB plasmids as EcoRI-BamHI fragments and subsequently the downstream DNA regions were inserted into the relevant plasmid as BamHI-HindIII fragments to give pDB4002 and pDB4003. Double recombination of either pDB4002 or pDB4003 with the *B. japonicum* genome led to the replacement of the *blr2808* gene (encoding a 418-aa protein) or the *blr2089* gene (encoding a 901-aa protein) for in-frame truncated versions encoding a 37- or 27-aa peptide respectively.

The *ntrABC* mutant was generated by double recombination of *B. japonicum* USDA110 genomic DNA with plasmids pDB4004 (Supplementary Table S1). To generate pDB4004, the upstream region of *blr2803* (773 bp) and the downstream region of *blr2805* (721 bp) were amplified by PCR using primer pairs *blr2803_up_For*/blr2803_up_Rev and *blr2805_down_For*/blr2805_down_Rev (Supplementary Table S2). Firstly, the PCR product containing the upstream region was digested with BamHI and XbaI restriction enzymes and cloned into pK18mobsacB. Then, the PCR product corresponding to the downstream region was inserted into the plasmid as an XbaI-PstI fragment to give plasmid pBD4004 (Supplementary Table S1). Double recombination of pBD4004 with the *B. japonicum* genome replaced *blr2803-05* genes for an in-frame truncated version encoding a 31-aa peptide.

All plasmids constructed for mutagenesis were sequenced and transferred via conjugation into *B. japonicum* USDA110 using *E. coli* S17-1 as donor strain. Double recombination events were favoured by first selecting single recombinants for kanamycin resistance and screening candidates by PCR. Then, selected clones containing the plasmid co-integrated in the genome were grown in PSY-agar medium supplemented with sucrose 10% (w/v) to select for double recombinants. Sucrose resistant colonies were checked for kanamycin sensitivity. Double recombinants were confirmed by PCR.

*B. japonicum* GRC131-4001 containing a double mutation in *bjgb* and *norC* was generated by transferring plasmid pJNOR43M2 [34] via conjugation into the *B. japonicum* *bjgb* mutant (Supplementary Table S1). Double recombinants were selected for kanamycin resistance and tetracycline sensitivity. The correct replacement of the WT *norC* gene by kanamycin resistance gene (*aphII*) insertion was checked by PCR.

*B. japonicum* GRPA1-4003 containing a double mutation in *napA* and *nasC* was generated by transferring plasmid pBG602Ω [26] via conjugation into the *B. japonicum* *nasC* mutant (Supplementary Table S1) using *E. coli* S17-1 as donor strain. Double recombination events were favoured by growth on agar plates containing sucrose. Mutant strains resistant to spectinomycin/streptomycin, but sensitive to kanamycin were checked by PCR for correct replacement of the WT fragment by the Ω interposon.

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The *bijg*, *flp* and *nasC* strains were complemented with pDB4014, pDB4015, pDB4017 expression constructs containing the corresponding intact genes (Supplementary Table S1). For this, *bijg*, *flp* and *nasC* genes were amplified by PCR using primer sets blr2807_Rev/blr2707_For, blr2808_Rev/blr2808_For and blr2809_For/blr2809_Rev (Supplementary Table S2). DNA fragments containing the relevant ORF and Shine–Dalgarno sequence were cloned separately into pTE3 vector [35]. All complementation constructs were sequenced and transferred via conjugation into the relevant *B. japonicum* mutant using *E. coli* S17-1 as donor strain. Complemented strains 4001-pDB4014, 4002-pDB4015 and 4003-pDB4017 (Supplementary Table S1) were confirmed by plasmid extraction and checked by restriction analyses and PCR.

**Analysis of gene expression by RT-PCR**

Total RNA was isolated from *B. japonicum* cells grown anaerobically to a *D* of ~0.4 (at 600 nm) in BN3 medium, as previously described [36]. First strand cDNA synthesis was performed with the SuperScript II reverse transcriptase (Invitrogen) according to the supplier’s guidelines, using 1 μg of total RNA and primer e (Supplementary Table S2). cDNA generated was used for amplification of putative intergenic regions between blr2805 and blr2809, using primers pairs a1/a2 to d1/d2 (Supplementary Table S2), essentially as described by Sambrook and Russell [32]. In negative controls, reverse transcriptase was omitted and for positive controls PCR was performed with *B. japonicum* USDA110 genomic DNA as template.

**β-Galactosidase assays**

β-Galactosidase activity was determined using permeabilized cells from at least three independently grown cultures assayed in triplicate for each strain and condition, as previously described [37]. The absorbance data at 420 and 600 nm were determined for all samples and cultures in a plate reader (SUNRISE Absorbance Reader, TECAN), using the software XFFluor4 (TECAN) and specific activities were calculated in Miller units.

**Determination of NO₃⁻ reductase and NO₂⁻ reductase activity**

*B. japonicum* was 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 to a *D* value of ~0.4 (at 600 nm) in fresh minimal medium of the same composition. Following 72 h incubation under relevant conditions, cells were harvested, washed with 50 mM Tris/HCl buffer (pH 7.5) to remove excess NO₂⁻ and then resuspended in 1 ml of the same buffer prior to assay for enzymatic activity. Methyl viologen-dependent NO₃⁻ reductase (MV-NR) and NO₂⁻ reductase (MV-NIR) activity was assayed essentially as described by Delgado et al. [26]. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 200 μM MV, 100 μl of cell suspension (with 0.02–0.04 mg of protein) and 10 mM KNO₃ or 100 μM NaNO₃, for MV-NR or MV-NIR activity respectively. Methyl viologen was reduced by the addition of freshly prepared sodium dithionite (dissolved in 300 mM NaHCO₃ solution) at a final concentration of 14.4 mM.

**Haem-staining analysis**

Aerobically grown *B. japonicum* cells were harvested by centrifugation, washed twice with BN3 medium and resuspended in 500 ml of fresh medium of the same composition. Microaerobic conditions were then established with 2% (v/v) initial O₂ concentration and cells were cultured for 48 h until a final *D* of ~0.5 (at 600 nm) was reached. Cells were disrupted using a French pressure cell (SLM Aminco, Jessup) and membranes were isolated as described previously [26]. Membrane protein aliquots were diluted in sample buffer [124 mM Tris/HCl, pH 7.0, 20% (v/v) glycerol, 5% (v/v) SDS and 50 mM 2-mercaptoethanol] and incubated at room temperature for 10 min. Membrane proteins were separated at 4 °C by SDS-PAGE [12% (v/v) acrylamide resolving gel with 2% (v/v) acrylamide stacking gel] and bovine liver catalase (250 units ml⁻¹) and Succinyl-CoA:3-oxoacid CoA-transferase (EC 2.3.1.9) (Sigma-Aldrich), 900 μl of cell suspension (4–5 mg protein), 100 μl of an enzyme mix containing *Aspergillus niger* glucose oxidase (40 units ml⁻¹) and bovine liver catalase (250 units ml⁻¹) (Sigma-Aldrich), 90 μl of 1 M sodium succinate and 100 μl of 320 mM glucose. Once a steady base line was obtained, 50 μl of a saturated NO solution (1.91 mM at 20°C) was added to the cuvette to start the reaction. Each assay was monitored until the NO detection had dropped to zero, i.e. when all NO was consumed.

**N₂O measurements**

*B. japonicum* cells were cultured as indicated above for NO consumption experiments, except that in addition to 2% (v/v) initial O₂, the headspace of the cultures also contained 10% (v/v) acetylene in order to inhibit N₂O reductase activity. After 96 h growth, gaseous samples were taken from the headspace of cultures. N₂O was measured using an HP 4890D gas chromatograph instrument equipped with an electron capture detector (ECD). The column was packed with Porapak Q 80/100 MESH and the carrier gas was N₂ at a flow rate of 23 ml min⁻¹. The injector, column and detector temperatures were 125, 60 and 375 °C respectively. The samples were injected manually using a Hamilton® Gastight syringe. Peaks corresponding to N₂O were integrated using GC ChemStation Software (Agilent Technologies®) and the concentrations of N₂O in each sample were calculated using N₂O standards (Air Liquid).

**RESULTS**

**Genetic basis for NO₃⁻ and NO₂⁻ assimilation in endosymbiotic denitrifying rhizobia**

*B. japonicum* USDA110 contains a putative assimilatory NO₃⁻ reductase encoded at blr2809 (Figure 1A) [19,20]. Experiments confirmed that *B. japonicum* is able to grow aerobically or anaerobically using NO₃⁻ as sole N-source with values for *μ*ₘₐₓ
Nitrate assimilation and nitric oxide detoxification in *B. japonicum*

Figure 1 Organization of regulatory and structural genes for the assimilatory NO$_3^-$/NO$_2^-$ reductase pathway in *B. japonicum*

(A) Schematic representation of the blr2803-5, blr2806-09 and blr4571-73 ORFs investigated in the present study. Putative intergenic regions probed by RT-PCR to determine the transcriptional architecture of the blr2806-09 region (i.e. nark-bjgb-flp-nasC) are labelled a–d. (B) The results for RT-PCR analysis obtained by agarose gel electrophoresis for regions a–d. Total RNA isolated from cells grown anaerobically with NO$_3^-$ served as the template for cDNA synthesis, whereas PCR amplifications using genomic DNA and without reverse transcriptase enzyme served as positive and negative controls respectively (as indicated above lanes).

Notably, blr2809 lies downstream of several putative ORFs with predicted roles in N-metabolism (Figure 1A). To investigate the transcriptional architecture of this region, RT-PCR experiments were performed to detect intergenic regions (a–d). Here, specific cDNA was obtained for all regions except ‘a’ (Figure 1B). These findings reveal that blr2806–09 constitute a transcriptional unit. Thus, in-frame deletion strategies for subsequent molecular genetics experiments were adopted to prevent possible polar effects on co-transcribed genes (see ‘Experimental’ section for details).

Analysis of the amino acid sequence of blr2809 suggests the protein is a member of the molybdobacterin bis-molybdopterin dinucleotide cofactor binding superfamily and contains consensus motifs for co-ordination of an N-terminal [4Fe-4S] cluster and a C-terminal [2Fe-2S] cluster. This general organization is similar to other assimilatory NO$_3^-$ reductases, including *P. denitrificans* NasC and *Klebsiella oxytoca* NasA from the α- and γ-proteobacterial clades, respectively [25]. Accordingly, we adopt the α-proteobacterial nomenclature, NasC, for the *B. japonicum* protein encoded at blr2809 hereafter. A *B. japonicum* strain that was mutated in in-frame deletion of nasC lost the capacity for aerobic or anaerobic growth with NO$_3^-$ as sole N-source (Figures 2A and 2B; Supplementary Table S3). However, this strain retained the ability to grow using NO$_2^-$ as sole N-source and displayed similar growth kinetics to WT [μ$_{max}$ (app) ∼ 0.03 h$^{-1}$] (Figure 3A; Supplementary Table S3).

The genome of *B. japonicum* also contains an ORF for a putative assimilatory NO$_3^-$ reductase (*nirA*) at blr4571, a distinct locus situated ∼2 Mb from nasC on the chromosome (Figure 1A). NirA contains canonical cysteine-rich motifs in central and C-terminal sequence regions for iron–sulfur co-ordination and formation of the sirohaem NO$_3^-$ reductase/sulfite reductase ferredoxin half-domain respectively. However, NirA lacks N-terminal FAD- and NAD(P)H-binding domains present in bacterial NirB-type NAD(P)H-dependent NO$_3^-$ reductases [25]. Deletion of *nirA* resulted in *B. japonicum* being unable to grow aerobically or anaerobically with either NO$_3^-$ or NO$_2^-$ as sole N-source (Figures 2A, 2B and 3A; Supplementary Table S3). The ability of WT and *nirA* cells to consume 1 mM NO$_2^-$ during incubation experiments was tested (Figure 3B, open symbols). Whereas all NO$_2^-$ was removed from minimal medium after ∼6 days by WT cells, no significant decrease in extracellular NO$_2^-$ was observed in *nirA* mutant cultures (Figure 3B). Conversely, NO$_2^-$ production experiments using 10 mM NO$_3^-$, as sole N-source, revealed that WT cells did not accumulate NO$_2^-$ in the extracellular medium (Figure 3B, closed symbols). However, accumulation of ∼1 mM NO$_2^-$ was observed following incubation of the *nirA* mutant with NO$_3^-$ (Figure 3B).

The putative flavoprotein (Flp), encoded at blr2808, contains canonical FAD- and NAD(P)H-binding domains typical of cytoplasmic NAD(P)H-dependent oxidoreductases present in several bacterial Nas operons [23] and is a strong candidate for mediating electron transfer to NasC and/or NirA. A *B. japonicum* flp mutant was unable to grow aerobically or anaerobically with NO$_3^-$ as the sole N-source (Figures 2A and 2B; Supplementary Table S3). However, the *flp* mutant displayed similar growth kinetics and yields [μ$_{max}$ (app) ∼ 0.03 h$^{-1}$, maximum D (at 600 nm) = 0.43 ± 0.08] to that observed for WT [μ$_{max}$ (app) ∼ 0.03 h$^{-1}$, maximum D (at 600 nm) = 0.51 ± 0.01] when cultured aerobically with NO$_3^-$ (Figure 3A; Supplementary Table S3). These findings suggest that Flp mediates electron transfer to NasC, but not to NirA. In order to confirm that deletion of *flp* did not influence expression...
of downstream genes, relevant strains were complemented with either pDB4017 (nasC) or pDB4015 (flp) constructs. The presence of pDB4017 and pDB4015 plasmids restored both aerobic and anaerobic growth of the nasC and flp mutants in the presence of NO₃⁻ to near WT levels, thereby verifying the phenotypes observed (Supplementary Table S3).

Deletion of the blr2803–05 ORFs, predicted to encode an NrtABC-type NO₃⁻ transporter, did not affect the capacity of the cells to grow with NO₃⁻ as sole N-source (Supplementary Table S3). Bioinformatics analysis of blr2806 revealed that it encodes a putative member of the MFS of membrane proteins, sharing 66% and 59% amino acid similarity with the NO₃⁻/NO₂⁻ antiporters E. coli NarK [40] and P. denitrificans NarK2 [41] respectively (Supplementary Figure S1). Thus, we term this MFS-type transporter NarK rather than the generic ‘nitrite extrusion protein’ genome annotation currently assigned (http://genome.kazusa.or.jp/rhizobase/).

A B. japonicum narK mutant showed improved growth kinetics and yields when cultured aerobically \([\mu_{\text{max}} \text{(app)}] \approx 0.09 \text{ h}^{-1}\), maximum \(D\) (at 600 nm) = 0.98 ± 0.05) or anaerobically \([\mu_{\text{max}} \text{(app)}] \approx 0.07 \text{ h}^{-1}\), maximum \(D\) (at 600 nm) = 0.89 ± 0.09) with NO₃⁻ as sole N-source when compared with aerobic \([\mu_{\text{max}} \text{(app)}] \approx 0.06 \text{ h}^{-1}\), maximum \(D\) (at 600 nm) = 0.73 ± 0.12) or anaerobic \([\mu_{\text{max}} \text{(app)}] \approx 0.04 \text{ h}^{-1}\), maximum \(D\) (at 600 nm) = 0.61 ± 0.03] growth of WT under the same conditions (Figures 2A and 2B; Supplementary Table S3). Furthermore, following 24 h aerobic growth, the narK mutant accumulated \(\sim 2\)-fold higher levels of intracellular NO₂⁻ than that accumulated by WT cells, i.e., 5.3 ± 0.7 compared with 2.2 ± 0.1 nmol NO₂⁻ mg-protein \(^{-1}\) for the narK and WT strains respectively (Supplementary Figure S2). The addition of L-glutamate to minimal growth medium restored the inability of the nasC, nirA and flp mutants to grow with NO₃⁻ under aerobic or anaerobic conditions (Supplementary Table S3). Under these conditions, growth yields obtained from the narK mutant were also similar to those obtained from WT cells (Supplementary Table S3). Collectively, these results confirm the importance of NarK, Flp, NasC and NirA for NO₃⁻ assimilation by B. japonicum.

The regulatory proteins encoded by bli4573 (nasT) and bli4572 (nasS) constitute a NO₃⁻/NO₂⁻ responsive two-component system, NasS-NasT, which has been recently reported in B. japonicum [22]. A B. japonicum nasT mutant strain showed significant growth attenuation compared with the WT cells when cultured aerobically with either NO₃⁻ (Figure 4A; Supplementary
Nitrate assimilation and nitric oxide detoxification in B. japonicum

A biochemical pathway for assimilation of NO$_3^-$ and NO$_2^-$

The biochemical basis of growth phenotypes observed for the various deletion strains was examined by enzymatic activity assay of whole cells, using dithionite-reduced methyl viologen, as an artificial electron donor. Here, MV-NR and MV-NIR activities were measured in WT and nasC, nirA, flp, bjgb and narK mutants, following aerobic incubation with NO$_3^-$ as sole N-source (Table 1). Since B. japonicum has periplasmic respiratory NO$_3^-$ reductase (NapABC) and NO$_2^-$ reductase (NirK) systems that might also use methyl viologen as an electron donor [26,42], control experiments using napA and nirK mutants were also performed in the present study. Importantly, and as expected, the respective MV-NR and MV-NIR activity levels observed in napA and nirK cells were similar to those observed in WT cells (Table 1), indicating that the contribution of the NapABC or NirK respiratory enzymes was not significant in cells cultured under aerobic conditions. This provided a solid platform for subsequent experiments.

Significantly, MV-NR activity was not detectable in nasC cells, but a similar level of MV-NIR activity was observed compared with WT cells. This was consistent with the loss of assimilatory NO$_3^-$ reductase expression, but not NO$_3^-$ reductase expression, in nasC cells (Table 1). MV-NR activity could be restored to WT levels in the nasC mutant, when the deletion was complemented with a corresponding plasmid-borne gene copy. Also, MV-NIR activity was absent from the nirA mutant (Table 1), consistent with the loss of assimilatory NO$_3^-$ reductase expression. However, the nirA mutant showed similar levels of MV-NR activity present in the bacterial strain following incubation with NO$_3^-$.

Additional experiments revealed that MV-NR levels of flp cells showed an apparent ~2-fold increase in activity compared with WT incubation with NO$_3^-$, but MV-NIR activity was relatively similar in both flp and WT cells (Table 1). That the absence of Flp (i.e. the proposed electron donor and partner to NasC) should increase MV-NR activity may result from modulation in catalytic activity of the isolated NasC protein. Alternatively, without Flp, the artificial chemical electron donor could have greater access to NasC and thus may enhance NO$_3^-$ reductase activity. Finally, as shown in Table 1, MV-NR and MV-NIR activities of bjgb or narK mutants were similar to those observed in WT cells.

Regulation of the narK-bjgb-flp-nasC operon and nirA by NasS-NasT

In order to test the involvement of the NasT regulatory protein in NO$_3^-$-dependent induction of the narK-bjgb-flp-nasC operon and nirA gene, we examined expression of narK-lacZ and nirA-lacZ transcriptional fusion constructs in WT and nasT mutant cells following aerobic culture in the presence or absence of the inducer NO$_3^-$ (Table 2). Whereas similar low levels of β-galactosidase activity were observed from both fusions in WT cells incubated without NO$_3^-$, the presence of this molecule induced expression of the narK-lacZ and nirA-lacZ transcriptional fusions by approximately 5- and 3-fold respectively. However, β-galactosidase activity from the narK-lacZ reporter was undetectable in the nasT strain regardless of whether NO$_3^-$ was present or not (Table 2). Although similar basal levels of nirA-lacZ expression were observed in WT and nasT cells incubated without NO$_3^-$, a decrease of approximately 2-fold was found in nasT compared with WT when cells were incubated in the presence of NO$_3^-$ (Table 2).

Table 1 MV-NR and MV-NIR activities of B. japonicum strains incubated aerobically in minimal medium with NO$_3^-$ as sole N-source

| B. japonicum str. | Genotype | Activities |
|-------------------|----------|------------|
| USDA 110          | WT       | 32.0 ± 5.2 | 6.9 ± 0.9 |
| GRPA1             | napA     | 32.1 ± 0.5 | –         |
| GRK038            | nirK     | –          | 7.5 ± 0.8 |
| 4003              | nasC     | n.d.       | 7.2 ± 1.2 |
| 4003-pDB4017      | nasC (pDB4017) | 30.5 ± 4.8   |
| 4011              | nirA     | 49.7 ± 1.8 | n.d.      |
| 4002              | flp      | 68.3 ± 6.7 | 6.1 ± 0.8 |
| 4001              | bjgb     | 28.4 ± 5.0 | 6.1 ± 0.4 |
| 4000              | narK     | 35.8 ± 2.2 | 10.9 ± 1.5 |

*MV-NR and MV-NIR activities are expressed as nanomoles of NO$_3^-$ produced or consumed min$^{-1}$mg protein$^{-1}$. Data are expressed as the mean value ± S.D. from at least two different cultures assayed in triplicate; –, not determined; n.d., not detectable.

Figure 4 Growth curves for the B. japonicum nasS and nasT mutants

Growth of WT (●), nasS (○) and nasT (□) strains was measured in minimal medium, under aerobic conditions, with either NO$_3^-$ (A) or NO$_2^-$ (B), as sole N-source. The results presented are the mean of two biological replicates assayed in triplicate.
### Table 2  \( \beta \)-Galactosidase activity for \( \text{nark-lacZ} \) and \( \text{narA-lacZ} \) fusions in \( \text{B. japonicum} \) WT, \( \text{nasS} \) or \( \text{nasT} \) strains

Cells were cultured under aerobic conditions, in minimal medium, with or without \( \text{NO}_3^- \) as sole N-source. Data are means \( \pm \) S.D. from at least three independent cultures, assayed in triplicate; n.d., not detectable.

| \( \text{B. japonicum} \) str. | Relevant genotype | \( -\text{NO}_3^- \) Miller units | \( +\text{NO}_3^- \) Miller units |
|-------------------------------|-------------------|--------------------------|--------------------------|
| 4009 WT: \( \text{nark-lacZ} \)  | 153 \( \pm \) 40 | 759 \( \pm \) 54 |
| 4012-4009 \( \text{nasS}:\text{nark-lacZ} \) | 972 \( \pm \) 132 | 897 \( \pm \) 66 |
| 4013-4009 \( \text{nasT}:\text{nark-lacZ} \) | n.d. | n.d. |
| 4018 WT: \( \text{nirA-lacZ} \) | 137 \( \pm \) 22 | 395 \( \pm \) 56 |
| 4012-4018 \( \text{nasS}:\text{nirA-lacZ} \) | 412 \( \pm \) 37 | 372 \( \pm \) 31 |
| 4013-4018 \( \text{nasT}:\text{nirA-lacZ} \) | 163 \( \pm \) 34 | 203 \( \pm \) 13 |

Additional studies to examine the role of \( \text{NasS} \) in \( \text{NasT} \)-dependent induction of the \( \text{nark-bjgb-flp-nasC} \) operon and \( \text{nirA} \) gene were also performed, using \( \text{nark-lacZ} \) or \( \text{narA-lacZ} \) reporters. Here, \( \beta \)-galactosidase assays revealed that, in the absence of \( \text{NO}_3^- \), the activity of each reporter fusion was significantly higher (approximately 6- and 3-fold for \( \text{narK-lacZ} \) and \( \text{nirA-lacZ} \) respectively) in \( \text{nasS} \) cells compared with WT cells (Table 2). These results imply that, in the absence of \( \text{NO}_3^- \), \( \text{NasS} \) is a repressor of \( \text{nark-bjgb-flp-nasC} \) and \( \text{nirA} \) transcription. When equivalent experiments were performed in WT and \( \text{nasS} \) cells that had been pre-exposed to \( \text{NO}_3^- \), expression levels for each reporter-fusion were very similar (Table 2).

Collectively, the reporter-fusion results suggest an inhibitory role for \( \text{NasS} \) in \( \text{NasT} \)-dependent induction of gene expression in \( \text{B. japonicum} \) and that \( \text{NO}_3^- \)-responsive control of both \( \text{nark-bjgb-flp-nasC} \) and \( \text{nirA} \) assimilatory gene expression is lost \( \text{in vivo} \) without \( \text{NasS} \). This mode of regulation is analogous to \( \text{NO}_3^-\) to \( \text{NO}_2^-\) conversion, which requires \( \text{NO}_3^-\) detoxification and nitrosative stress defence systems for bacterial survival [43,44]. To investigate the role of Bjgb in NO-metabolism, the nitrosative stress agent SNP was added (at 1 mM final concentration) to microaerobic \( \text{B. japonicum} \) cultures following growth in minimal medium with \( \text{L-glutamate} \) (BG) as sole N-source. Growth of WT cells was not significantly perturbed, whereas addition of SNP resulted in transient growth arrest of \( \text{bjgb} \) and \( \text{flp} \) strains that was restored after 24 h (Figure 5A). Perhaps most significantly, a \( \text{norC} \) or a \( \text{bjgb;norC} \) double mutant showed a substantially longer period of growth inhibition of approximately 7 days following addition of SNP to cultures (Figure 5A). The effect of SNP on cell viability was also assayed by performing viable cell counts on samples taken at intervals spanning a 5-h period following addition of SNP to cultures. Although WT cell viability was not significantly affected, addition of SNP caused a \( \sim60\% \) decrease in cell survival for \( \text{norC} \) or \( \text{bjgb} \) cultures after 2 h (Figure 5B). The most prominent effect was observed with the \( \text{bjgb;norC} \) double mutant, which was the most sensitive to nitrosative stress. Here, approximately 80 \% of cells were killed within 1–2 h following SNP exposure (Figure 5B). Furthermore, the addition of SNP provoked a \( \sim40\% \) decrease in \( \text{flp} \) viability after 2 h incubation. These results revealed the importance of Bjgb and Flp for protection against nitrosative stress in \( \text{B. japonicum} \) under free-living conditions. NO is a product of SNP breakdown and a similar sensitivity of \( \text{bjgb} \) or \( \text{flp} \) mutants to NO was observed using spermine NONOate as an NO-generating compound (result not shown). Importantly, complementation with pDB4014 (harbouring a functional plasmid-borne copy of \( \text{bjgb} \)) allowed the \( \text{bjgb} \) mutant to grow anaerobically with \( \text{NO}_3^- \) to near WT levels (Supplementary Table S3). This confirmed that the growth phenotype observed for the \( \text{bjgb} \) mutant was not caused by a downstream effect on \( \text{flp} \) gene expression.
Table 3 NO consumption activity and N₂O levels for B. japonicum WT, bjgb, flp, norC and bjgb:norC strains cultured in BSN3 minimal medium under 2% (v/v) initial O₂.

Data are expressed as the means ± S.D. from at least two different cultures assayed in triplicate; n.d., not detectable.

| B. japonicum str. | Genotype | NO consumption activity (nmol h⁻¹ mg⁻¹ protein⁻¹) | N₂O (mM) |
|------------------|----------|-----------------------------------------------|-----------|
| USDA110          | WT       | 155 ± 29                                      | 1.04 ± 0.26 |
| 4001             | bjgb     | 384 ± 60                                      | 2.34 ± 0.16 |
| 4002             | flp      | 101 ± 17                                      | 0.88 ± 0.03 |
| GRC131           | norC     | 97 ± 14                                       | n.d.       |
| GRC131-4001      | bjgb:norC| 92 ± 18                                       | n.d.       |

NO formed during NO₃⁻ assimilation induces nor gene expression

To further investigate the role of Bjgb and Flp in NO metabolism, the ability of B. japonicum bjgb and flp strains to consume NO was analysed. Here, cells were incubated in BSN3 medium, with 2% initial O₂ and NO consumption rates were determined using an NO-electrode (Supplementary Figure S3). A ~2.5-fold increase in NO consumption was observed in the bjgb mutant compared with the WT strain (Table 3). This increase was not observed in the flp mutant, which showed NO consumption rates marginally lower than that observed in WT cells (Table 3; Supplementary Figure S3). NO consumption in the norC or the bjgb:norC mutants was approximately 1.6- and 1.7-fold lower respectively, compared with that observed in WT cells (Table 3; Supplementary Figure S3). The presence of residual activity in the bjgb:norC implies that under our experimental conditions, another enzyme(s) or perhaps a chemical process may be involved in NO consumption. The ability of bjgb cells to produce N₂O following incubation in BSN3 medium with 2% initial O₂ was also investigated. The bjgb mutant produced approximately 2.5-fold more N₂O than WT cells. By contrast, the level of N₂O produced by the flp mutant was comparable to WT (Table 3). Given that N₂O production was not detected for either the norC or the bjgb:norC mutants, this suggested the NorCB enzyme was the main source of N₂O in vivo.

To test whether the higher levels of NO consumption and N₂O production observed by the bjgb mutant were due to an induction of NorCB expression, norC transcription and relative abundance of NorC in membrane extracts were analysed, using a norC-lacZ transcriptional fusion and haem staining SDS-PAGE respectively. Firstly, a ~2-fold increase in norC-lacZ expression was observed in the bjgb mutant compared with WT (Figure 6). Given that the norC promoter is highly sensitive to N-oxides, including NO, an induction of β-galactosidase activity implies that Bjgb may act as a net sink for NO in WT cells. By contrast, β-galactosidase activity of the norC-lacZ transcriptional fusion was similar for both the nasC and the napA mutants, being approximately 3-fold lower compared with WT levels (Figure 6). Activity of the norC-lacZ transcriptional fusion was essentially abolished in the nasC:napA double mutant, implying that NO₁⁻ reduction by NasC or NapA was the source of NO required for norC-lacZ expression (Figure 6).

SDS-PAGE analysis of membranes (that were normalized for total protein) by haem staining was used as a qualitative assay for expression of the NorC cytochrome. In bjgb cells, NorC levels were significantly increased relative to WT (Figure 6 inset; compare lanes 1 and 2). However, a clear decrease in NorC expression was observed in the nasC mutant compared with WT (Figure 6 inset; compare lanes 1 and 3). Furthermore, haem staining failed to detect NorC expression in membranes prepared from either the napA or the nasC:napA mutant (Figure 6 inset; compare lane 1 with lane 4 or 5).

DISCUSSION

Defining the key components and transcriptional architecture of NO₃⁻ and NO₂⁻ assimilation in B. japonicum

A series of molecular genetics studies have established that genes encoded at two distinct loci, bli2806–09 and bli4571–73 of the B. japonicum genome (http://genome.kazusa.or.jp/rhizobase/), encode structural and regulatory components of a combined assimilatory NO₃⁻ reductase and NO detoxification system (Figure 1). RT-PCR experiments demonstrate that the narK-bjgb-flp-nasC genes (present at bli2806–09 respectively) constitute a transcriptional unit. However, three putative genes (bli2803–05) predicted to encode a NO₁⁻ transport system (similar to NrtABC, reviewed in [45]) and that lie immediately upstream of the narK operon are transcribed from a different promoter. The nasTS-nirA gene cluster (present at bli4571–73 respectively) lies some 2 Mb from the narK operon in the genome and encodes a NO₃⁻/NO₂⁻ responsive two-component regulatory system, NasS-NasT [22] and a putative ferredoxin-dependent NO₂⁻ reductase (NirA).

A role for the bjgb (bli2807) gene product in NO detoxification has been described [3,20], but the functions of other putative proteins encoded within the narK operon and biochemical components for the assimilatory NO₃⁻ reductase pathway in B. japonicum were unknown. In the present work, we have demonstrated that the assimilatory NO₃⁻ reductase (we rename herein as NasC) is encoded by bli2809 and is essential for NO₂⁻-dependent growth. The second core cytoplasmic enzyme component of the NO₁⁻ assimilation pathway is NirA, which is required for growth on either NO₃⁻ or NO₂⁻ as sole N-source. Consistent with our findings, it has recently been demonstrated that NirA (encoded by bli4571) is required for utilization of...
\[ \text{NO}_3^- \text{ or NO}_2^- \text{ as sole N-source in } B. \text{japonicum} \] 46]. NO\textsubscript{3}^- dependent induction of nasC (as part of the narK operon) and nirA expression is mediated by the two-component regulator NasS-NasT, an observation that is consistent with the role of this system in other α-proteobacteria [21].

Phenotypic analyses of a mutant lacking Flp (encoded by \( bJr2808 \)) suggest that Flp mediates electron transfer to NasC, but not to NirA. Consecutive genes from the same operon encode Flp and NasC, but lie in a different genetic locus to \( bJr4571 \) (nirA). This genetic organization may explain the requirement of Flp for \( \text{NO}_3^- \) assimilation but not for \( \text{NO}_2^- \) assimilation, which instead is ferredoxin dependent (Figure 7).

In contrast with B. japonicum, in P. denitrificans the regulatory and structural elements for a cytoplasmic \( \text{NO}_3^- \)/\( \text{NO}_2^- \) reductase system comprise a large gene cluster, \( \text{nasTSABGHC} \) [23]. The absence of a \( \text{nasG} \) homologue in either the narK operon or the nirA cluster in B. japonicum is notable. NasG may mediate electron flux to both the \( \text{NO}_3^- \) reductase and the \( \text{NO}_2^- \) reductase in other bacteria to prevent accumulation of excess \( \text{NO}_3^- \) by \( \text{NO}_3^- \) reduction in the cytoplasmic compartment [23,25]. Instead, for B. japonicum, genes encoding systems for \( \text{NO}_2^- \) transport and NO-detoxification are present within the operon encoding the \( \text{NO}_2^- \) reductase, which generates \( \text{NO}_3^- \).

Sequence comparison of \( bJr2806 \) with homologous proteins from diverse bacterial phyla suggests that this gene encodes an MFS-type \( \text{NO}_3^- \)/\( \text{NO}_2^- \) antiporter with similarity to E. coli NarK. The capacity of a B. japonicum narK mutant to accumulate \( \text{NO}_2^- \) inside the cell demonstrates the involvement of NarK in \( \text{NO}_2^- \) export. Further, phenotypic analyses reveal that NarK is not the main system for cytoplasmic \( \text{NO}_3^- \) import, as narK cells were still able to grow on \( \text{NO}_3^- \). Instead, the narK mutant showed enhanced growth compared to WT cells with \( \text{NO}_3^- \) as sole N-source, either under aerobic or anaerobic conditions. These observations imply that NarK acts to lower cytoplasmic \( \text{NO}_3^- \) levels by exporting \( \text{NO}_2^- \) to the periplasm and this process may involve corresponding import of \( \text{NO}_3^- \) (Figure 7) [40]. In this respect, it is significant that B. japonicum NarK performs a very different role to the MFS-type \( \text{NO}_3^- / \text{NO}_2^- \) transporter NasA, which supplies \( \text{NO}_3^- \) to the cytoplasmic \( \text{NO}_3^- / \text{NO}_2^- \) reductase pathway in other α-proteobacteria [23]. Instead, by counteracting \( \text{NO}_2^- \) accumulation, the B. japonicum NarK protein may thus represent a first level of protection to mitigate the production of cytotoxic NO by adventitious reduction of \( \text{NO}_3^- \) within the cytoplasm [43]. However, as a consequence, in WT cells NarK may also lower substrate availability for NirA and thus limit growth on \( \text{NO}_3^- \).

Deletion of \( bJr2803-05 \) that bioinformatics analyses had predicted to collectively encode an NtrABC family transporter, did not affect the ability of B. japonicum to assimilate \( \text{NO}_3^- \) as sole N-source. Therefore, the main route(s) for assimilatory \( \text{NO}_3^- \) import remains to be established. Although \( bJr2803-05 \) are not required for \( \text{NO}_3^- \) assimilation, there are other NtrABC-like candidates present on the chromosome (e.g. \( bJr5732-34 \)) that may facilitate \( \text{NO}_3^- \) import to the cytoplasm.

**A modular detoxification system for NO generated during \( \text{NO}_3^- \) assimilation**

In general, Nas systems have a high degree of structural plasticity, yet most contain proteins for transport and reduction of \( \text{NO}_3^- \) and \( \text{NO}_2^- \) [23,25,45,47,48]. In the present work, a novel \( \text{NO}_3^- \) assimilation system that also includes proteins for NO-detoxification is reported. The \( \text{narK-bjgB-flp-nasC} \) operon in B. japonicum encodes the sdHb BjgB [3,20], which is homologous to the N-terminal haem-containing domain of E. coli Fhh (Hmp) as well as the sdHbs from V. stercoraria (Vgb) and Campylobacter jejuni (Cgb) [20].

Deletion of \( bjgB \) had a strong negative affect on O\textsubscript{2}-limited growth with \( \text{NO}_3^- \) as sole N-source, relative to WT, which implies a role for BjgB in protecting B. japonicum cells from nitrosative stress. Importantly, in the absence of BjgB, \( \text{NO}_2^- \) respiring cells were also highly sensitive to exogenous NO. Since growth of the \( bjgB \) mutant was not affected under aerobic conditions, the role of BjgB may be restricted to anaerobic \( \text{NO}_3^- \)–dependent growth. However, our data suggest that the contribution of BjgB to \( \text{N}_2\text{O} \) production in vivo is low. These observations are consistent with studies performed in E. coli, which reveal Hmp can reduce NO to \( \text{N}_2\text{O} \) under anaerobic conditions, but with a much lower rate compared with the activity of the FHHd NorV [44]. Furthermore, expression of the respiratory NorCB is significantly up-regulated in the \( bjgB \) mutant, relative to WT (see Figure 6), in response to increased intracellular NO levels that arise during \( \text{NO}_3^- \)–dependent growth. This result suggests that increased NorCB expression may counteract accumulation of cytotoxic NO and may partially compensate for the absence of the cytoplasmic BjgB NO-detoxification system to maintain cell viability, albeit with a detrimental impact on anaerobic growth. Consequently, the bulk of the \( \text{N}_2\text{O} \) produced by the \( bjgB \) mutant can be attributed to NorCB activity, which is increased by \( \sim 2\)-fold relative to WT levels.

In E. coli Hmp, the FAD prosthetic group within the C-terminal NADH-reductase domain provides electrons from NAD(P)H that are required to reduce the NO-bound haem active site and complete the catalytic cycle. Aside from NO dioxygenation, Hmp has also been shown to perform slower reduction of NO to \( \text{N}_2\text{O} \) under anoxic conditions, which operates at approximately 1% of the rate observed for aerobic dioxygenase activity [49–52]. In the case of Cgb (an sdHb family protein that like BjgB lacks the reductase domain present in the Fhh Hmp), the electron–donor
protein remains to be identified. However, recent heterologous expression studies of Cgb in E. coli have reported a minor role for the NADH-(flavo)rubredoxin oxidoreductase NorW [53]. In B. japonicum, the enhanced sensitivity of the flp mutant to chemical NO-donors suggests that Flp may supply electrons from NAD(P)H that are required for Bjb activity (Figure 7).

Sources of NO: NasC and NapA activity is responsible for elevated NorCB expression

In eukaryotes, NO synthase (NOS) enzymes have been well described as the main NO-forming pathway for cell signalling and anti-microbial host defence [54]. By contrast, NO-formation in prokaryotes has been considered a by-product of denitrification, anaerobic ammonium oxidation and other related respiratory pathways [55–58]. However, NO is now increasingly recognized as a key substrate for ‘non-respiratory’ pathways in bacteria, e.g. those that protect against nitrosative stress and the link between NO-detoxification and pathogenicity has been the focus of several studies (reviewed by Maia and Moura [56,59]). The biochemical basis for NO-formation during anaerobic bacterial respiration has been shown to result from enzymic reduction of the pseudo-substrate NO$_2^-$ by the respiratory membrane-bound NO$_2^-$ reductase, Nar [43,60,61]. Furthermore, a small contribution (less than 3%) has been attributed to the periplasmic enzyme, Nap [43,61]. In the context of this present study, the potential contribution of cytoplasmic NO$_2^-$ reduction to NO formation, by NasC, during NO$_3^-$/NO$_2^-$ assimilation has not yet been investigated.

In the denitrifying endosymbiotic bacterium B. japonicum, reduction of NO$_2^-$ by the periplasmic copper-dependent NO$_2^-$ reductase NirK is the main NO-forming process, which occurs during anaerobic NO$_2^-$ respiration [1,42]. Many studies have proposed that NO activates transcription of nor genes and that this control is mediated by regulatory proteins designated NNR/NnrR and DNR (reviewed by Spiro [18,62]). In the present study, we demonstrate that cells lacking the periplasmic respiratory NO$_2^-$ reductase NapA, where NO synthesis from denitrification is blocked, results in very low expression of NorCB. Perhaps our most important finding was that, in addition to NapA, the assimilatory NO$_2^-$ reductase (NasC) is also responsible for generating NO, as induction of NorCB was significantly lowered and completely abolished, relative to WT, in the nasC and nasCnapA mutant strains respectively (Figure 6). Therefore, the importance of NasC not only in NO$_2^-$ assimilation but also in NO production has been demonstrated.

Co-expression of bjb, flp and nasC that constitute a combined NO$_2^-$ assimilation/NO-detoxification system may represent a novel method by which bacteria maintain cytoplasmic NO homeostasis and protect against nitrosative stress imposed during NO$_2^-$-dependent growth, where pathways for both respiratory denitrification and NO$_2^-$/NO$_3^-$ assimilation are active (Figure 7). Although co-regulation between similar NO-forming and consuming systems has been proposed in Aspergillus nidulans [63], to our knowledge, this is the first time where this mechanism has been reported in bacteria. Finally, should production of NO exceed concentrations that can be contained by Bjb-Flp, a ‘safety’ mechanism exists to enhance expression of NorCB to drive reduction of excess NO to N$_2$O.

AUTHOR CONTRIBUTION

Juan Cabrera, Ana Salas, María Torres, Eulogio Bedmar, David Richardson, Andrew Gates and María Delgado designed the research. Juan Cabrera, Ana Salas, David Richardson, Andrew Gates and María Delgado analysed the data. Andrew Gates and María Delgado wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Dr Hans-Martin Fischer (Institute of Microbiology, ETH Zürich, Switzerland) for providing the pSUP3535 plasmid.

FUNDING

This work was supported by European Regional Development Fund (ERDF) co-financed grants from Ministerio de Economía y Competitividad, Spain (grant numbers: AGL2010-18607 and AGL2013-45087-R to M.J.D.); the Junta de Andalucía [grant number P1212-AGR1968 (to E.J.B.); the Biotechnology and Biological Sciences Research Council [grant number BB/M0256X/1 to A.J.G.]); and the Royal Society International Exchanges Programme, U.K. [grant number IE140222 (to A.J.G. and M.J.D.)]. J.J.C. was supported by a fellowship from the Consejo Superior de Investigaciones Científicas (CSIC) JAE programme. D.J.R. is a Royal Society Wolfson Foundation Merit Award holder.

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