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Purification and characterisation of *Azospirillum brasilense* N-truncated NtrX protein

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Abstract

The NtrX protein has been identified as a transcriptional activator of genes involved in the metabolic control of alternative nitrogen sources, acting as a member of a two-component regulatory system. The *in silico* analysis of the NtrX amino acid sequence shows that this protein contains an N-terminal receiver domain, a central AAA+ superfamily domain and a C-terminal DNA binding domain. To over-express and purify this protein, the *ntrX* gene of *Azospirillum brasilense* lacking the first eight codons was cloned into the vector pET29a+. The NtrX protein was over-expressed as an S.Tag fusion protein induced by L-arabinose in the *Escherichia coli* strain BL21AI and purified by ion exchange and affinity chromatography. The ATPase activity of NtrX was measured by coupling the ATP conversion to ADP with NADH oxidation. The ATPase activity of NtrX was stimulated in the presence of *A. brasilense* σ^{54}/NtrC-dependent promoter of the *glnBA* gene. Phosphorylation by carbamyl-phosphate also stimulated ATPase, in a manner similar to the NtrC protein. Together our results suggest that NtrX is active in the phosphorylated form and that there may be a cross-talk between the NtrYX and NtrBC regulatory systems in *A. brasilense*.

Keywords: NtrX; Nitrogen fixation; *Azospirillum brasilense*; Two-component system

The *ntrYX* genes, which encode the NtrY and NtrX proteins, were identified in the diazotrophic bacterium *Azospirillum brasilense* downstream from the *nifR3ntrBntrC* operon [1,2]. The two proteins are probably involved in extracellular sensing of the nitrogen status and in the regulation of the nitrogen metabolism. The signaling pathway may involve the phosphorylation of the response regulator NtrX by the protein kinase NtrY, acting as a two-component regulatory system [2,3].

The function of the NtrX protein was first studied in *Azorhizobium caulinodans*. In this organism, a mutant strain in *ntrX* gene was defective in using nitrate as nitrogen source, had reduced *nifA* expression under nitrogen fixation conditions and a severely disturbed symbiotic phenotype [4]. In *A. brasilense*, the NtrX protein is probably a transcriptional activator of genes involved in nitrate utilization and other alternative nitrogen sources [3,2].

The N-terminal region of NtrX contains a sensor domain with a sequence motif probably involved in phosphate and magnesium binding. A conserved aspartate residue is also present is this domain suggesting that *A. brasilense* NtrX is probably activated by phosphorylation by the cognate histidine kinase NtrY [5]. The central region of NtrX has an AAA+ domain (ATPases associated with a variety of cellular activities), involved in ATP hydrolysis, open-complex formation and oligomerisation. The AAA+ domain contains a classical Walker A (P-loop) and Walker B motifs, which are probably responsible for ATP binding and ATPase activity. Furthermore, the GAFTGA motif is present, which is responsible for interaction with the sigma-54 RNA polymerase [6,7]. The DNA binding domain is located in the C-terminal region [5]. Together, the domain

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Table 1
Bacterial strains and plasmids

Strain and Plasmids	Genotype	Source/References
E. coli		
DH10B	*F-mcrA* Δ *mrr-hsdRMS-mcrBC*	Life technologies
BL21λ (DE3) codonplus	*F-dcm ompT hsdS* ($r_B^- m_B^-$)*gal* (λDE3)	Stratagene
RosettaBlue (DE3) pLysS	*endA1 hsdR17*(rK12–mK12+) *supE44 thi-1 recA1 gyrA96 relA1 lac* pLysS RARE2	Novagen
C41 (DE3)	*ompT hsdS* (r_B m_B) *gal* λ (DE3) containing one non-characterised mutation at least	[8]
C43 (DE3)	*ompT hsdS* (r_B m_B) *gal* λ (DE3) containing two non-characterized mutations at least, derived from C41(DE3)	[8]
BL21AI	*F-ompT hsdSB* (rB-mB-) *gal dcm araB*::T7RNAP-*tetA*	Invitrogen
Plasmids		
pMCA1	pTZ18R vector containing a 2.0 kb *Eco*RI/*Sph*I fragment of C-terminal region of *ntrY* gene and *ntrX* gene of *A. brasilense* FP2	[5]
pET29a+	Expression vector/T7 promoter Km^R S.Tag fusion	Novagen
pETM-x1	Sequence encoding N-truncated NtrX protein of *A. basilense* FP2 in pET29a+	This work
pSPL46	pSPORT2 carrying a 4.85-kb *Eco*RI–*Bgl*II containing part of *ntrC* and the *ntrYX* genes of *A. brasilense* FP2	[2]
PLHglnBTZ	Amp^R (pTZ19R) *Xho*I–*Xba*I fragment derived from pLHglnBET3 containing *glnBp1p2*	[9]

structure of NtrX suggests that it regulates gene expression by binding at promoter sites located upstream from σ^{54}-dependent genes.

In this work we described the purification of the native soluble form of *A. brasilense* NtrX overproduced in *Escherichia coli* strain BL21AI and characterized its ATPase activity.

Materials and methods

Bacterial strains and plasmids

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

Cloning of N-truncated ntrX gene into the pET29a+ expression vector

Plasmid pMCA1 was constructed by cloning the 2.0 kb *Eco*RI/*Sph*I fragment containing part of the *ntrY* gene and the whole *ntrX* gene into pTZ18R. The 1.5 kb *Sal*I/*Eco*RI fragment from this plasmid was subsequently cloned into the vector pET29a+ digested with *Bgl*II/*Eco*RI. To allow ligation, the *Sal*I and *Bgl*II cohesive ends of the insert and vector, respectively, were flushed by previous treatment with T4 DNA polymerase. This plasmid was denominated pETM-x1 and it was capable to express the N-truncated NtrX fused to an S-Tag in the N-terminal region (Fig. 1).

Over-expression of the A. brasilense N-truncated NtrX S.Tag fusion protein

Plasmid pETM-x1 was introduced into different *E. coli* strains (Table 1) carrying the T7 RNA Polymerase gene. To determine the condition for NtrX expression, transformants were inoculated into 10 mL of Luria–Bertani medium (LB)[1] supplemented with kanamycin 50 μg/mL and tetracycline 10 μg/mL, grown at 30 °C with aeration in a rotatory shaker at 150 rpm to an $OD_{600} = 0.3$, and 0.5 mmol/L IPTG, 0.5% lactose or 0.2% L-arabinose were added as inducers. The cultures were incubated for 4 h at 30 °C. After harvesting, the cells were re-suspended in lysis buffer (50 mmol/L Tris–HCl, pH 8.0, 500 mmol/L NaCl, 0.1 mmol/L EDTA, and 50% glycerol). The cell suspension (2 mL) was sonicated 3 times for 30 s at 20% breadth (Ultrasonic Processor XL, Heat Systems); between the sonication cycles the cell suspension was kept on ice for 1 min. The crude extract was centrifuged at 13,000*g* for 15 min at 4 °C. The supernatant was separated and the pellet was re-suspended in 1 volume of lysis buffer. The same volume of the supernatant and re-suspended pellet were mixed with 1 volume of Laemmli loading buffer, boiled for 3 min and loaded onto the SDS–PAGE electrophoresis [10].

Purification of A. brasilense N-truncated NtrX S.Tag fusion protein

Fresh *E. coli* BL21AI pETM-x1 transformants were inoculated into 5 L of Luria–Bertani medium (LB) with appropriate antibiotics. Cells were grown at 30 °C and 150 rpm. At $OD_{600} = 0.3$, L-arabinose (0.2%) was added and the culture incubated for 4 h at 30 °C. After harvesting, the cells were re-suspended in 6 mL lysis buffer. The cell suspension was sonicated 8 times for 30 s at 20% breadth (Ultrasonic Processor XL, Heat Systems); between the sonication cycles the cell suspension was kept on ice for 1 min. The crude extract was centrifuged at 13,000*g* for 15 min at 4 °C and diluted 10-fold with buffer A (50 mmol/L Tris–HCl, pH 8.0, 50 mmol/L NaCl, 0.1 mmol/L EDTA, and 10% glycerol). The resulting preparation (60 mL) containing the S-tagged NtrX protein was loaded onto a 30-mL Q-Sepharose column (Amersham Biosciences) equilibrated with buffer A. The protein was eluted with 150 mL of a linear NaCl gradient (0.05–1 M). Fractions containing the protein were pooled and dialyzed against buffer A and loaded onto a 5-mL

[1] *Abbreviations used:* LB, Luria–Bertani medium; EBP, enhancer-binding proteins.