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

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 β-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.

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 brasiliense, 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 σ54-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 σ70 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 gltB 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 EcoRI fragment of 6.3 kb containing part of ntrB and the whole of ntrC, ntrY and ntrX genes into pTZ18R. The 5.1-kb SalI fragment from pTH6E3 was subsequently cloned into pSUP202, producing pSH5S1. The Km-lacZ cassette from pKOK6.1 was then inserted into the NsiI site of pSH5S1 in both orientations, producing plasmids pLKIII and pLK015 (Figure 1 and Table 1).

To construct pL46 the 4.85-kb BglII/EcoRI fragment from pTH6E3 was cloned into pLAFR3.18 digested with BamHI and EcoRI (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 37°C and 200 rpm. *A. brasilense* strains were grown at 30°C 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 NaNO₃ for 24 h at 30°C.

Analytical assays

β-Galactosidase activity was determined as described by Miller (25) in *E. coli* cultures grown in liquid NFDM (26). The nitrogen source was NH₄Cl (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 NH₄Cl).

Table 1. Bacterial strains and plasmids.

<table>
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<th>Strain or plasmid</th>
<th>Genotype(s) or Phenotype(s)</th>
<th>Reference</th>
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<td>Δ(lac-pro) F′ lacIQ2 ΔM15 pro AB⁺</td>
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<td>RP4-2 Tc::Mu Km::Tn7 Tra::recA⁻</td>
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<td>ET8556</td>
<td>ntrC Nal⁺ Δlac</td>
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<td><em>A. brasilense</em></td>
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<tr>
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<td>Sp7 ATCC 29145 Nif⁺ Smr Nal⁺</td>
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</tr>
<tr>
<td>FP9</td>
<td>ntrC⁻ Smr Nal⁺</td>
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<td>MLY84</td>
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<td><strong>Plasmids</strong></td>
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<td>Present study</td>
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<td>pTH6E3</td>
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<td>Cb' Tc' Cmᵣ oriT</td>
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<td>Tc' transcriptional fusion vector containing lacZ gene</td>
<td>P. Woodley</td>
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<td>Cb' lacZ</td>
<td>Life Technologies</td>
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<td>plK015</td>
<td>pSH551 derivative with ntrY::Km-lacZ</td>
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<td>pL46</td>
<td>pLAFR3.18 carrying a 4.85-kb EcoRI/BglII from pTH6E3 containing part of ntrC and the ntrYX genes of <em>A. brasilense</em></td>
<td>Present study</td>
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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 BglII/EcoRI fragment derived from pTH6E3, containing the \textit{A. brasilense} \textit{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 \textit{A. brasilense} \textit{ntrY} and \textit{ntrX} were deposited at the EMBL-GenBank under the accession number AF426449.

Results and Discussion

Identification and sequencing of the \textit{ntrYX} genes

The 4.85-kb insert of plasmid pSPL46 was fully sequenced and showed the pres-
ence of two complete open reading frames (ORF1 and ORF2) downstream from the \textit{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 \textit{A. brasilense} genes (29). The probable start codon was located 183 bp downstream from the \textit{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 poly-
peptide of 777 amino acids, with a high degree of identity with the NtrY protein of \textit{A. caulinodans} (41\%) and of \textit{C. crescentus} (40\%).

Analysis of the \textit{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 \textit{A. caulinodans} NtrY (1), and to those of the \textit{E. coli} and \textit{Salmonella typhimurium} chemoreceptor proteins.

![Hydropathy graph of the N-terminal region of the NtrY proteins from Azo-
rhizobium caulinodans (A; EMBL-GeneBank accession number X63841) and from Azos-
spirillum brasilense (B). Arrows indicate the equivalent putative transmembrane re-
geons. The hydropathy profiles were deter-
mined 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 σ54 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

NtrY/NtrX of Azospirillum brasilense
tein of *A. brasilense*, Asp 54 (14). The *A. brasilense* NtrX protein displays structural domains characteristic of the regulator part-
er of two-component regulatory systems, namely the receiver domain, the ATP-bind-
ing catalytic domain, the RNA polymerase $\sigma_{54}$ factor interaction domain and the helix-
turn-helix DNA-binding motif. The degree of identity of the receiver, ATP-binding and 
RNA polymerase $\sigma_{54}$ factor interaction do-
main 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. brasi-
lese* may constitute a two-component regu-
lar system in which NtrY could serve as a 
sensor and NtrX as a regulator protein.

A $\sigma_{70}$-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 can-
not be ruled out due to the absence of a 
transcription terminator sequence down-
stream from the *ntrC* gene.

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 con-
taining plasmid pCF2 (*A. brasilense* pnifA:: 
lacZ fusion with its native promoter) alone 
or together with plasmid pSPL46 (*A. brasi-
lese ntrYX genes expressed from their own 
promoter) were assayed for $\beta$-galactosidase 
activity, under conditions of ammonium def-
iciency or excess. The results showed that 
the presence of the *ntrYX* genes had no effect 
on the expression of the pnifA::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 β-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.

**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 ntrX genes, similar to the nifR3ntrBC operon, are apparently not required for the expression of the nifA gene in A. brasilense (11,14,15).

Acknowledgments

We thank M.G. Yates for a critical reading of this manuscript.

References


