DNA Microarray-Based Identification of Genes Regulated by NtrC in *Bradyrhizobium japonicum*

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The *Bradyrhizobium japonicum* NtrBC two-component system is a critical regulator of cellular nitrogen metabolism, including the acquisition and catabolism of nitrogenous compounds. To better define the roles of this system, genome-wide transcriptional profiling was performed to identify the NtrC regulon during the response to nitrogen limitation. Upon cells perceiving low intracellular nitrogen, they stimulate the phosphorylation of NtrC, which induces genes responsible for alteration of the core glutamine synthetase/glutamate synthase nitrogen assimilation pathway, including the genes for the glutamine synthetases and PII proteins. In addition, genes responsible for the import and utilization of multiple nitrogen sources, specifically nitrate and nitrite, were upregulated by NtrC activation. Mutational analysis of a candidate nitrite reductase revealed a role for NtrC in regulating the assimilation of nitrite, since mutations in both *ntrC* and the gene encoding the candidate nitrite reductase abolished the ability to grow on nitrite as a sole nitrogen source.

The alphaproteobacterium *Bradyrhizobium japonicum* engages in a complex symbiotic interaction with soybean, leading to the development of nitrogen-fixing root nodules. During establishment of the symbiotic interaction, the bacteria infect cells within the developing plant nodule and differentiate into the nitrogen-fixing bacteroid state. Nitrogen fixation by symbiotic bacteroids requires expression of the nitrogenase enzyme complex (1). In many diazotrophic proteobacteria, the *nifA* gene, whose protein product controls expression of the *nif* genes, is regulated by the NtrBC two-component regulator (2). However, in *B. japonicum*, *nifA* expression is regulated by a redox status mediated by the activity of the RegSR proteins (3). Concurrent with upregulation of the *nif* genes during bacteroid development is the downregulation of functions related to the assimilation and catabolism of nitrogenous compounds, including glutamine synthetase activity (4). *B. japonicum* assimilates ammonia via the activity of the glutamine synthetase/glutamate synthase pathway (5, 6). *B. japonicum*, in a manner consistent with that of many rhizobia, contains two isoforms of glutamine synthetase, GSI and GSII, encoded by the *glnA* and *glnII* genes, respectively. The two isoforms are under the control of different regulatory mechanisms; specifically, *glnA* is controlled posttranslationally by reversible adenylation, while *glnII* is regulated at the level of gene expression.

Regulation of nitrogen metabolism in *B. japonicum* is controlled, in part, via the *ntr* regulatory system, which monitors the intracellular ratio of glutamine to α -ketoglutarate (7–9). Fluctuations in this ratio are reflected in the uridylylation state of the PII regulatory proteins encoded by *glnB* and *glnK*. During nitrogen limitation, uridylylated PII stimulates the activation of glutamine synthetase and the phosphorylation of the nitrogen regulatory protein NtrC via its interaction with the histidine kinase NtrB. In contrast to typical histidine kinases, NtrB is not membrane associated and monitors cellular nitrogen status via its interaction with the PII proteins. The *B. japonicum* NtrC protein is a member of the NtrC class of enhancer binding proteins, which regulate gene expression in response to nitrogen limitation. In *B. japonicum*, NtrC is required for utilization of nitrate as a sole N source (8), suggest-

ing a critical role for NtrC during free-living growth. On the other hand, a *B. japonicum* *ntrC* mutant formed a fully functional symbiosis with soybean, indistinguishable from that formed by the wild type (8). These results are similar to those obtained for a *Sinorhizobium meliloti* *ntrC* mutant (10) and indicate that NtrC does not play a critical role in the symbiosis. In addition, the alternative sigma factor RpoN is important for expression of the NtrC-dependent regulatory system (11, 12). Interestingly, there are two functional *rpoN* genes (*rpoN1* and *rpoN2*) identified in *B. japonicum* (11). *rpoN1* expression is activated under microaerobic conditions (i.e., 2% O₂), while *rpoN2* is negatively autoregulated (11).

DNA microarray-based transcriptional profiling was used to define the NtrC regulon for a number of organisms, including *Escherichia coli* (13), *S. meliloti* (14), and *Pseudomonas putida* (15). The results of these experiments provided insight into the global regulatory function of NtrC in these organisms and suggested that NtrC is essential for expression of genes related to the transport and catabolism of nitrogenous compounds, as well as regulation of central metabolic networks. Here, we report the isolation and characterization of several additional *B. japonicum* *ntr* mutants, as

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TABLE 1 Bacterial strains and plasmids used in this study^a

Strain or plasmid	Relevant genotype or characteristic(s)	Source or reference
<i>B. japonicum</i> strains		
USDA 110	Wild type	USDA, Beltsville, MD
<i>Bj</i> Δ4486u	<i>nifR</i> mutant, Kan ^r	This study
<i>Bj</i> Δ <i>ntrB</i> u	<i>ntrB</i> mutant	This study
<i>Bj</i> Δ <i>ntrC</i>	<i>ntrC</i> mutant, Kan ^r	This study
<i>Bj</i> Δ <i>ntrBC</i>	<i>ntrBC</i> mutant, Kan ^r	This study
<i>Bj</i> Δ4571	bll4571 mutant, Kan ^r	This study
<i>Bj</i> Δ <i>ntrC</i> -C	<i>Bj</i> Δ <i>ntrC</i> containing pRK311- <i>nifR</i> - <i>ntrBC</i>	This study
<i>Bj</i> Δ4571-C	<i>Bj</i> Δ4571 containing pRK311-4571	This study
<i>E. coli</i> strains		
DH5α	<i>supE44</i> Δ <i>lacU169</i> (Φ80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Bethesda Research Laboratory
BW25141	F [−] Δ(<i>araD</i> - <i>araB</i>)567 Δ <i>lacZ</i> 4787(:: <i>rrnB3</i>) Δ(<i>phoB</i> - <i>phoR</i>)580 LAM <i>galU95</i> Δ <i>uidA3</i> :: <i>pir</i> ⁺ <i>recA1</i> <i>endA9</i> (deleted and reinserted)::FRT <i>rph-1</i> Δ(<i>rhaD</i> - <i>rhaB</i>)568 <i>hsdR514</i>	CGSC
Plasmids		
pRK2073	RK2 Tra ⁺ Sp ^r Sm ^r	24
pHP45Ω-Tc	Source of tetracycline cassette	19
pKnockout-Ω	Suicide vector, Sp ^r Sm ^r	18
pKnockout-G	Suicide vector, Gm ^r	18
pKOTc	Suicide vector, Tet ^r	This study
pKOTc2	Suicide vector, Tet ^r	This study
pKD4	Source of FRT-flanked kanamycin resistance template	20
pKD78	λ <i>Red</i> genes, Cm ^r	CGSC
pCP20	Source of yeast flippase	22
pTE3	Tet ^r	23
pTE3-FLP	FLP expressed from the <i>trp</i> promoter of pTE3, Tet ^r	This study
pTE3-FLP RBS	FLP with an artificial RBS expressed from the <i>trp</i> promoter of pTE3, Tet ^r	This study
pKOTc2-4486	PCR clone, blr4486 with or without a 500-bp flanking sequence, Tet ^r Ap ^r	This study
pKOTc2-4486ΔKm	Deletion construct for blr4486, Tet ^r Ap ^r Kan ^r	This study
pKOTc-ntrBC	PCR clone, <i>ntrBC</i> with or without a 500-bp flanking sequence, Tet ^r Ap ^r	This study
pKOTc-ntrBΔKm	Deletion construct for <i>ntrB</i> , Tet ^r Ap ^r Kan ^r	This study
pKOTc-ntrCΔKm	Deletion construct for <i>ntrC</i> , Tet ^r Ap ^r Kan ^r	This study
pKOTc-ntrBCΔKm	Deletion construct for <i>ntrBC</i> , Tet ^r Ap ^r Kan ^r	This study
pKOTc2-4571	PCR clone, bll4571 with or without a 500-bp flanking sequence, Tet ^r Ap ^r	This study
pKOTc2-4571ΔKm	Deletion construct for bll4571, Tet ^r Ap ^r Kan ^r	This study
pRK311	Tet ^r	25
pRK311- <i>nifR3ntrBC</i>	<i>nifR3ntrBC</i> with a 500-bp 5′ flanking sequence in pRK311, Tet ^r	This study
pRK311-4571	bll4571 and bll4570 with a 500-bp 5′ flanking sequence, Tet ^r	This study

^a CGSC refers to the *E. coli* Genetic Stock Center (<http://cgsc.biology.yale.edu/index.php>). RBS, ribosome binding site.

well as a DNA microarray characterization of the NtrC-mediated transcriptional response to nitrogen limitation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains used in this study are listed in Table 1. *B. japonicum* strain USDA 110 was routinely maintained at 30°C with shaking (200 rpm) in MYB medium (pH 6.8), consisting of 0.3 g of K₂HPO₄, 0.3 g of KH₂PO₄, 0.5 g of NH₄NO₃, 0.1 g of MgSO₄·7H₂O, 4 ml of glycerol, 0.5 g of yeast extract, 1.3 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, 1.1 g of 2-(*N*-morpholino)ethane sulfonic acid, 1 ml of 10 mM FeCl₃, and 1 ml of trace element solution (lacking FeSO₄) (16) per liter of deionized water. Cultures for microarray experiments were grown in MMB, a defined medium identical to MYB but lacking yeast extract and containing 10 mM sodium glutamate (MMB glutamate, low nitrogen) or 10 mM sodium glutamate plus 10 mM NH₄Cl (MMB glutamate plus ammonium, high nitrogen) as the nitrogen source(s), rather than NH₄NO₃. Antibiotic concentrations of

150 μg ml^{−1} kanamycin, 100 μg ml^{−1} tetracycline, and 30 μg ml^{−1} chloramphenicol were used for *B. japonicum* when necessary. *E. coli* cultures were routinely grown on Luria-Bertani medium (17) at 37°C with antibiotic concentrations as follows when necessary: 100 μg ml^{−1} kanamycin, 100 μg ml^{−1} spectinomycin, 100 μg ml^{−1} streptomycin, 100 μg ml^{−1} ampicillin, 20 μg ml^{−1} tetracycline, and 30 μg ml^{−1} chloramphenicol.

Plasmids. Plasmids used in this study are listed in Table 1. Plasmid pKOTc was constructed by replacing the Ω cassette of pKnockoutΩ (18) with the tetracycline resistance cassette from pHP45-Tc (19) by recombinational cloning. Plasmid pKnockoutΩ was transformed via electroporation into *E. coli* strain BW25141 harboring pKD78, and in the resulting strain, the λ *Red* genes were induced and competent cells were prepared as described previously (20, 21). Briefly, cells were cultured in 25 ml super optimal broth (SOB) medium (17) containing 10 mM L-arabinose to induce the λ *Red* genes at 30°C, with shaking, until an optical density at 600 nm (OD₆₀₀) of 0.4 to 0.6 was reached. Cells were pelleted, washed two times with cold 10% glycerol, and frozen at −80°C until use.

A PCR product generated from amplification of the tetracycline resistance cassette from pHP45-Tc with the primers Omega_AgeI_F (5'-GCTGAACCGGTCTAGGCCACTAACTAA-3') and Omega_AgeI_R (5'-CAGTAACCGGTTGATTGATTGAGCAAGC-3') was DpnI treated and purified. The PCR product was then electroporated into BW25141 containing pKD78 and pKnockout Ω . A tetracycline-resistant and streptomycin-sensitive recombinant was selected and its genotype confirmed by restriction digestion. Plasmid pKOTc2 was constructed by PCR amplification of the tetracycline resistance gene from pHP45-Tc with primers Tet_F_BglII (5'-TGCGCAGATCTTAAGCTTTAATGCGGTAGTT-3') and Tet_R_BglII (5'-GTACTAGATCTATTACAGTCGAGGTGGCCCG-3'), followed by digestion of the PCR product with BglII. The resulting fragment was then inserted into the BglII site within the gentamicin resistance gene of pKnockout-G (18).

Construction of pTE3-FLP and pTE3-FLPRBS was performed by PCR amplification of the yeast flippase (FLP) from pCP20 (22) using the primers Flp_R_BamHI (5'-CTGAAGGATCCTTATATGCGTCTATTTATG-3') and Flp_F_PstI (5'-ACGTACTGCAGATGCCACAATTTGGTATAT-3') or Flp_F_RBS_PstI (5'-ACGTACTGCAGTAAGGAGGATATTCATATG-3'), where the PstI and BamHI sites are underlined. The resulting PCR products were digested with PstI and BamHI and cloned into the PstI/BamHI sites of pTE3 (23).

Construction of *B. japonicum* mutants. *B. japonicum* mutants were produced by marker exchange following double homologous recombination of a deletion cassette (see Fig. S1 in the supplemental material). To prepare deletion cassettes, genomic DNA encompassing the deletion target and approximately 500 bp of the 5' and 3' flanking sequences was PCR amplified using Phusion Hot Start high-fidelity DNA polymerase (Finnzymes, Keilaranta, Finland) per the manufacturer's instructions. PCR primers for amplification of *B. japonicum* genomic DNA and flanking sequence are listed in Table S1 in the supplemental material. Following the addition of 3' adenine overhangs, the PCR products were cloned into the XcmI site of either pKOTc or pKOTc2. The resulting genomic clones were introduced by electroporation into *E. coli* BW25141 containing pKD78. Induction of the λ Red genes and preparation of competent cells was performed as described above. A PCR product for disruption of the targeted gene was generated by first amplifying the flippase recognition target (FRT)-flanked kanamycin resistance gene from pKD4 using primers PS1 (5'-GTGTAGGCTGGAGCTGCTTC-3') and PS2 (5'-CATATGATATCCTCCTTAG-3'), corresponding to priming sites 1 and 2 of pKD4 as described previously (20). The resulting PCR product was purified and DpnI treated. Twenty nanograms of this PCR product was used as the template for a second PCR using the 60-mer oligonucleotide primers listed in Table S1 designed to add 40 bases to the 5' ends of PS1 and PS2, which are homologous to the regions immediately flanking the deletion target. The PCR was performed using Phusion Hot Start high-fidelity DNA polymerase (Finnzymes, Keilaranta, Finland) per the manufacturer's instructions with the following amplification profile: 98°C for 30 s; 2 cycles of 98°C for 10 s, 54°C for 30 s, and 72°C for 45 s; 28 cycles of 98°C for 10 s, 68°C for 30 s, and 72°C for 45 s; and 72°C for 5 min. The resulting PCR product was purified with the Wizard SV kit (Promega, Madison, WI, USA).

The PCR-amplified kanamycin resistance genes were then electroporated into BW25141 containing pKD78 and the targeted genomic clone. Kanamycin-resistant recombinants were selected at 37°C. Deletion constructs were confirmed by PCR using the forward or reverse primers from the initial PCR cloning in combination with one of two primers located inside the kanamycin resistance gene, PSk1 (5'-CAGTCATAGCCGAATAGCCT-3') (20) and PSkt' (5'-GGATTCATCGACTGTGGCCG-3'). Following confirmation, deletion constructs were retransformed into DH5 α and moved into wild-type *B. japonicum* by triparental mating with the helper plasmid pRK2073 (24). Double-recombination mutants were selected by resistance to kanamycin and sensitivity to tetracycline and confirmed by PCR.

The FRT-flanked kanamycin resistance marker was removed from

mutant *B. japonicum* strains by introduction of the FLP gene (22) carried in pTE3-FLP(RBS) by triparental mating with the helper plasmid pRK2073. Tetracycline-resistant transformants in which the kanamycin gene was lost were identified by colony PCR and sensitivity to kanamycin. In the resulting unmarked mutant strains, pTE3-FLP(RBS) was removed via multiple rounds of subculture in the absence of selection, followed by identification of tetracycline-sensitive colonies.

Construction of complemented strains. To complement the *B. japonicum* USDA 110 Δ ntrC (*Bj* Δ ntrC) mutant strain, plasmid pRK311-nifR3-ntrBC was created by PCR amplification of the *nifR3-ntrBC* operon from *B. japonicum* genomic DNA with primers *nifR*-F(PstI) (5'-CAACTGCAGGCGTCGTGGATCAGGAC-3') and *ntrC*-R(BamHI) (5'-AAAGGATCCAGGCGGCGAGCTAGAAGT-3'), where the PstI and BamHI sites are underlined. PCR products were digested with PstI and BamHI and cloned into the PstI and BamHI sites of pRK311 (25). To complement the *Bj* Δ 4571 mutant strain (with the entire coding sequence of *bll4571* deleted), plasmid pRK311-4571 was constructed by PCR amplification of *B. japonicum* genomic DNA with the primers *bll4571*-comp F (NsiI) (5'-TATATGCATCTCTACGCCAGATGGTG-3) and *bll4571*-comp R (BglII) (5'-GCAAGATCTTAGTAGACGTCAGCCTG-3'), where the NsiI and BglII sites are underlined. PCR products were digested with NsiI and BglII and cloned into the PstI and BamHI sites of pRK311. The resulting plasmids, pRK311-nifR3ntrBC and pRK311-4571, were introduced into *Bj* Δ ntrC and *Bj* Δ 4571, respectively, to complement them by triparental mating with the helper plasmid pRK2073. The complemented strains were named *Bj* Δ ntrC-C and *Bj* Δ 4571-C, respectively (Table 1).

qRT-PCR analysis. *B. japonicum* strains were cultured in 20 ml of MMB glutamate or MMB glutamate plus ammonium to an OD₆₀₀ of 0.4 to 0.6. Cells were harvested and RNA was extracted as described previously (26–28). Synthesis of cDNA and quantitative reverse transcription-PCR (qRT-PCR) was performed using 3 μ g of total RNA as described previously (26–28). Data for three independent biological replicates were generated and normalized to the expression of the *bll7457* gene, encoding the histidyl-tRNA synthetase HisS.

DNA microarray analysis. The whole-genome DNA oligonucleotide microarrays used were described previously (26). One-hundred-milliliter cultures of *Bj*110 or *Bj* Δ ntrC were cultured at 30°C with shaking at 180 rpm in MMB glutamate or MMB glutamate plus ammonia to an OD₆₀₀ of 0.4 to 0.6. Cells were harvested by centrifugation at 8,000 $\times$ g at 4°C for 8 min following the addition of a 1/10 volume of 5% phenol (pH 4.3) in ethanol. Supernatants were decanted, and cell pellets were frozen in liquid nitrogen and stored at –80°C until use. RNA extraction, cDNA synthesis, cDNA labeling, and DNA microarray hybridizations were performed as described previously (26, 29). A total of 4 μ g of cDNA was used for each sample hybridized. Image acquisition and data processing were performed as described previously (29). The raw intensities were adjusted for background using the normexp background correction method of the limma software (30) and then normalized by the joint lowess normalization method using R/maanova software (1.16.0 version; Jackson Laboratory, Bar Harbor, ME; <http://churchill.jax.org/software/rmaanova.shtml>). After normalization of each array, all 48 channels (2 channels per array) were median centered so that the median log-scale expression levels were all zero. The 1,280 empty and control spots were removed from the downstream analysis to reduce the dimension. A linear mixed-effects model was applied to the normalized log₂-scale expression measures separately for each gene using R/maanova software (1.16.0 version). Each linear mixed-effects model included array effect, dye effect, spot effect, biological replicate effect, and condition effect, where the array, spot, and biological replicate effects were treated as random effects. Here the spot effect was included to account for duplicated spots on the arrays. Since the condition effect referred to the combination effect of bacterial strains and culture conditions, there were four levels for the condition effect as shown in Fig. 1: wild type at the high nitrogen level (A), wild type at the low nitrogen level (B), *Bj* Δ ntrC at the high nitrogen level (C), and *Bj* Δ ntrC at the low nitrogen level (D). *t* tests were conducted to identify significantly differ-

FIG 1 Overview of the DNA microarray experiment. Numbers refer to the total number of genes differentially expressed in each comparison. *Bj110*, wild type; *BjΔntrC*, *ntrC* mutant; Glu, glutamate.

entially expressed genes for four pairwise comparisons between the four levels of the condition effect, namely, B/A, B/D, A/C, and D/C comparisons. *P* values from the *t* statistics were obtained via the *mate* statement in the R/maanova software, and a *q* value as a false discovery rate (FDR) (31) was computed for each *P* value. An FDR of 5% was used to produce lists of differentially expressed genes.

Characterization of the mutant strain *BjΔ4571*. The growth of *BjΔ4571* on nitrite was examined by producing growth curves in MMB amended with 2 mM nitrite as the sole nitrogen source. Absorbance measurements at 600 nm were recorded for triplicate 20-ml cultures grown at 30°C with shaking at 180 rpm.

Nitrite concentrations were analyzed by the Griess reaction as described previously (32). Briefly, cells grown in MMB glutamate were washed once and resuspended in MMB lacking nitrogen to an OD₆₀₀ of 0.5. Nitrite was added to a final concentration of 900 μM, and the cells were incubated at 30°C with shaking at 180 rpm. To assay nitrite concentration, 150-μl aliquots were removed from the cultures and centrifuged at 13,000 × *g* for 5 min. The supernatants were removed and diluted appropriately. To 50 μl of diluted supernatant, 50 μl of 1% sulfanilamide (in 5% phosphoric acid) was added, and the mixture was incubated in the dark for 10 min. Following the addition of 50 μl of 0.1% *N*-(1-naphthyl) ethylenediamine and subsequent incubation for 10 min in the dark, absorbance at 530 nm was recorded and nitrite concentrations were determined from a standard curve generated from samples of known concentration.

GEO accession number. Microarray data are accessible via the NCBI Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) by GEO series accession number GSE66091.

RESULTS AND DISCUSSION

Construction and analysis of a series of *B. japonicum ntr* mutants. The *B. japonicum ntrC* gene resides in the *nifR3-ntrB-ntrC* operon with the gene for the cognate histidine kinase, *ntrB*, and an open reading frame (ORF) of unknown function, *nifR3* (Fig. 2). This genetic arrangement is a common feature among the alpha-proteobacteria (4, 33). Two *B. japonicum ntrC* mutants (*Bj3028* and *Bj27147*) were constructed previously and partially characterized (8). Because these mutants were isolated many years ago, a series of additional mutations were created in *nifR3* (*blr4486*), *ntrB*

FIG 2 Schematic representation of the *nif* and *ntr* mutants. Dashed lines mark the regions deleted from each mutant.

TABLE 2 Analysis of nitrogen source utilization by the wild type and *B. japonicum* mutant strains^a

Strain	Result with indicated nitrogen source	
	Nitrate	Nitrite
<i>Bj110</i>	+	+
<i>BjΔ4486u</i> (<i>nifR3</i> mutant)	+	+
<i>BjΔntrBu</i>	+	+
<i>BjΔntrC</i>	—	—
<i>BjΔntrBC</i>	—	—
<i>BjΔntrC-C</i>	+	+
<i>BjΔ4571</i> (<i>nirA</i> mutant)	—	—
<i>BjΔ4571-C</i>	+	+

^a Cultures were grown in MMB supplemented with 2 mM nitrate or nitrite as the sole nitrogen source. Plus and minus signs refer to growth and no growth, respectively.

(*blr4487*), and *ntrC* (*blr4488*) in the *B. japonicum* USDA 110 wild-type background and are represented schematically in Fig. 2. The mutants were created utilizing a marker replacement methodology described by Datsenko and Wanner (20), with plasmid pKD4 as the source of kanamycin resistance. The mutagenesis strategy is outlined in Fig. S1 in the supplemental material. Utilization of this mutagenesis strategy confers two primary advantages. First, the regions targeted for deletion can be specifically defined and are not limited to the presence or absence of restriction enzyme target sites. Furthermore, the incorporation of flippase recognition target (FRT) sequences flanking the antibiotic resistance marker facilitates removal of the marker in the mutant strain. This allows the design of nonpolar mutations and recycling of the antibiotic resistance marker, for which there are a limited number of useful markers in *B. japonicum*. In the present study, the kanamycin resistance gene was removed from each of the single mutants. As a result, both marked and unmarked versions of each strain exist and were used as indicated in the text. Unmarked strains are indicated with a “u” following the strain designation (i.e., *BjΔntrBu* versus *BjΔntrB*).

Previous work with strains *Bj3028* and *Bj27147* indicated that *B. japonicum ntrC* mutants were compromised in the utilization of nitrate as a sole nitrogen source (8). Mutants carrying deletions of *ntrC*, *BjΔntrC* and *BjΔntrBC*, were unable to utilize nitrate as a sole nitrogen source (Table 2). In contrast, the *nifR3* and *ntrB* mutants, *BjΔ4486u* and *BjΔntrBu*, were able to utilize nitrate as a sole nitrogen source. Complementation of *BjΔntrC* with plasmid pRK311-*nifR3-ntrBC* (*BjΔntrC-C*) restored the strain’s ability to grow on nitrate (Table 2). Utilization of nitrate as a nitrogen source requires reduction of nitrate to nitrite via the activity of an assimilatory nitrate reductase. Therefore, the ability of the *ntr* mutants to grow on nitrite as a nitrogen source was also examined (Table 2). *BjΔntrC* and *BjΔntrBC* mutants were unable to utilize nitrate or nitrite as a sole nitrogen source. However, the *BjΔ4486u* and *BjΔntrBu* mutants were able to utilize nitrite as a sole nitrogen source. These results indicate that *NtrC* is the key component of the *nifR3-ntrB-ntrC* operon required for nitrate and nitrite utilization in *B. japonicum* and that the *ntrC* mutant phenotype is consistent with that found in other organisms (10, 34).

Previous reports indicated that *ntrC* mutants are defective for induction of *glnII* expression under aerobic nitrogen-limiting conditions (8). As shown in Fig. 3A, qRT-PCR analysis confirmed the upregulation of *glnII* under the low-nitrogen condition (10

FIG 3 *glnII* regulation in the wild type and *ntr* mutants. (A) Differences (fold changes) between the levels of expression of *glnII* in the wild type under the high-nitrogen condition and in the *ntrC* mutant under the low-nitrogen condition; (B) differences between the levels of expression of *glnII* in the wild type and mutant strains under low-nitrogen conditions. Comp, complemented. Numbers above bars indicate average fold changes from three replicates, with standard errors shown by 1 bars.

mM glutamate), compared to its level of expression under the high-nitrogen condition (10 mM glutamate plus 10 mM ammonium), for the wild type but not the *BjΔntrC* mutant. Furthermore, direct comparison of *Bj110* to *BjΔntrC* under low-nitrogen conditions indicated a much higher expression of *glnII* in the wild type (Fig. 3B). Similar results were obtained with the *ntrBC* double mutant, *BjΔntrBC* (data not shown). However, the complemented strain *BjΔntrC-C* restored *glnII* expression to wild-type levels (Fig. 3B). Interestingly, *glnII* expression in a nonpolar *ntrB* mutant, *BjΔntrBu*, was only 2.5-fold lower than that in the wild type. *BjΔntrBu* expresses *ntrC* at wild-type levels (data not shown). These results indicate that *ntrC* is expressed in the *BjΔntrBu* mutant and that *BjΔntrBu* is only partially compromised in the NtrC response to nitrogen limitation, consistent with the phenotypic analysis described above.

Genetic analysis of a series of *ntr* mutants in *Rhodobacter capsulatus* indicated that NtrC, but not NtrB, was essential for utilization of N_2 and urea as sole nitrogen sources (33). However, an *ntrB ntrY* double mutant was defective for utilization of N_2 and urea (33). NtrY is the histidine kinase of the *ntrXY* two-component regulatory system that resides immediately downstream of the *nifR3-ntrBC* operon in both *B. japonicum* and *R. capsulatus*. Utilization of nitrate and nitrite by *BjΔntrBu* suggests that a cross talk among the *ntrBC* and *ntrXY* two-component systems similar to that described for *R. capsulatus* may exist in *B. japonicum*.

DNA microarray analysis of the *B. japonicum* NtrC regulon.

FIG 4 Functional classifications of differentially expressed genes upregulated under either the low-nitrogen or the high-nitrogen condition. Transcript changes that were greater than 2-fold in the *B. japonicum* low-nitrogen versus *B. japonicum* high-nitrogen comparison are noted.

To identify genes responding to nitrogen limitation in an NtrC-dependent manner, a series of pairwise DNA microarray comparisons among the wild-type and *ntrC* mutant strains under both high- and low-nitrogen conditions were performed as depicted in Fig. S1 in the supplemental material. Tables S2 to S5 in the supplemental material contain the complete list of differentially expressed genes for each comparison. Analysis of NtrC activity often involves a comparison of cells grown on an organic nitrogen source with cells grown on an inorganic nitrogen source (8, 14, 15). The addition of an inorganic nitrogen source (i.e., ammonium) results in dephosphorylation and subsequent inactivation of NtrC. Comparison of the wild-type *B. japonicum* strain grown under conditions of high (glutamate and ammonia) nitrogen and low (glutamate only) nitrogen was performed to identify transcripts that respond to nitrogen limitation (i.e., B/A in Materials and Methods and Fig. 1). This comparison included genes that are NtrC-dependently regulated, as well as those that are not NtrC dependent. Therefore, a second comparison of *BjΔntrC* under high- and low-nitrogen conditions was performed to identify transcripts that respond to nitrogen limitation in an NtrC-independent manner (i.e., D/C in Materials and Methods and Fig. 1). Likewise, direct comparison of *Bj110* to *BjΔntrC* was performed under both high- and low-nitrogen conditions.

Examination of the comparison of wild-type *B. japonicum* grown under high- and low-nitrogen conditions (i.e., B/A) revealed the differential expression of 168 transcripts at a 2-fold cutoff, with an FDR of 5%. A breakdown of this comparison by functional category is presented in Fig. 4. The transition of *Bj110* from high to low nitrogen results in changes in gene expression for several functional categories. Most interesting is the strong up-regulation of genes involved in transport and regulatory functions, indicating that the cells alter the composition of proteins related to nitrogen acquisition and ammonia metabolism. Furthermore, genes related to energy metabolism and amino acid biosynthesis are downregulated under the low-nitrogen condition, suggesting that the cells respond to nitrogen limitation by altering the biosynthetic capabilities of the cell.

To validate microarray data, we performed qRT-PCR analysis. Thirteen genes were chosen based on fold induction (both up- and downregulation) and functional categories (see Fig. S2 and Table

TABLE 3 NtrC-activated genes in a nitrogen status-dependent manner induced in the wild type under low-nitrogen conditions^a

Gene ID ^b	Gene product description	Fold change	
		B/A ^c	B/D ^d
blr1448	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	48.7	38.5
<u>blr0612</u>	Nitrogen regulatory protein PII	24.8	18.1
blr4169	Glutamine synthetase II	13.1	10.0
<u>blr6148</u>	Bmp family protein	10.5	6.7
blb6154	Unknown protein	7.4	3.0
blr2803	ABC transporter nitrate-binding protein	6.4	5.2
blr0613	Ammonium transporter	6.1	7.5
<u>blb5738</u>	Unknown protein	6.1	3.8
<u>blb4571</u>	Ferredoxin-nitrite reductase, putative	6.0	5.1
blb3763	Unknown protein	5.6	4.7
blb6424	Drug resistance transporter, Bcr/CflA family, putative	5.2	2.3
blb7538	Hypothetical protein	5.1	2.2
blb3286	Bacterial extracellular solute-binding domain protein, putative	4.6	2.3
blr6158	Bmp family protein	3.8	2.6
<u>blb3347</u>	Cytosine/purine/uracil/thiamine/allantoin permease family protein, putative	3.6	3.7
blr0606	Nitrogen regulatory protein PII	3.5	9.9
<u>blr3337</u>	Heme-binding lipoprotein, putative	3.3	2.6
<u>blr4948</u>	Nitrogen regulatory protein PII	3.0	5.2
blr7922	ABC transporter substrate-binding protein	3.0	2.9
blr3004	Squalene-hopene cyclase	2.9	2.5
<u>blr1036</u>	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein, putative	2.9	2.7
blr7887	CvpA family protein	2.8	2.0
blr3321	Putative aspartate aminotransferase-related protein	2.7	2.1
blb5737	Putative acyl coenzyme A dehydrogenase	2.3	2.0
blb3318	Taurine ABC transporter, ATP-binding protein	2.3	2.0
blr1455	Urease, beta subunit	2.2	2.4
<u>blb0897</u>	Soluble pyridine nucleotide transhydrogenase	2.2	2.1
blr2243	Unknown protein	2.2	2.4
blr5575	Putrescine ABC transporter, permease protein, putative	2.1	2.2
blr2099	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	2.1	2.0
blb1798	Hypothetical protein	2.1	3.4
blb2045	Unknown protein	2.1	4.8

^a Genes were upregulated under both conditions (i.e., B/A and B/D) of nitrogen limitation, and the wild type was compared to the *BjΔntrC* mutant.

^b An underlined gene identifier (ID) indicates that its promoter region contains the putative RpoN sigma factor binding site.

^c B/A refers to a comparison of the *B. japonicum* wild type grown under low-nitrogen conditions and the *B. japonicum* wild type grown under high-nitrogen conditions.

^d B/D refers to a comparison of the *B. japonicum* wild type grown under low-nitrogen conditions and the *BjΔntrC* mutant grown under low-nitrogen conditions.

S1 in the supplemental material). In general, there was a strong positive correlation ($R^2 = 0.90$) between the microarray and qRT-PCR data. To extract NtrC-activated genes from the microarray data, we selected 32 genes that were upregulated under both conditions of nitrogen limitation (i.e., B/A) in *Bj110* and compared them to those in *BjΔntrC* (i.e., B/D) (Table 3). In contrast, only one NtrC-repressed gene, blb6809, encoding a hypothetical protein, was identified under both conditions (expression levels indicated -21 - and -2.9 -fold changes in B/A and B/D, respectively). Of these NtrC-dependent genes, 12 genes were found to have putative RpoN promoter consensus sequences in their upstream regions by motif search analysis (Table 3) (unpublished data). In addition to NtrC-related genes, 48 genes regulated independently of NtrC (upregulated, 27 genes; downregulated, 21 genes) under nitrogen limitation were observed in the D/C comparison (Table 4 and Table S5 in the supplemental material).

Regulation of glutamine synthetase and the PII proteins. Assimilation of nitrogen in rhizobia occurs primarily via the activity of the GS/GOGAT (glutamine synthetase/glutamate synthase) pathway (4). Unlike enteric bacteria that contain only a single glutamine synthetase, rhizobia typically contain two or three glu-

tamine synthetase enzymes (4). The *B. japonicum* genome encodes three glutamine synthetase enzymes, two copies of *glnA* (blr4949 and blr4835), and the *glnII* (blr4169) gene. Among them, only *glnII* was induced in an NtrC-dependent manner under low-nitrogen conditions (Table 3). Neither copy of *glnA* was differentially regulated by the change in nitrogen status or the introduction of the *ntrC* mutation (see Tables S2 to S5 in the supplemental material). This result suggests that *glnII* might be the gene primarily controlled by NtrC at the transcriptional level to activate the GS/GOGAT system when *B. japonicum* encounters nitrogen starvation. The lack of transcriptional regulation of the two *glnA* genes is not surprising since GlnA is known to be regulated posttranslationally in *B. japonicum* by reversible adenylation (35, 36). It was also proposed that the rhizobial *glnII* isoform is likely important for assimilation of ammonium generated intracellularly during nitrogen starvation (4). Consistently with this model and previous results (8), our findings suggest different levels of regulation of expression of three glutamine synthetase enzymes in *B. japonicum*. In addition to investigating the GS/GOGAT pathway, it might be interesting to investigate *gdhA*, encoding glutamate dehydrogenase, repressed by NtrC in *P. putida* (13), which is not

TABLE 4 Genes induced under nitrogen limitation independently of NtrC^a

Gene ID	Gene product description	Fold change (D/C) ^b
blr1889	Sugar ABC transporter, periplasmic sugar-binding protein, putative	6.89
bl15155	Hypothetical protein	4.30
blr5803	Sulfonate ABC transporter, permease protein SsuC	4.01
blr1893	3-Oxoacyl (acyl carrier protein) reductase (EC 1.1.1.100)	3.94
blr1891	Sugar ABC transporter, permease protein	3.79
blr1890	Sugar ABC transporter, permease protein	2.91
bsr2847	Unknown protein	2.58
bl13639	Acetyl coenzyme A carboxylase, biotin carboxyl carrier protein	2.56
blr1895	Maltose/maltodextrin ABC transporter, ATP-binding protein	2.49
bl17395	Hypothetical protein	2.43
blr4988	Unknown protein	2.35
blr5556	Hypothetical protein	2.31
bl12292	Probable plastocyanin	2.28
blr2344	Purine-binding chemotaxis protein, putative	2.22
bsr5172	Unknown protein	2.17
blr8273	Transcriptional regulator, LysR family, putative	2.16
bl18202	Unknown protein	2.12
blr4995	Hypothetical protein	2.12
bl13386	Transcriptional regulatory protein	2.11
bl16754	Hypothetical protein	2.11
bl11568	Unknown protein	2.10
bsr5798	Unknown protein	2.09
blr3288	Amidase family protein	2.07
blr7050	Unknown protein	2.06
blr8090	Unknown protein	2.06
blr6347.1n	Hypothetical protein	2.03
blr4224	Unknown protein	2.00

^a Genes that are upregulated in the *BjΔntrC* mutant strain under low-nitrogen conditions (i.e., the D/C comparison).

^b D/C refers to a comparison of the *BjΔntrC* mutant grown under low nitrogen and the *BjΔntrC* mutant grown under high nitrogen.

present in the annotated *B. japonicum* genome. The locus blr7995, initially annotated as a hypothetical protein, likely encodes a putative NAD-dependent glutamate dehydrogenase since it contains a highly conserved bacterial NAD-glutamate dehydrogenase domain (pfam05088). However, the expression of blr7955, unlike that of glutamine synthetase, was not NtrC dependent.

The expression of GlnII in *Rhizobium leguminosarum* was demonstrated to be dependent upon the glutamine synthetase translation inhibitor protein GstI (37). GstI is a small 63-amino-acid protein in *R. leguminosarum* that serves to regulate the translation of *glnII* mRNAs in response to nitrogen status, and the expression of *gstI* mRNA is repressed by phosphorylated NtrC (37). A *B. japonicum* homolog of *gstI*, bsl4167, is downregulated 3.8-fold under nitrogen limitation (see Table S2 in the supplemental material). The *gstI* gene of *B. japonicum* lies upstream of *glnII* (blr4169) and is transcribed in the opposite orientation in a manner similar to that of *R. leguminosarum*. However, the presence of an additional predicted ORF (bl14168) of unknown function between *gstI* and *glnII*, which is not present in *R. leguminosarum*, suggests that transcriptional control of *B. japonicum* *gstI*

may differ from that in *R. leguminosarum*. Interestingly, *gstI* is also 3.8-fold upregulated in the wild type compared to in *BjΔntrC* under high-nitrogen conditions (see Table S4 in the supplemental material); no difference was observed under low-nitrogen conditions (Table S3), suggesting that NtrC may be necessary for maximal *gstI* expression.

In contrast to *glnII*, which was upregulated, blr6742, a putative glutamate synthase homologous to *gltD*, was downregulated under the low-nitrogen condition (see Table S2 in the supplemental material). A similar regulation was observed in *S. meliloti* under nitrogen limitation (14). The regulation of blr6742 appears to be independent of NtrC, as differential regulation was not observed when the wild type was compared to the *ntrC* mutant under low-nitrogen conditions (Table S3). The NtrC-independent regulation of *gltD* was also observed in *S. meliloti* (14). Based upon the differential regulation of *glnII* and a *gltD* homolog in *B. japonicum*, it appears that, as in *E. coli* (33), the GS/GOGAT pathway is manipulated during nitrogen limitation to maintain nitrogen assimilation for biosynthetic purposes by directing the GS/GOGAT pathway toward glutamine production.

The PII proteins of *B. japonicum* presumably control the phosphorylation of NtrC and the adenylation of GlnA, but not GlnII (38). Uridylation of the PII proteins by GlnD under nitrogen limitation disrupts the interaction of PII with NtrB, thereby allowing the phosphorylation and activation of NtrC. *B. japonicum* contains three PII proteins, two copies of *glnK*, *glnK1* (blr0606) and *glnK2* (blr0612), and a single copy of *glnB* (blr4948), which are all induced under low-nitrogen conditions in an NtrC-dependent manner (Table 3). Interestingly, one of the PII proteins, GlnK2, is more strongly induced than the other two PII proteins (Table 3). The *glnK2* gene, adjacent to and likely cotranscribed with the putative ammonium transporter *amtB*, was also demonstrated to be regulated by NifA and RpoN (39), as well as induced in bacteroids (40). Examination of the PII proteins of *Rhodospseudomonas palustris* indicated that *glnK2* is expressed predominantly under nitrogen-limiting conditions (41), in agreement with the data presented here, and likely serves to regulate the expression of the ammonium transporter AmtB (blr0613), which is also NtrC dependent and upregulated under low-nitrogen conditions (Table 3).

Regulation of transport functions. In addition to genes involved in ammonium assimilation and the perception of the intracellular nitrogen status being regulated, a number of transport-related genes are NtrC regulated. Among 32 NtrC-activated genes, 12 were categorized as having transport and substrate binding functions (Table 3 and see Tables S2 to S3 in the supplemental material). Included in this list are blr1448, blr6148, blr2803, blr0613, bl16424, bl13286, blr6158, blr3337, blr7922, blr1036, bl13318, and blr5575.

As indicated in Table 3, expression of blr1448 responds most strongly to NtrC activation. This gene encodes an ABC transporter substrate-binding protein with homology to *urtA*, a component of the high-affinity urea transporter characterized in the cyanobacterium *Anabaena* sp. strain PCC 7120 (42). Under physiological conditions, urea diffuses freely across membranes, and therefore, energy-dependent transport systems for urea uptake typically operate only under nitrogen limitation and have high affinities for urea, facilitating the utilization of urea present in low concentrations in the environment (42).

Once urea is transported into the cytosol, the hydrolysis of urea into ammonia and carbon dioxide should be catalyzed by an enzyme called urease. Expression of *blr1455* (*ureB*), encoding the urease beta subunit, was NtrC dependent under low nitrogen (Table 3), which suggests that following urea uptake, *B. japonicum* utilizes the urease system to support the use of this nitrogen resource. However, we did not detect the regulation of other genes, such as *ureA*, encoding the alpha subunit, and *ureI*, encoding the urea channel, in the urease system. The full complement of urease genes in *C. glutamicum* (43) and *Pseudomonas putida* (15) were upregulated in response to nitrogen limitation. A possible explanation for the lack of induction of the full urease system in *B. japonicum* under nitrogen limitation is the induction of two genes (*blr1601* and *blr1602*) encoding substrate binding and permease proteins of an ABC transporter under low nitrogen (see Table S2 in the supplemental material). These two genes have homology to the *nikA* and *nikB* genes of *E. coli* and *Brucella suis*. The *nikA* and *nikB* genes are components of a nickel transport system, and NikA was demonstrated to be required for full activity of the nickel-dependent urease of *B. suis* (44). Furthermore, *blr1601* is also 8-fold upregulated under hydrogen-oxidizing conditions during the chemoautotrophic growth of *B. japonicum* (29) when nickel is required for derepression of the nickel-dependent hydrogenase (45). The medium used for the *B. japonicum* DNA microarray experiments described here was not supplemented with nickel. Therefore, the lack of expression of the urease system in the absence of nickel may reflect the nickel-dependent derepression of the hydrogenase system. Interestingly, recent evidence suggests a role for *blr1601* and *blr1602* in determining the host range of *B. japonicum* (46). Both genes were identified as upregulated in bacteroids obtained from siratro compared to bacteroids from either soybean or cowpea (46). Mutation of the region encompassing *blr1601* and *blr1602* resulted in a reduction in symbiotic nitrogen fixation in siratro, but not in other hosts (46). Presently, the substrates transported by this set of genes remain unclear. However, the apparent induction of genes potentially related to urea and nickel transport suggests that the utilization of urea (or nitrogen compounds metabolized via urea) may be an important adaptation to nitrogen limitation in *B. japonicum*.

Nitrate and nitrite assimilation. The inability of the *BjΔntrC* mutant to utilize nitrate or nitrite as a sole nitrogen source suggests that NtrC controls the expression of genes essential for the assimilation of these compounds. The respiratory denitrification pathway of *B. japonicum*, including those of dissimilatory nitrate and nitrite reductases, supports anaerobic growth using nitrate as a terminal electron acceptor and is well characterized (47). None of the reported components of the *B. japonicum* denitrification pathway were induced under the low-nitrogen condition. The components of this system are part of the oxygen-responsive regulon (47) in *B. japonicum* and, hence, may not be part of the NtrC-controlled regulon. However, *blr2803* (*nrtA*), encoding an ABC transporter nitrate-binding protein, was upregulated in an NtrC-dependent manner (Table 3), suggesting that *nrtA* may be a key gene under NtrC control for nitrate assimilation.

An additional gene, *bl4571* (*nirA*), encoding a putative ferredoxin-nitrite reductase, was also upregulated more than 5-fold under the low-nitrogen conditions in an NtrC-dependent manner (Table 3). However, we did not observe the induction of any nitrate reductase by NtrC. The discrepancy between the NtrC-dependent expression of nitrite reductase and nitrate reductase

FIG 5 (A) Growth curves of *B. japonicum* strains cultured on 2 mM nitrite as the sole nitrogen source; (B) nitrite consumption in *B. japonicum* cultures incubated in the presence of 900 μ M nitrite. Symbols and strains used are as follows: ■, *B. japonicum* wild type; □, *BjΔntrC*; ○, *BjΔ4571*; ●, *BjΔ4571-C*. Error bars represent standard deviations from three biological replicates.

might be explained by the fact that glutamate added under the low-nitrogen condition might be converted to alpha-ketoglutarate and ammonium by glutamate dehydrogenase (e.g., *Blr7995*) with NAD as a cofactor. As a by-product, the ammonium can be converted to nitrite by NirA, whose catalytic function is reversible in the reaction, although the majority of ammonia may be used for macromolecular, biosynthetic processes. On the other hand, assimilatory nitrate reduction may be suppressed because both ammonia and nitrite are already available.

To investigate the function of NirA, a *B. japonicum nirA* mutant, strain *BjΔ4571*, was generated by deletion of the entire coding sequence of *bl4571*. The *BjΔ4571* mutant was unable to utilize nitrate or nitrite as a sole nitrogen source (Table 2). The symbiotic phenotype of *BjΔ4571* was indistinguishable from that of the wild-type parent (data not shown). Complementation of *BjΔ4571* with plasmid pRK311-4571 restored the ability of the mutant strain to utilize nitrate and nitrite, with growth curves comparable to those of the wild type (Fig. 5A). To analyze nitrite consumption, the wild-type, *BjΔntrC*, *BjΔ4571*, and *BjΔ4571-C* strains were grown on glutamate under low-nitrogen conditions and cells were harvested and resuspended in MMB containing 900 μ M nitrite. Nitrite concentrations were analyzed from culture supernatants for a period of 24 h. As shown in Fig. 5B, the wild-type and complemented *BjΔ4571-C* strains consumed all the nitrite present in the medium within the 24-h period. In contrast, no nitrite was consumed by the *ntrC* and *nirA* mutants (Fig. 5B). The inability of *BjΔ4571* to utilize nitrite and the homology to characterized NirA proteins suggest that the *B. japonicum nirA* homolog, *bl4571*, encodes an assimilatory nitrite reductase.

Conclusions. The results presented here provide a framework for understanding the transcriptional changes that occur in *B. japonicum* in response to nitrogen limitation. The NtrBC two-component regulatory system controls the expression of a portion of genes responding to nitrogen limitation. Interestingly, *ntrB* seems to be dispensable for the function of NtrC. Nitrogen limitation induces the expression of genes required for nitrogen assimilation, including components of the GS/GOGAT pathway, as well as PII proteins acting to regulate the pathway. Expression of a large number of transport-related proteins was also induced. However, the substrates for these transporters are unknown, and it is difficult to predict their cellular functions based strictly on the gene annotations. Finally, genes involved in nitrate and nitrite acquisition and reduction were also upregulated in an NtrC-dependent fashion. Among these genes, the microarray analysis led to the identification and characterization of the *B. japonicum nirA* gene, encoding assimilatory nitrite reductase.

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