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Identification and characterization of the two-component NtrY/NtrX regulatory system in *Azospirillum brasilense*

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Abstract

Two *Azospirillum brasilense* open reading frames (ORFs) exhibited homology with the two-component NtrY/NtrX regulatory system from *Azorhizobium caulinodans*. These *A. brasilense* ORFs, located downstream to the *nifR3ntrBC* operon, were isolated, sequenced and characterized. The present study suggests that ORF1 and ORF2 correspond to the *A. brasilense ntrY* and *ntrX* genes, respectively. The amino acid sequences of *A. brasilense* NtrY and NtrX proteins showed high similarity to sensor/kinase and regulatory proteins, respectively. Analysis of *lacZ* transcriptional fusions by the ß-galactosidase assay in *Escherichia coli ntrC* mutants showed that the NtrY/NtrX proteins failed to activate transcription of the *nifA* promoter of *A. brasilense*. The *ntrYX* operon complemented a *nifR3ntrBC* deletion mutant of *A. brasilense* for nitrate-dependent growth, suggesting a possible cross-talk between the NtrY/X and NtrB/C sensor/regulator pairs. Our data support the existence of another two-component regulatory system in *A. brasilense*, the NtrY/NtrX system, probably involved in the regulation of nitrate assimilation.

Key words
- *Azospirillum brasilense*
- Two-component regulatory system
- Nitrogen regulation
- *ntrYX* genes
- *ntrBC* genes

Introduction

The *ntrYX* genes were identified and sequenced and their probable physiological function was characterized in *Azorhizobium caulinodans* ORS571 (1). In this symbiotic nitrogen-fixing bacterium, the NtrY and NtrX proteins constitute a two-component regulatory system apparently involved in nitrogen fixation and metabolism (1). The NtrY protein was homologous with sensor transmembrane proteins while NtrX exhibited a high degree of homology with positive regulatory proteins, such as NtrC. The expression of the *ntrYX* operon was depressed in an *ntrC* mutant grown in the presence of nitrate, suggesting a possible interaction between the *ntrYX*/*ntrBC* systems in *A. caulinodans* (1). *A. caulinodans ntrC* or *ntrX* mutants were also unable to activate the expression of the *nifA* gene, implying that NtrC and NtrX proteins could be involved in *nifA* expression. The authors suggested that the *ntrYX* and *ntrBC* genes were involved in nitrogen metabolism in *A. caulinodans* and that the NtrY/NtrB sensors could cross-talk with the NtrX/NtrC regulators to activate trans-

cription initiation from *ntr*-dependent promoters (1). The *ntrYX* genes were also found in other microorganisms such as *Acetobacter diazotrophicus* (*Gluconacetobacter diazotrophicus*) (2), *Caulobacter crescentus* (3), *Sinorhizobium meliloti* (4), *Mesorhizobium loti* (5), *Rickettsia prowazekii* (6), *Neisseria meningitidis* (7) and *Zymomonas mobilis* (8). The function of these genes in these bacteria remains unknown.

Regulation of nitrogen fixation in *Azospirillum brasilense*, a free-living bacterium, is still under investigation. Pedrosa and Yates (9) suggested a mechanism of regulation similar to that observed in *Klebsiella pneumoniae* after they isolated a *nifA* (FP10) and two *ntrC* (FP8 and FP9) mutants, which were unable to fix nitrogen. However, Liang et al. (10), sequencing the *A. brasilense nifA* gene, did not find NtrC-binding sites or a σ^{54}-type promoter in the upstream region of this gene. An essential region for *nifA* promoter activity was identified between nucleotides -67 and -47 from the *nifA* transcription start site (11). A sequence resembling a σ^{70} recognition site occurs in this region and may constitute the *nifA* gene promoter (11). Oxygen inhibited expression of the *nifA* gene, but only partial repression by ammonium was observed (10). On the other hand, when oxygen and ammonium were present repression of *nifA* expression reached high levels (80-90%) (11). In *glnB* mutants of *A. brasilense* the NifA protein is synthesized in an inactive form, suggesting the involvement of the PII protein in the regulation of NifA activity by ammonia (12). In the presence of ammonia, NifA is in an inactive form since its N-terminal domain has been suggested to auto-inhibit its activity (13). On the other hand, under ammonium-limiting conditions, the PII protein was necessary to maintain the NifA protein in the active form, apparently by preventing the N-terminal inhibition. The mechanism by which PII prevents N-terminal inhibition under conditions of ammonia limitation is still unknown.

The NtrB/NtrC system in *A. brasilense* is involved in regulation of nitrate utilization (9,14,15), switch-off of nitrogenase by ammonium (9,16) and (methyl) ammonium transport (17). Deletion or mutation of the *ntrBC* genes did not abolish nitrogenase activity but reduced it to half of that observed in the wild-type strain (14,15), suggesting the probable involvement of a second system in the regulation of nitrogen fixation.

In the present study, we found the *ntrYX* genes in *A. brasilense* located downstream from the *nifR3ntrBC* operon. The genes were sequenced completely and the translation start codon of the *ntrX* gene was shown to overlap the 3' end of the *ntrY* gene. Their gene products were highly homologous to the NtrY and NtrX proteins from other organisms. The *ntrYX* genes complemented a *nifR3ntrBC* deletion mutant of *A. brasilense* for nitrate-dependent growth.

Material and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in the present study are listed in Table 1.

Plasmid pTH6E3 was constructed by cloning an *Eco*RI fragment of 6.3 kb containing part of *ntrB* and the whole of *ntrC*, *ntrY* and *ntrX* genes into pTZ18R. The 5.1-kb *Sal*I fragment from pTH6E3 was subsequently cloned into pSUP202, producing pSH5S1. The Km-*lacZ* cassette from pKOK6.1 was then inserted into the *Nsi*I site of pSH5S1 in both orientations, producing plasmids pLKIII and pLK015 (Figure 1 and Table 1).

To construct pL46 the 4.85-kb *Bgl*II/*Eco*RI fragment from pTH6E3 was cloned into pLAFR3.18 digested with *Bam*HI and *Eco*RI (Table 1). In this construction, the *ntrC* gene lacks its N-terminal region and its orientation of transcription is opposite to that of the *lacZ* promoter, and the *ntrYX* genes are expressed from their native promoter, as in pTH6E3.

Media and growth conditions

Escherichia coli was grown in Luria-Bertani medium (24) at 37ºC and 200 rpm. *A. brasilense* strains were grown at 30ºC in liquid or semi-solid NFbHPN medium (15). The antibiotics used were ampicillin (200 µg/ml), chloramphenicol (30 µg/ml), kanamycin (50 µg/ml), nalidixic acid (10 µg/ml), tetracycline (10 µg/ml), and streptomycin (100 µg/ml). Nitrate-dependent growth in *A. brasilense* was monitored in liquid NFbHPN medium containing 10 mM $NaNO_3$ for 24 h at 30ºC.

Analytical assays

ß-Galactosidase activity was determined as described by Miller (25) in *E. coli* cultures grown in liquid NFDM (26). The nitrogen source was NH_4Cl (20 mM) and serine (100 µg/ml) was added to cultures without ammonium. The NFDM medium was supplemented with 50 µg/ml L-glutamine and 1 mM IPTG. *A. brasilense* cultures were grown in NFbHPN medium under conditions of ammonium deficiency (5 mM L-glutamate) or excess (20 mM NH_4Cl).

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype(s) or Phenotype(s)	Reference
Strain		
E. coli		
DH5α	*supE44* Δ*lacU*169 (f80 *lac*ZΔM15)	18
7118	Δ(*lac-pro*) F'*lacqI*^{q}Z DM15 *pro AB*$^+$	19
S17.1	RP4-2 Tc::Mu Km::Tn7 Tra$^+$*recA*$^-$	20
ET8556	*ntrC* Nal^r Δ*lac*	21
A. brasilense		
FP2	Sp7 ATCC 29145 Nif$^+$ Sm^r Nal^r	9
FP9	*ntrC*$^-$ Sm^r Nal^r	9
HDK1	FP2 Δ*nifR3ntrBC*::*lacZ*-Km	15
FP2.R	FP2 *nifA*::Tn5-B20. Chromosomal *nifA*::*lacZ* fusion. Nal^r Sm^r Km^r	11
FP9.R	FP2 *nifA*::Tn5-B20. Chromosomal *nifA*::*lacZ* fusion. Nal^r Sm^r Km^r *ntrC*$^-$	11
MLY9	FP2 *ntrYX*::*lacZ*-Km	Present study
MLY84	FP2 *ntrYX*::Km-*lacZ*	Present study
Plasmids		
pTZ18R/19R	Cb^r *lacZ* f1	22
pLAFR3.18	cos IncP-1 Tc^r pUC18 multiple cloning site	15
pHM9	21.5 kb from *A. brasilense* in pLAFR3	15
pSH5S1	5.1-kb/*Sal*I DNA fragment from pHM9 containing part of *ntrB* and the *ntrCntrY* genes of *A. brasilense* in pSUP202	Present study
pTH6E3	6.3-kb/*Eco*RI DNA fragment from pHM9 containing part of *ntrB* and the *ntrCntrYntrX* genes of *A. brasilense* in pTZ18R	Present study
pKOK6.1	promotorless *lacZ*-Km^r cassette. Cb^r Km^r	23
pSUP202	Cb^r Tc^r Cm^r *oriT*	20
pPW452	Tc^r, transcriptional fusion vector containing *lacZ* gene	P. Woodley
pCF2	*A. brasilense nifA*::*lacZ* fusion in pPW452	11
pSPORT2	Cb^r *lacZ*	Life Technologies
pLKIII	pSH5S1 derivative with *ntrY*::*lacZ*-Km	Present study
pLK015	pSH5S1 derivative with *ntrY*::Km-*lacZ*	Present study
pTL46	pTZ19R carrying a 4.85-kb *Eco*RI/*Bgl*II from pTH6E3 containing part of *ntrC* and the *ntrYX* genes of *A. brasilense*	Present study
pSPL46	pSPORT2 carrying a 4.85-kb *Eco*RI/*Bgl*II from pTH6E3 containing part of *ntrC* and the *ntrYX* genes of *A. brasilense*	Present study
pL46	pLAFR3.18 carrying a 4.85-kb *Eco*RI/*Bgl*II from pTH6E3 containing part of *ntrC* and the *ntrYX* genes of *A. brasilense*	Present study

DNA manipulations and sequencing

Isolation of plasmid DNA, gel electrophoresis and cloning experiments were carried out as described by Sambrook et al. (24). The 4.85-kb *Bgl*II/*Eco*RI fragment derived from pTH6E3, containing the *A. brasilense ntrYX* genes, was cloned into the vector pSPORT2 producing plasmid pSPL46 (Table 1). The inserted fragment was fully sequenced in both directions. Double-stranded DNA was sequenced with the Thermosequenase kit (Amersham Pharmacia Biotech, Uppsala, Sweden) or the Big Dye kit (Applied Biosystems ABI 310 sequencer, Foster City, CA, USA). The database was searched using the Blast program (27) and DNA or protein sequences were compared using the Clustal W program (28). The sequences of *A. brasilense ntrY* and *ntrX* were deposited at the EMBL-GenBank under the accession number AF426449.

Results and Discussion

Identification and sequencing of the *ntrYX* genes

The 4.85-kb insert of plasmid pSPL46 was fully sequenced and showed the pres-

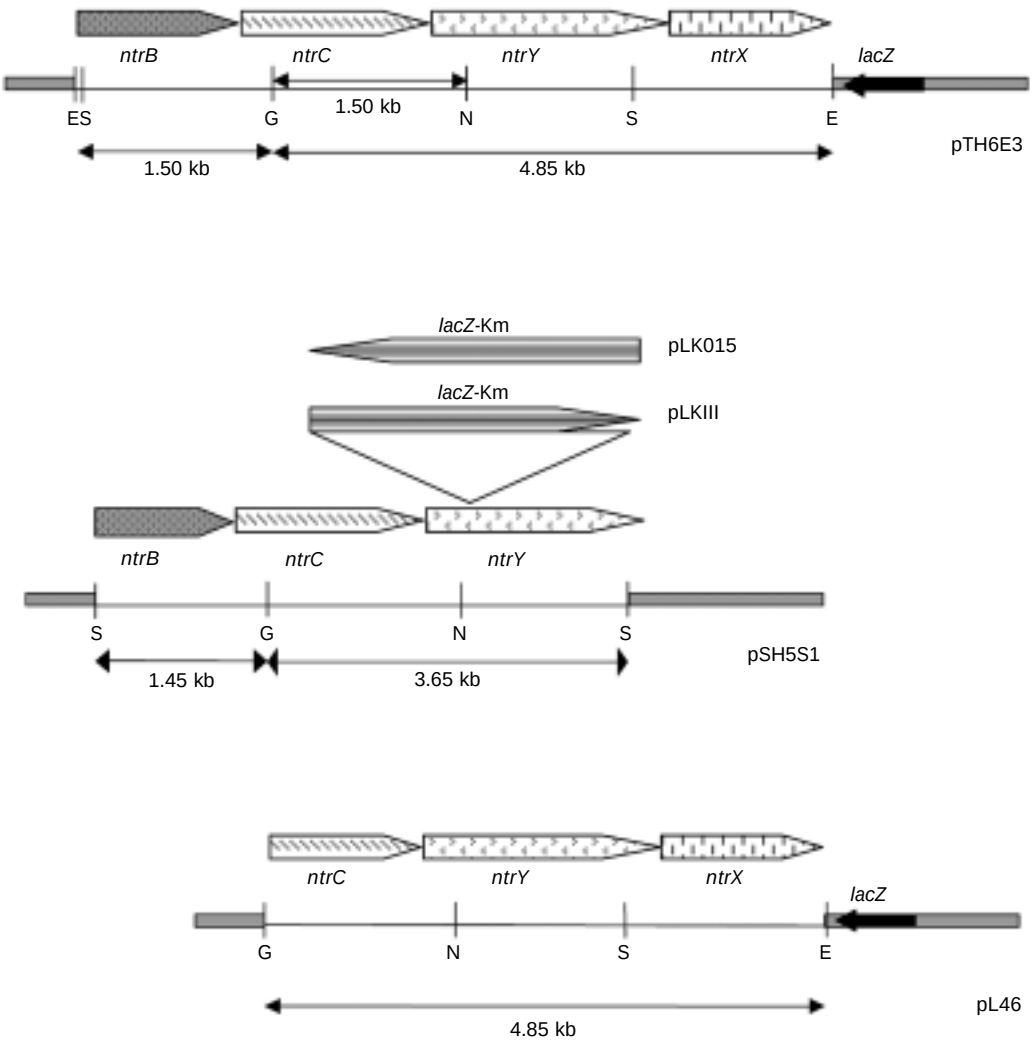


Figure 1. Genetic and physical maps of the DNA region containing the *ntrBC/ntrYX* genes of *Azospirillum brasilense*. The vectors of plasmid pTH6E3, pSH5S1 and pL46 are pTZ18R, pSUP202 and pLAFR3.18, respectively. Vector maps are not shown. Plasmids pLKIII and pLK015 were used to produce mutants MLY9 and MLY84, respectively, and contained the *lacZ*::Km cassette inserted into the *Nsi*I site of *ntrY* in the direction indicated. Restriction enzymes are: E, *Eco*RI; G, *Bgl*II; N, *Nsi*I, and S, *Sal*I.

ence of two complete open reading frames (ORF1 and ORF2) downstream from the *nifR3ntrBC* operon. ORF1 contains 2331 nucleotides plus the stop codon TGA, and has a high G + C content in the third base position (89.3%), characteristic of *A. brasilense* genes (29). The probable start codon was located 183 bp downstream from the *ntrC* gene termination codon, with a potential ribosome-binding site (GGA) 3 bp upstream from the ATG initiation codon. This ORF1 translated into a hydrophobic polypeptide of 777 amino acids, with a high degree of identity with the NtrY protein of *A. caulinodans* (41%) and of *C. crescentus* (40%).

Analysis of the *A. brasilense* NtrY protein hydropathy graph (Figure 2), determined according to Kyte and Doolittle (30), revealed four hydrophobic regions in the N-terminus equivalent to the putative transmembrane regions of *A. caulinodans* NtrY (1), and to those of the *E. coli* and *Salmonella typhimurium* chemoreceptor proteins

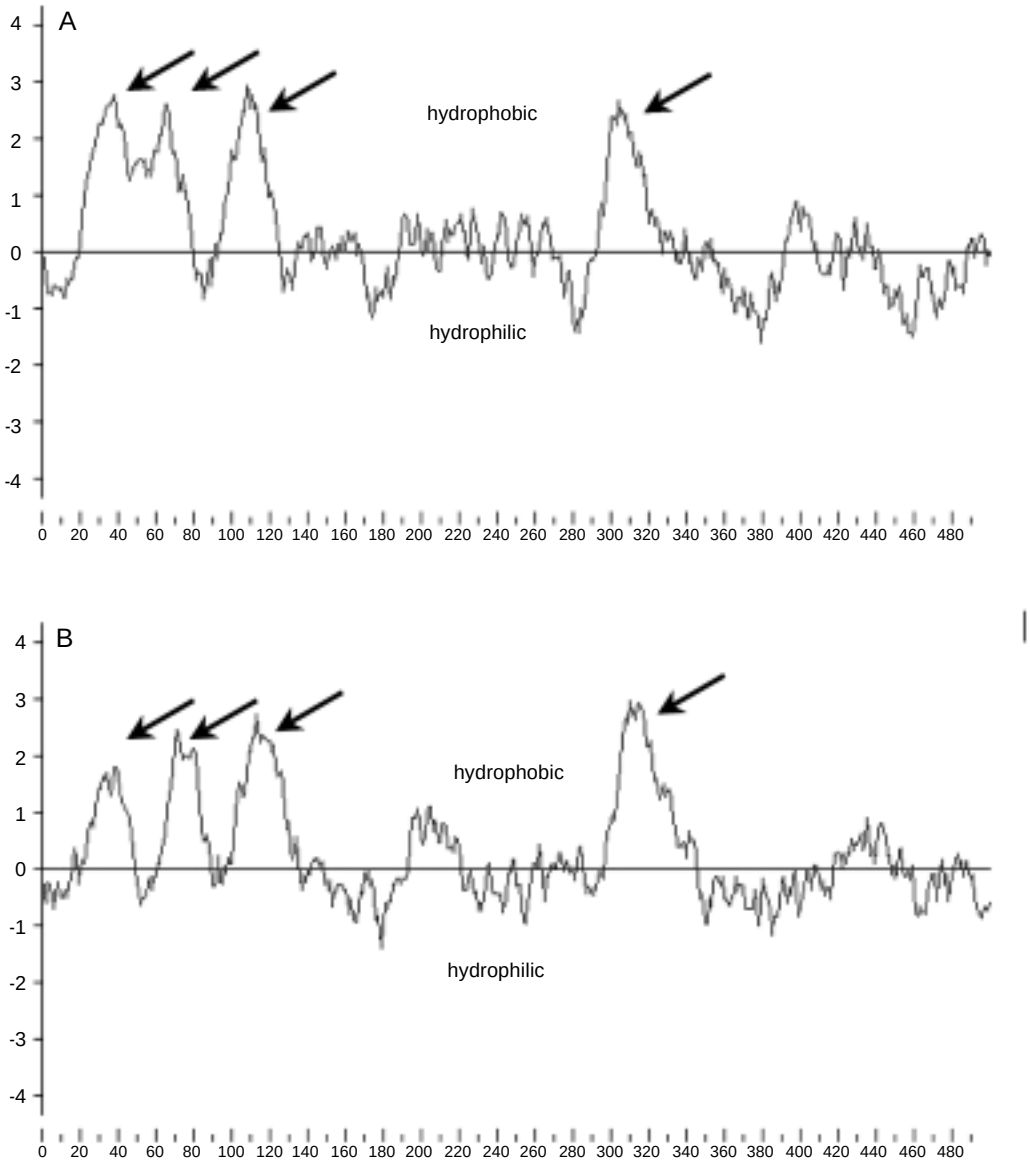


Figure 2. *A*, Hydrophobicity graph of the N-terminal region of the NtrY proteins from *Azorhizobium caulinodans* (A; EMBL-GeneBank accession number X63841) and from *Azospirillum brasilense* (B). Arrows indicate the equivalent putative transmembrane regions. The hydropathy profiles were determined according to Kyte and Doolittle (30).

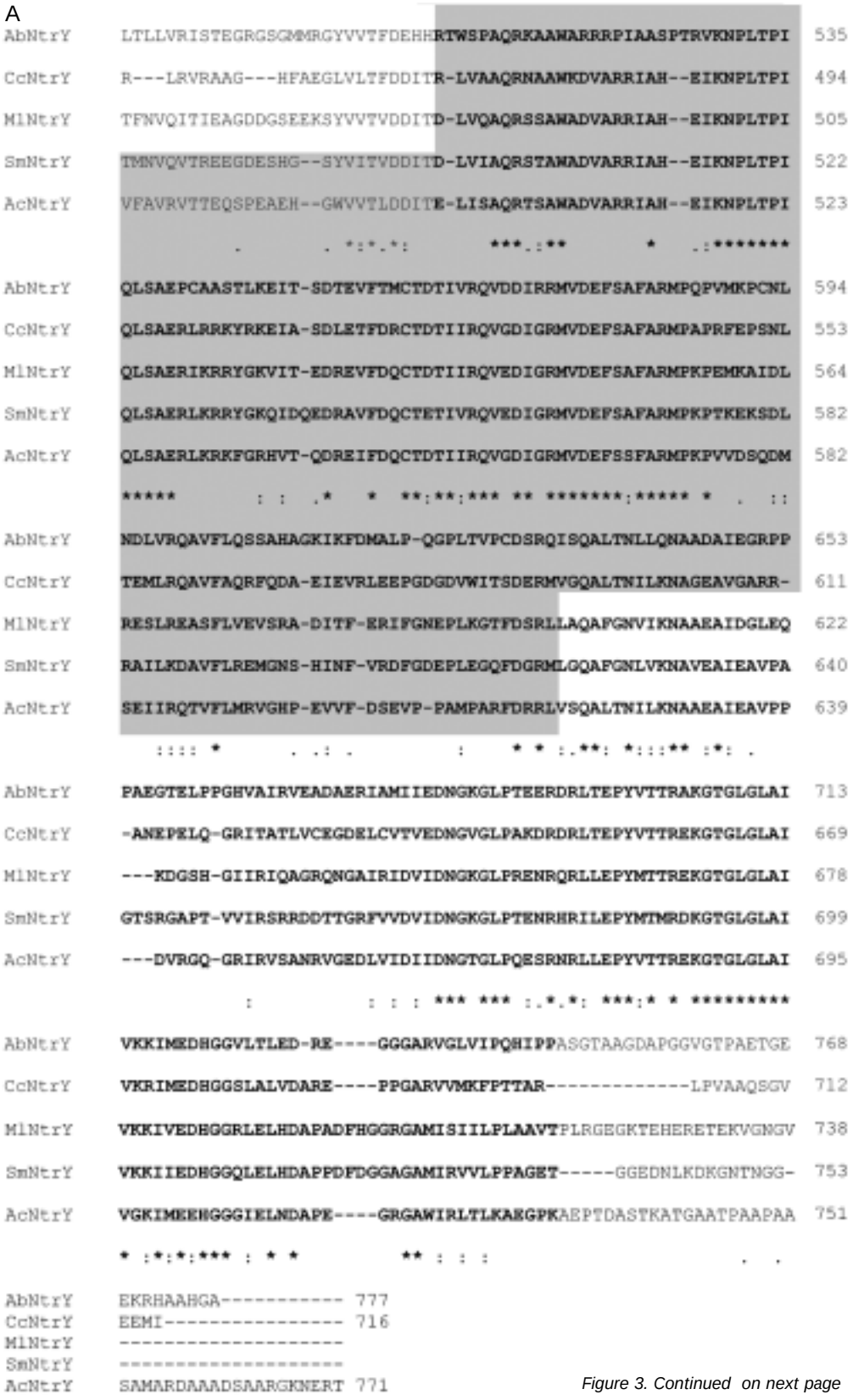
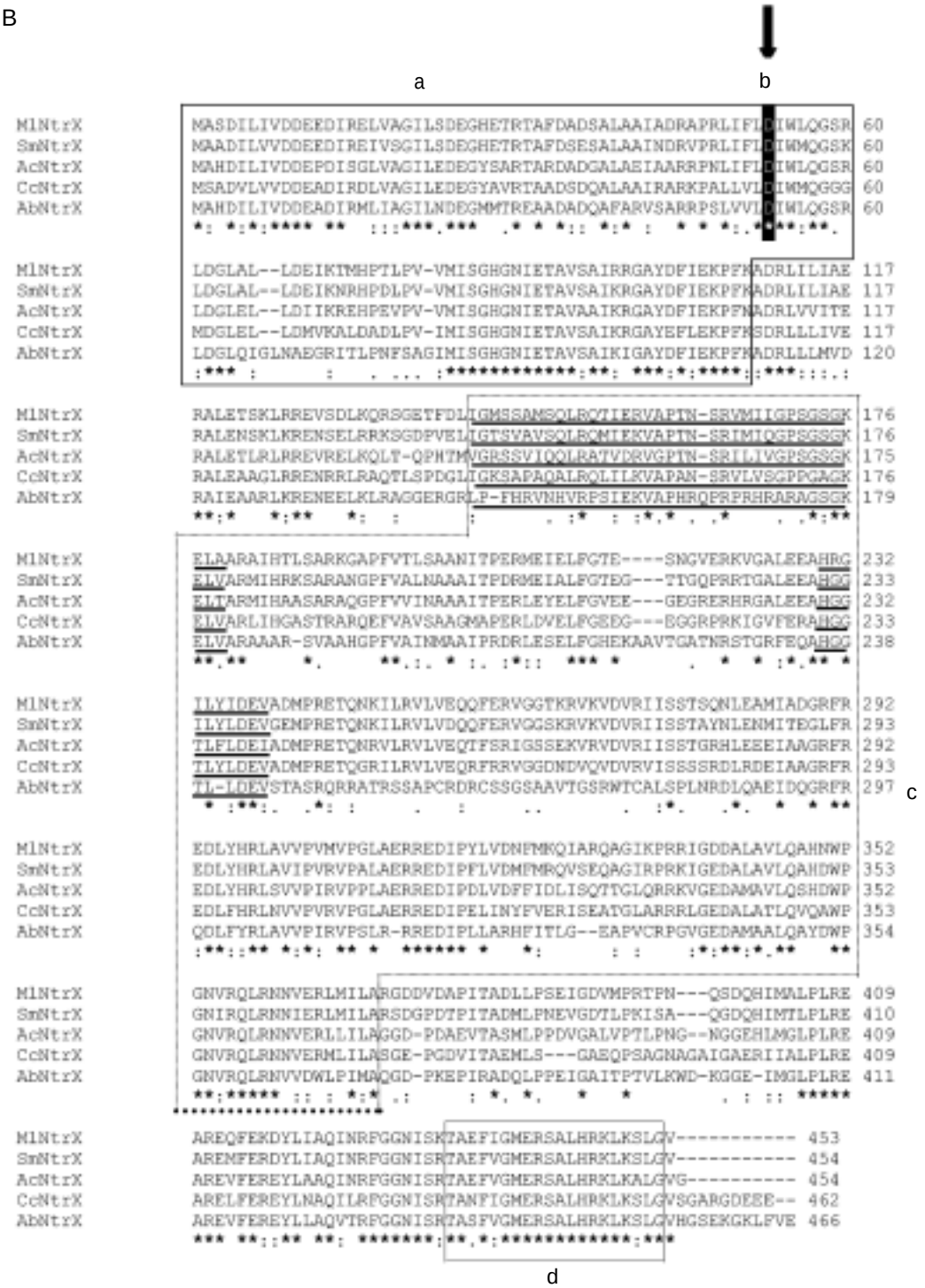


Figure 3. *A*, Amino acid sequence comparison of sensor domains from different NtrY proteins. The sensor region is contained in the grey box. *B*, Amino acid sequence comparison of *Azospirillum brasilense* and *Azorhizobium caulinodans* NtrX proteins. The boxes show the receiver domain (a) and helix-turn-helix DNA-binding motif (d). The dotted boxes represent: ATP-binding site (underlined), RNA polymerase σ^{54} factor interaction domain (c). The black box and the arrow indicate the phosphorylation site (b). Identical amino acids are indicated by an asterisk, conserved substitution by a colon, and semi-conserved substitution by a dot. Ac = *Azorhizobium caulinodans*; Ab = *Azospirillum brasilense*; Cc = *Caulobacter crescentus*; Sm = *Sinorhizobium meliloti*; Ml = *Mesorhizobium loti.*

Figure 3. Continued on next page

Figure 3. Continued

B



(31,32). The C-terminus of the *A. brasilense* NtrY protein (Figure 3A) showed a high degree of identity (35%) with the conserved C-termini of homologous proteins from *A. caulinodans*, *C. crescentus*, *M. loti* and *S. meliloti*.

ORF2 encoded a 466-amino acid protein with a high degree of identity (52%) to *A. caulinodans* NtrX (Figure 3B), and a lower identity (32%) to NtrC proteins of other organisms including *A. brasilense*. The aspartic acid residue at position 53 in the *A. brasilense* NtrX is probably the site of phosphorylation, since it is conserved in all NtrX proteins (Figure 3B). This site is equivalent to the phosphorylation site of the NtrC protein of *A. brasilense*, Asp 54 (14). The *A. brasilense* NtrX protein displays structural domains characteristic of the regulator partner of two-component regulatory systems, namely the receiver domain, the ATP-binding catalytic domain, the RNA polymerase σ^{54} factor interaction domain and the helix-turn-helix DNA-binding motif. The degree of identity of the receiver, ATP-binding and RNA polymerase σ^{54} factor interaction domains of the NtrX protein of *A. brasilense* was on average 31%, while that of the helix-turn-helix DNA-binding motif was 89% to homologous proteins of *A. caulinodans*, *C. crescentus*, *M. loti* and *S. meliloti*. These data indicate that the *ntrYX* loci of *A. brasilense* may constitute a two-component regulatory system in which NtrY could serve as a sensor and NtrX as a regulator protein.

A σ^{70}-type promoter was identified 26 bp upstream from the start codon of NtrY (TTGGCA-N18-TATCAT). Machado et al. (15) sequenced the N-terminal region of NtrY downstream from the *ntrC* gene and reported constitutive promoter activity located in the intergenic region in an *E. coli* background. These results suggest that the *ntrYX* operon is expressed from its own promoter although readthrough from an upstream promoter cannot be ruled out due to the absence of a transcription terminator sequence downstream from the *ntrC* gene.

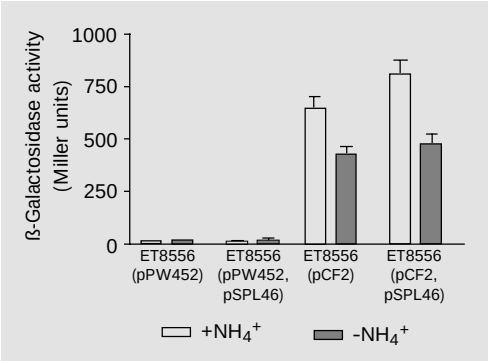


Figure 4. Expression of the *nifA* promoter in the presence of the NtrY/NtrX proteins in an *ntrC*⁻ *Escherichia coli* strain ET8556. The cultures were grown for 24 h in NFDM minimal medium. The nitrogen source was NH_4Cl (20 mM); cultures without ammonium were supplemented with serine (100 µg/ml). The expression of the *nifA* promoter was determined by ß-galactosidase activity. The results are the average of 3 independent experiments run in 3 replicates and bars indicate the standard deviation.

Table 2. Effect of *ntrYX* genes on the expression of chromosomal *nifA::lacZ* fusions in *Azospirillum brasilense* strains.

Strain and plasmid	ß-Galactosidase activity (Miller units)	
	20 mM NH_4Cl	5 mM L-glutamate
FP2 (wild type)	1.6 ± 0.4	2.8 ± 0.3
FP9 (*ntrC* mutant)	1.1 ± 0.1	2.1 ± 0.2
FP2.R (*nifA*::*lacZ* chromosomal)	50.6 ± 3.9	144.0 ± 7.2
FP2.R (pL46)	56.8 ± 4.3	174.7 ± 19.1
FP9.R (*ntrC*, *nifA*::*lacZ* chromosomal)	50.9 ± 6.9	140.6 ± 28.1
FP9.R (pL46)	81.9 ± 6.9	163.3 ± 11.0

Cultures were grown in liquid NFbHPN medium in the presence (20 mM) or absence of NH_4Cl, for 24 h. L-glutamate (5 mM) was added to cultures grown in the absence of NH_4Cl. Expression of the *nifA::lacZ* chromosomal fusion was determined by ß-galactosidase activity. The results are the average of three independent experiments run in duplicate ± SD.

Effect of the *ntrYX* gene products on the expression of an *A. brasilense nifA* fusion in an *E. coli* mutant strain

E. coli ET8556 (*ntrC*⁻) transformants containing plasmid pCF2 (*A. brasilense* p*nifA::lacZ* fusion with its native promoter) alone or together with plasmid pSPL46 (*A. brasilense ntrYX* genes expressed from their own promoter) were assayed for ß-galactosidase activity, under conditions of ammonium deficiency or excess. The results showed that the presence of the *ntrYX* genes had no effect on the expression of the p*nifA::lacZ* (pCF2)

fusion (Figure 4), suggesting that the NtrY/NtrX proteins are not involved in the expression of the *nifA* promoter in *A. brasilense* (Figure 4). Previous results showed that there is an active promoter immediately upstream from the *ntrY* gene (15).

Effect of *ntrYX* genes on the expression of chromosomal *nifA::lacZ* fusions in *A. brasilense* strains

A. brasilense FP2.R (*nifA::lacZ*) and FP9.R (*ntrC*, *nifA::lacZ*) transconjugants containing pL46 (*ntrYX* expressed from its own promoter) were grown under conditions of ammonium deficiency or excess, and assayed for ß-galactosidase activity. No effect of the *ntrYX* genes was observed in either of the *Azospirillum* strains (Table 2), consistent with the results for the *E. coli* ET8556 background. The data suggested that the NtrY/NtrX pair has no effect on the expression of the *nifA* promoter in *A. brasilense*. The function for this regulatory pair in this organism is different from the proposed involvement in nitrogen metabolism in *A. caulinodans* (1). Our findings agree with those of Kaminski and Elmerich (33) who disputed the functions ascribed to the NtrY/NtrX proteins of *A. caulinodans* by Pawlowski et al. (1).

Attempts to construct *ntrY* mutants of *A. brasilense*

Plasmids carrying Km cassette insertions in the *ntrY* genes in both orientations were constructed (Figure 1; Table 1). The wild-type *A. brasilense* strain FP2 was transformed with plasmids pLKIII and pLK015 in separate experiments and plated onto a selective medium containing kanamycin plus ammonium chloride (20 mM) to isolate *ntrY* mutants. Transformants resistant to kanamycin and sensitive to chloramphenicol (MLY9 and MLY84, respectively), indicating that the mutated gene had recombined into the chromosome by double-crossover events, were isolated and analyzed by hybridization. The hybridization results (data not shown) showed that the Km cassette was inserted into the chromosome of these mutants, however, at a site different from the *ntrY* gene. These results were surprising since this method of mutagenesis had previously yielded *ntrBC* mutants of *A. brasilense* (15). The reasons for our failure to obtain *A. brasilense ntrY* or *ntrX* mutants are not known. It is possible that NtrY and/or NtrX have pleiotropic effects affecting metabolic pathways involved in cell survival.

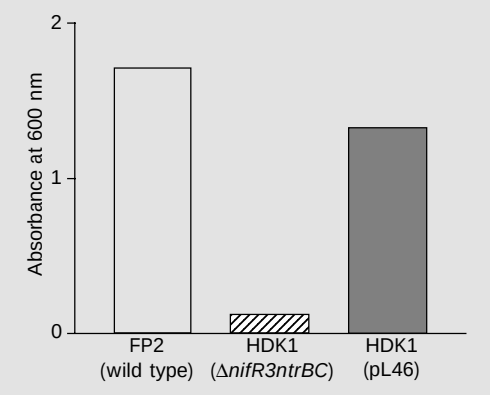


Figure 5. Complementation of the Nar$^-$ phenotype of an *Azospirillum brasilense nifR3ntrBC* mutant. The cultures were incubated for 24 h in liquid NFbHPN medium with 10 mM $NaNO_3$ as the sole nitrogen source. Nitrate-dependent growth was determined by absorbance of the culture at 600 nm. The results are from a representative experiment.

The *ntrYX* genes complement the Nar$^-$ phenotype of an *A. brasilense nifR3ntrBC* mutant

The *A. brasilense nifR3ntrBC* deletion mutant (HDK1) failed to grow on nitrate as sole nitrogen source and was complemented by the *ntrBC* genes (15). The same mutant HDK1 was complemented for nitrate-dependent growth by the *A. brasilense ntrYX* genes carried by plasmid pL46 (Figure 5). Taken together, these results corroborate previous observations that suggested that in *A. brasilense* the *ntrYX* pair was interchangeable with the *ntrBC* pair with respect to nitrate-dependent growth (34).

In this study, we sequenced and identified the *ntrYX* genes as a second two-component system in *A. brasilense*. The *A. brasi-*

lense NtrY protein contains probable transmembrane segments located in its N-terminus and may be involved in sensing the extracellular nitrogen concentration. The NtrX protein is suggested to be a transcriptional activator of alternative nitrogen assimilation pathways such as nitrate in *A. brasilense*. The *ntrYX* genes, similar to the *nifR3ntrBC* operon, are apparently not required for the expression of the *nifA* gene in *A. brasilense* (11,14,15).

Acknowledgments

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