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Characterization of a novel *Azorhizobium caulinodans* ORS571 two-component regulatory system, NtrY/NtrX, involved in nitrogen fixation and metabolism

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Summary. *Azorhizobium caulinodans* ORS571 *nifA* regulation is partially mediated by the nitrogen regulatory gene *ntrC*. However, the residual *nifA* expression in *ntrC* mutant strains is still modulated by the cellular nitrogen and oxygen status. A second *ntrC*-homologous region, linked to *ntrC*, was identified and characterized by site-directed insertion mutagenesis and DNA sequencing. Tn*5* insertions in this region cause pleiotropic defects in nitrogen metabolism and affect free-living as well as symbiotic nitrogen fixation. DNA sequencing and complementation studies revealed the existence of a bicistronic operon (*ntrYX*). NtrY is likely to represent the transmembrane 'sensor' protein element in a two-component regulatory system. NtrX shares a high degree of homology with NtrC proteins of other organisms and probably constitutes the regulator protein element. The regulation of the *ntrYX* and *ntrC* loci and the effects of *ntrYX*, *ntrY* and *ntrX* mutations on *nifA* expression were examined using β-galactosidase gene fusions. NtrY/NtrX were found to modulate *nifA* expression and *ntrYX* transcription was shown to be partially under the control of NtrC.

Key words: *Azorhizobium caulinodans* ORS571 – *nifA* regulation – Nitrogen regulation – Two-component regulatory system – Nitrogen fixation and metabolism

Introduction

Azorhizobium caulinodans ORS571 (Dreyfus et al. 1988; referred to as ORS571 in this report) occupies a unique position among the *Rhizobiaceae*: it can fix nitrogen in both stem and root nodules induced on its host, the tropical leguminous shrub *Sesbania rostrata* (Dreyfus and Dommergues 1981) and has the capacity to grow on dinitrogen (N_2) in the free-living state, at an unusually high temperature (37° C) and O_2 concentration (~ 3%; Dreyfus et al. 1983; Gebhardt et al. 1984; see de Bruijn 1989). It, therefore, constitutes a unique rhizobial strain in which it is possible to compare the regulation of nitrogen fixation (*nif*/*fix*) and assimilation genes in the free-living and symbiotic states (de Bruijn et al. 1987, 1988, 1990).

Expression of *nif* genes in ORS571 has been shown to be strictly controlled by the *nif*-specific regulatory gene *nifA*, both in the free-living and symbiotic states (Donald et al. 1986; Pawlowski et al. 1987; Ratet et al. 1988b). The regulatory circuitry controlling ORS571 *nifA* expression has been shown to have elements in common with that found in *Klebsiella pneumoniae*, where *nifA* transcription is controlled by the *ntr* (*nit*rogen *r*egulation) system, consisting of the NtrA, NtrB and NtrC proteins (see Gussin et al. 1986; Magasanik 1988; de Bruijn et al. 1990). NtrB/NtrC represent a two-component regulatory system (see Albright et al. 1989), composed of a cytosolic N-sensor protein (NtrB), which phosphorylates and thereby activates a DNA-binding regulatory protein (NtrC) under N-limiting conditions. NtrA (RpoN) is an alternative sigma factor, initiating transcription from *ntr*-controlled promoters in the presence of the corresponding regulatory DNA-binding proteins, e.g. NtrC or NifA (see Gussin et al. 1986; Magasanik 1988; Thöny and Hennecke 1989; Albright et al. 1989; Buck 1990; de Bruijn et al. 1990). The RNA polymerase – NtrA complex recognizes a characteristic promoter element ($^{-24}$GG/$^{-12}$GC; see Thöny and Hennecke 1989).

A mutation in the ORS571 *ntrC* gene causes a severe reduction in free-living nitrogen fixation and affects *nifA* transcription. In addition, ORS571 *ntrC* mutant strains are drastically altered in their nitrate utilization capacity and symbiotic properties (Pawlowski et al. 1987; de Bruijn et al. 1988; Ratet et al. 1989). The latter feature is peculiar to ORS571, since in *Rhizobium meliloti* and *Bradyrhizobium japonicum*, *ntrC* does not modulate symbiotic behavior (Szeto et al. 1987; Martin et al. 1988; see de Bruijn et al. 1990).

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The *R. meliloti nifA* gene is oxygen-controlled (induced microaerobically) via the two-component regulatory system FixL/FixJ, consisting of a transmembrane O_2 sensor (FixL) and a transcriptional activator, which has been postulated to act independently of NtrA (FixJ; David et al. 1988; Hertig et al. 1989; Gillis-Gonzales et al. 1991; see de Bruijn and Downie 1991). Expression of *nifA* in *B. japonicum* is enhanced microaerobically due to autoactivation of the *fixRnifA* operon by NifA (Thöny et al. 1989). The ORS571 *nifA* promoter is also oxygen-controlled via the *fixLJ* system and NifA-mediated autoregulation has been suggested (de Bruijn et al. 1988; Ratet et al. 1989; Kaminski and Elmerich 1991). Three conserved DNA motifs are present in the ORS571 *nifA* 5′ upstream region, which may be involved in nitrogen and oxygen control (de Bruijn et al. 1988; 1990; Nees et al. 1988; Ratet et al. 1989), namely a putative −24/−12 promoter element ($^{-24}$GG-N10-GC; see Thöny and Hennecke 1989; presumably involved in *ntr* control), an upstream activating sequence (UAS, GGT-N_{10}-ACA TGT-N10-ACA is the consensus sequence, see Buck 1990), which may interact with NifA and a sequence found in the promoters of *E. coli* genes induced during anaerobiosis by the Fnr regulatory protein (TTGAT-N_4-ATCAA; *fnr* box, see Spiro and Guest 1990).

ORS571 contains several regions homologous to *nifA*/*ntrC* in its genome, two of which have been characterized (Pawlowski et al. 1987). Since the reduced level of *nifA* expression remains under oxygen and nitrogen control in ORS571 *ntrC* mutants (Ratet et al. 1989), the presence of additional functional *ntrC*-homologous or related regulatory genes, such as those described for *Rhodobacter capsulatus* (Kranz and Haselkorn 1988), has been postulated. Here we report the characterization of an *nifA*/*ntrC* homologous region (*ntrX*) by DNA sequencing, as well as by Tn*5* and miniMu-*lac* mutagenesis (de Bruijn 1987; Ratet et al. 1988a). *ntrX*::Tn*5* insertions were found to affect *nifA-lac* expression, nitrogen fixation and assimilation. *ntrX* was found to be part of a bicistronic operon (*ntrYX*), which shares homology with two-component regulatory systems and itself appears to be *ntr*-controlled. Preliminary reports of these results were presented at the 7th and 8th International Nitrogen Fixation Congresses in Cologne, FRG, and Knoxville, USA (de Bruijn et al. 1988; 1990).

Materials and methods

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

Media and chemicals. Media and antibiotic concentrations used have been described by Pawlowski et al. (1987). Nitrogen sources were added to LSO medium (Elmerich et al. 1982) to a final concentration of 0.2%.

DNA isolation and manipulations. These procedures were carried out as described by Maniatis et al. (1982) and Meade et al. (1982).

DNA sequence analysis. Appropriate restriction fragments were subcloned in the polylinker of M13mp18/19 or pUC18/19 (Yanisch-Perron et al. 1985) and deletion clones were constructed using the exonuclease III/nuclease S1 method described by Henikoff (1984). Sequencing reactions were carried out using the chain termination method (Sanger et al. 1977, 1980) with *Taq*I-polymerase (Promega) according to the instructions of the manufacturer. The *ntrX*::MudIIPR46 junction sequence was determined using the standard M13 primer. DNA sequences and deduced amino acid sequences were analyzed using the software package of the University of Wisconsin Computer Group (Devereux et al. 1984). The 'codonpreference' calculation was based on the codon usage found in the ORS571 *nifA* and *nifH* genes (Ratet et al. 1989; Norel and Elmerich 1987). For data bank searches, the protein sequence databases NRBF (National Biomedical Research Foundation, Washington, USA) and Swiss-Prot (EMBL, Heidelberg, FRG) and the nucleic acid sequence databases EMBL data library (EMBL, Heidelberg, FRG) and GenBank (IntelliGenetics, Mountain View, Calif., USA) were used.

Tn5 mutagenesis, conjugation and gene replacement (homogenotization). These experiments were performed as described by de Bruijn (1987).

Construction of Tn5 insertion mutants. The deletion cosmids pLRSC1 Δ3, Δ12, and Δ28 (Fig. 1) were mutagenized with Tn*5* and nine new Tn*5* insertions within or flanking the *ntrC* and *ntrYX* regions were introduced into the ORS571 chromosome via gene replacement (I34, I284, I289, XY126, YX1215, YX1219, X2816, I2852; Fig. 1). Tn*5* insertion I15 was constructed as described for C6 and C7 (Pawlowski et al. 1987).

Construction of miniMu-lac gene fusions. Genes were subcloned in pJRD184 and mutagenized in *E. coli* MC4100(Mucts)(MudIIPR46) as described by Ratet et al. (1988a). After infection of Mu8820(Muc$^+$), Lac$^+$ colonies (blue on medium containing 5-bromo-4-chloro-3-β-*D*-indolyl-thiogalactoside, X-Gal) were selected for further analysis and the miniMu-*lac* insertion site was mapped by restriction endonuclease analysis. In order to construct a *ntrC-lac* gene fusion, plasmid pPR66 (Fig. 1) was mutagenized, a Lac$^+$ derivative carrying MudIIPR46 within the *ntrC* locus was selected (pCmM4; Fig. 7) and shown to be unable to complement ORS571C6 for growth on nitrate. An *Xho*I-*Hin*dIII fragment of pCmM4, carrying the gene fusion but lacking the *S*-end of Mu (Fig. 7), was subcloned into pJRD184, yielding pCmMS4, and cointegrated into the chromosome of strains ORS571, YX1219 and X2816 to generate merodiploid strains (ORS571CmMS4, YX1219CmMS4 and X2816CmMS4). To construct a *ntrX-lac* fusion, a MudIIPR46 insertion in the 3′ end of *ntrX* on pRSX31 (Fig. 1) was selected (pXmM3) and a *Hin*dIII-*Xho*I deletion derivative was generated for stable cointegration (pXmMS3; Fig. 7). The DNA sequence of the MudIIPR46 insertion site was determined

Table 1. Bacterial strains, phages, and plasmids

Strain	Relevant characteristics[a]	Reference
A. caulinodans		
ORS571	Wild type; Cb^R	Dreyfus et al. (1988)
ORS571A5, A7	*nifA*::Tn*5*; Cb^R, Km^R	Pawlowski et al. (1987)
ORS571C6, C7	*ntrC*::Tn*5*; Cb^R, Km^R	Pawlowski et al. (1987)
ORS571C46	*ntrC*::MudIIPR46, phenotype like ORS571C6; Cb^R, Gm^R	P. Ratet, unpublished
ORS571I15	Tn*5* insertion upstream of *ntrC*; Cb^R, Km^R	This work and Pawlowski et al. (1987)
ORS571I34, I284, I289	Tn*5* insertions between *ntrC* and *ntrYX*; Cb^R, Km^R	This work
ORS571YX126	Tn*5* insertion in *ntrY* locus; Cb^R, Km^R	This work
ORS571YX1215, YX1219	*ntrY*::Tn*5*; Cb^R, Km^R	This work
ORS571X2816	*ntrX*::Tn*5*; Cb^R, Km^R	This work
ORS571Y5	*ntrY*::Km^R; Cb^R, Km^R	This work
ORS571C46Y5	*ntrY*::Km^R, *ntrC*::MudIIPR46; Cb^R, Km^R, Gm^R	This work
ORS571I2852	ORF-2::Tn*5*; Cb^R, Km^R	This work
ORS571A54	$nifA^+$ *nifA*::MudIIPR46′; Cb^R, Gm^R	Ratet et al. (1989)
ORS571CmMS4	$ntrC^+$ *ntrC*::MudIIPR46′; Cb^R, Gm^R	This work
YX1219CmMS4	$ntrC^+$ *ntrC*::MudIIPR46′, *ntrY*::Tn*5*; Cb^R, Km^R, Gm^R	This work
X2816CmMS4	$ntrC^+$ *ntrC*::MudIIPR46′, *ntrX*::Tn*5*; Cb^R, Km^R, Gm^R	This work
ORS571XmMS3	$ntrX^+$ *ntrX*::MudIIPR46′; Cb^R, Gm^R	This work
ORS571C6XmMS3	$ntrX^+$ *ntrX*::MudIIPR46′, *ntrC*::Tn*5*; Cb^R, Km^R, Gm^R	This work
E. coli		
HB101	*recA* strain for cloning experiments; Sm^R	Boyer and Roulland-Dussoix (1969)
TB1	F′ (*traC36, proAB, lacF, lacZ*Δ*M15*), Δ(*lac, pro*), *supE, thi, recA, srl::Tn10* (Tc^R), used for M13 infection and *lacZ* α-complementation	B. Barrell, unpublished
MC4100(Mucts) (MudIIPR46)	MC4100(Mucts) lysogen for mini-Mu MudIIPR46 lysate production	Ratet et al. (1988a)
M8820 (Muc^+)	Recipient strain for mini-Mu transduction	Casadaban (1975)
Plasmid/phage		
pLAFR1	*cos*, Tra^-, mob^+, IncP; Tc^R	Friedman et al. (1982)
pRK2013	mob^+, Tra^+, IncN; Km^R	Ditta et al. (1980)
pRK290	mob^+, Tra^-, IncP; Tc^R	Ditta et al. (1980)
pWB5	pRK290 derivative containing *nptI* and polylinker; Tc^R, Km^R	W. Buikema and F.M. Ausubel, unpublished
pACYC184	mob^+, Tra^-, IncW; Tc^R, Cm^R	Chang and Cohen (1978)
pJRD184	mob^-, Tra^-, IncN; Tc^R, Ap^R	Heusterspreute et al. (1985)
pUC18/19	mob^-, IncN; used for cloning and plasmid sequencing; Ap^R	Yanisch-Perron et al. (1985)
M13mp18/19	used for cloning and single strand DNA sequencing	Yanisch-Perron et al. (1985)
pPR66	6.8 kb *Xba*I-*Eco*RI fragment of pLRSC1 cloned in pJRD184; Ap^R	P. Ratet, unpublished
pPR54	ORS571 *nifA*::MudIIPR46′ fusion cloned in pJRD184 for cointegration into the ORS571 chromosome; Tc^R, Gm^R (ORS571)	Ratet et al. (1989)
pLRSC1	pLAFR1 derivative; Tc^R; ORS571 *ntrC-ntrYX* region	Pawlowski et al. (1987)
pLRSC1Δ3, 12, 28	*Eco*RI deletion derivatives of pLRSC1	This work
pLRSC1Δ34	Tn*5* insertion in pLRSC1Δ3	This work
pLRSC1Δ126, 1215, 1219	Tn*5* insertions in pLRSC1Δ12	This work
pLRSC1Δ281, 2816	Tn*5* insertions in pLRSC1Δ28	This work
pRSX16	3 kb *Eco*RI fragment of pLRSC1 cloned in pACYC184; Tc^R	This work
pRSX16-Km^R	Fragment carrying the *nptII* gene of Tn*5* cloned in the *Bgl*II site of pRSX16; Tc^R	This work
pWB-Y5	Insert of pRSX16 containing the *nptII* gene of Tn*5* subcloned in pWB5; Km^R, Tc^R	This work
pRSX31	9.4 kb *Cla*I-*Sma*I fragment of pLRSC1 cloned in pJRD184; Ap^R	This work
pCmMS4	ORS571 *ntrC*::MudIIPR46′ fusion (pCmM4) subcloned in pJRD184 for cointegration into the ORS571 chromosome; Tc^R, Cm^R (*E. coli*), Gm^R (ORS571)	This work
pXmMS3	ORS571 *ntrX*::MudIIPR46′ fusion (pXmM3) subcloned in pJRD184 for cointegration into the ORS571 chromosome; Tc^R, Cm^R (*E. coli*), Gm^R (ORS571)	This work

[a] The phenotypes of ORS571 mutant strains are listed in Table 2

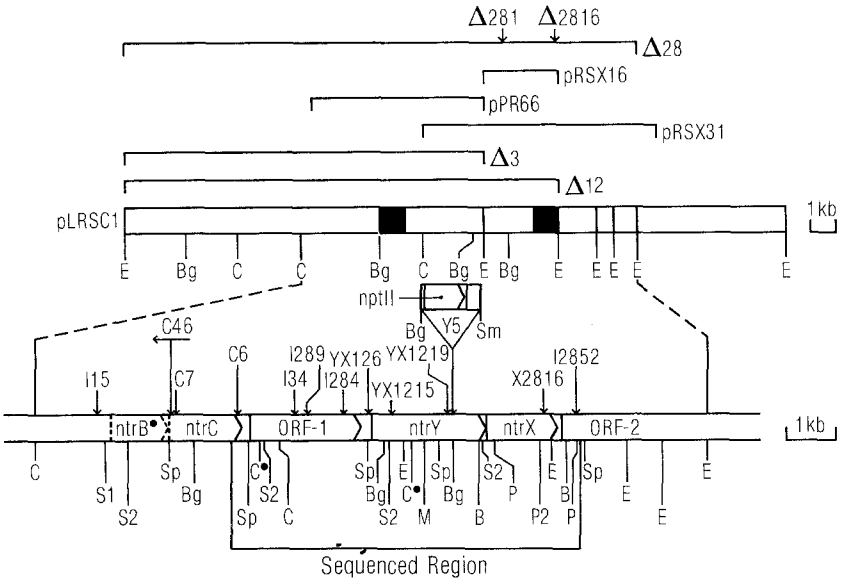


Fig. 1. Physical and genetic map of the ORS571 *ntrBC*-ORF-1-*ntrYX*-ORF2′ region. *nifA*/*ntrC* hybridizing regions are shown in *black*. The start point of the *ntrC* gene has not been determined by DNA sequencing, but is based on our studies on Tn*5* insertion mutants (e.g. C7 and I15) as well as the published lengths of *ntrC* genes from other rhizobia (Szeto et al. 1987; Nixon et al. 1986), and is therefore denoted by a *dotted line* in the expanded map. The position and extent of the putative *ntrB* gene (*ntrB*′) are hypothetical and based on partial sequence information (unpublished) and the position of the Tn*5* insertion I15. The *vertical arrows* indicate the positions of Tn*5* insertions and the *horizontal arrow* (C46) the position and orientation of the MudIIPR46 insertion (Direction and transcription of the *lac* operon). The restriction enzyme designations are as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I (C* is only recognized in *E. coli dam* strains); E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; P, *Pst*I; P2, *Pvu*II; Sp, *Sph*I; S1, *Sst*I; S2, *Sst*II; Xb, *Xba*I; Xh, *Xho*I

(see Fig. 2) and the *ntrX-lac* fusion was cointegrated into strains ORS571 and C6, to yield strains ORS571XmMS3 and C6XmMS3, respectively.

Construction of ORS571C46. Cosmid pLRSC1 (Fig. 1) was mutagenized with MudIIPR46 (Ratet et al. 1988a) and the resulting pLRSC1::MudIIPR46 plasmids were conjugally transferred to ORS571. Transconjugants were selected on gentamycin (MudIIPR46 marker). Due to the high degree of instability of pLRSC1::MudIIPR46 plasmids in ORS571 (P. Ratet, unpublished observation), predominantly integrations of these plasmids into the chromosome were obtained. A double crossover at the resident *ntrC* locus, resulting in a chromosomal *ntrC*::MudIIPR46 insertion mutation, was identified by examining growth on nitrate and ammonium as sole N-sources. Thus strain ORS571C46 was obtained, carrying an MudIIPR46 insertion immediately adjacent to *ntrC*::Tn*5* C7 (Fig. 1).

Construction of ORS571Y5 and C46Y5. The *Bgl*II-*Sma*I fragment of Tn*5*, containing the neomycin phosphotransferase gene lacking its promoter and part of the bleomycin resistance gene (Beck et al. 1982; Genilloud et al. 1984; see Fig. 1) was inserted into the *Bgl*II site of pRSX16. The *Eco*RI insert of the resulting plasmid, pRSX16-Km, was subcloned in pRK290 to form pRK-Y5, conjugally transferred to ORS571 and used to construct a chromosomal *ntrY* insertion by gene replacement (ORS571Y5; Fig. 1). The ORS571C46Y5 double mutant was constructed by transferring plasmid pRK-Y5 into strain ORS571C46 and selecting for transconjugants resulting from a gene replacement event.

Enzyme assays. Nitrogenase activity was determined using the C_2H_2 (acetylene) reduction assay, as described by Pawlowski et al. (1987). Galactosidase activity of free-living bacteria was estimated on plates containing 5-bromo-4-chloro-3-indolyl-β-*D*-galactoside (X-Gal) or determined by quantitative β-galactosidase assays, as described by Miller (1972) and Pawlowski et al. (1987). *In situ* staining of nodules for β-galactosidase activity was performed by incubating nodule segments in a 1:3 mixture of X-Gal:Z buffer (Miller 1972).

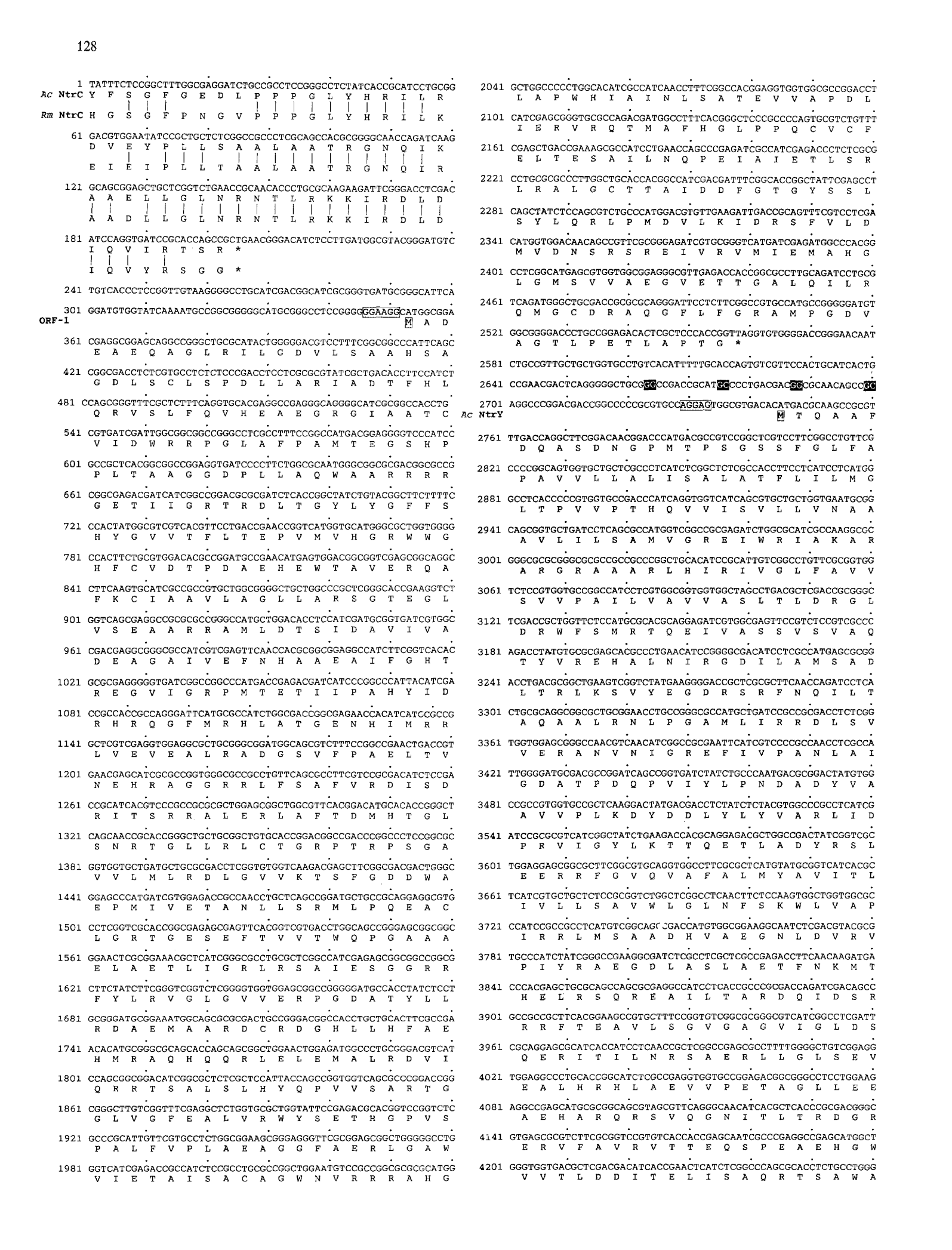
Plant experiments. Nodulation and symbiotic nitrogen fixation were examined as described by Pawlowski et al. (1987).

Results

Identification and sequencing of a second ntrC-*homologous region*

The DNA sequence of an *ntrC*/*nifA* homologous region on cosmid pLRSC1 (*ntrX*; Fig.1; Pawlowski et al. 1987; de Bruijn et al. 1988) was determined and the results are shown in Fig. 2. In the *ntrX* region, an open reading frame (ORF) was identified, preceded by a second open reading frame, which was designated *ntrY*. Two additional ORFs were found flanking the *ntrYX* region, designated ORF-1 and ORF-2 (see Figs. 1 and 2). The 3′ region of the *ntrC* gene was also sequenced. All five ORFs represent protein coding regions with a >90% probability, as determined by analyzing the sequence with the 'testcode' (Fickett 1982) and 'codonpreference' (Gribskov et al. 1984) programs (data not shown).

The *ntrY* ORF (positions 2745–5060) encodes a 771 amino acid (84.26 kDa) protein. It contains three methionines in its N-terminal region, only one of which is preceded by a putative Shine-Dalgarno sequence (positions 2729–2733; Shine and Dalgarno 1974). The C-terminal region of the NtrY shows significant homology with the conserved C-termini of sensor proteins of two-component regulatory systems (see Fig. 3; Albright et al. 1989). An analysis of the hydropathy plot of NtrY revealed three strongly hydrophobic regions, two of which (Fig. 4, arrows) share homology with transmembrane domains of *E. coli* and *Salmonella typhimurium* chemore-


Ac NtrC
Rm NtrC
ORF-1
Ac NtrY

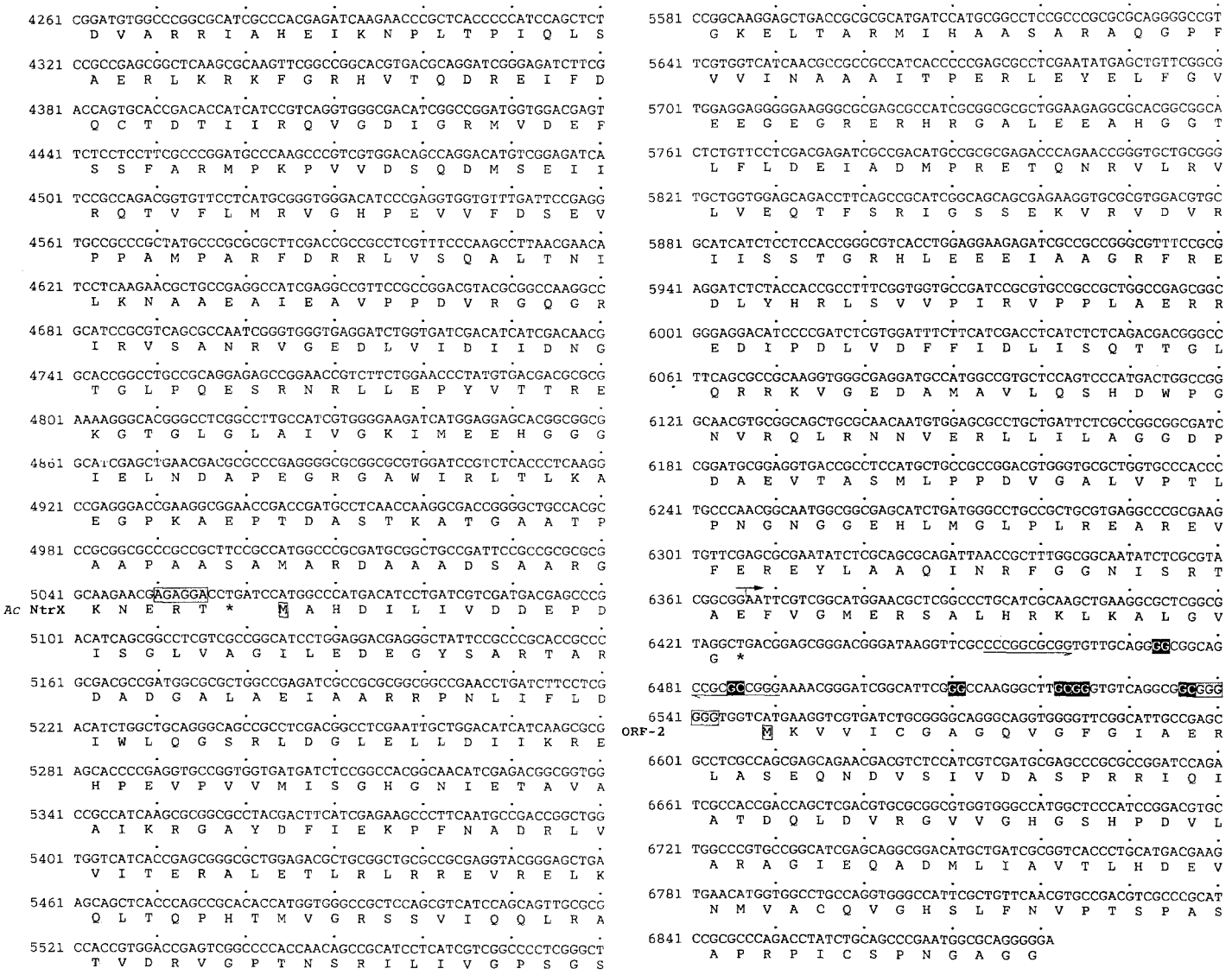


Fig. 2. DNA sequence of the ORS571 *ntrC'*-ORF-1-*ntrYX*-ORF-2' region. The 3' end of *ntrC* extends to position 207, and the amino acids identities between ORS571 NtrC (top amino acid sequence) and *R. meliloti* NtrC (bottom amino acid sequence; Szeto et al. 1987) are indicated by *vertical lines*. ORF-1 extends from position 353 to 2560, *ntrY* from position 2745 to 5060, *ntrX* from position 5064 to 6427. ORF-2 begins at position 6549. The putative ribosome-binding sites and the probable N-terminal methionines are *boxed*. Stop codons are indicated by an *asterisk*. Inverted repeat structures are indicated by *horizontal arrows*. The putative −24/−12 promoter elements (*ntr* boxes) are highlighted in *black*. The position of the *ntrYX-lac* fusion is indicated by the *horizontal arrow* at position 6366

ceptor proteins (Krikos et al. 1983; Russo and Koshland 1983; data not shown), suggesting that NtrY may be a transmembrane protein (see Nixon et al. 1986).

The *ntrX* ORF (position 5064–6428) encodes a 455 amino acid (50.2 kDa) protein, preceded by a putative Shine-Dalgarno sequence (positions 5050–5055). NtrX shows a high degree of homology with NtrC proteins of different organisms (Fig. 5). Therefore the *ntrYX* loci reveal typical features of a two-component regulatory system, in which NtrY would serve as the sensor and NtrX as the modulator protein. Upstream of *ntrY* (between ORF-1 and *ntrY*), two motifs similar to the −24/−12 consensus sequences found in NtrA-dependent promoters ($^{-24}$GG/GC^{-12}; Thöny and Hennecke 1989) were identified (GG-N_{10}-GC; positions 2663–2676 and 2687–2700). Downstream of *ntrX*, a palindromic structure, possibly representing a transcription terminal signal, is present (positions 6454–6490). The polypeptide encoded by the 3' part of the *ntrC* gene shares a high degree of homology with NtrC proteins of other rhizobia, such as *R. meliloti* (Fig. 2).

The protein encoded by ORF-1 (positions 353–2560; protein 1) shows homology with the product of an open reading frame within the mercury resistance region of plasmid R100 and transposon Tn*501* (URF-2; Brown et al. 1986). The physiological significance of URF-2 is unknown, but it is not involved in conferring mercury resistance (Brown et al. 1986). The C-terminal part of the ORF-1 product shows strong homology with the TnpM protein of transposons Tn*21* and Tn*501*, which

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Ec EnvZ  224  AAGVKQLADDRTLLMAGVSHDLRTPLTRIRLATEMMSEQ.....
Ec CpxA  229  VTALERMMTSQQRLLSDISHELRTPLTRLQLGTALL.....
Rm FixL  225  ELARLARLNEMGEMASTLAHELNQPLSAIANYSHGCTRLLRDMDD
At VirA  455  RLEHAQRLEAVGTLAGGIAHEFNNILGSILGHAE.LAQNSV....
Bp NtrB  125  QLTHRSAARSVIALAAMLAHEIKNPLSGIRGAAQLLEQQAS....
Ac NtrY  495  ELISAQRTSAWADVARRIAHEIKNPLTPIQLSAERLKRKFGRHVT

Ec EnvZ  ......DGYLAE......SINKDIEECNAI....IE.QFIDYLRTGQEMP
Ec CpxA  RRRSGESKELERIETEAQRLDSMINDLLVMSRNQQKNALVSETIKANQLW
Rm FixL  AVATRIREALEEVASQSLRAGQIIKHLREFVTKGETEKAPEDIRKLVEES
At VirA  SRTSVTRRYIDYIISSGDRAMLIIDQILTLSRKQER......MIKPFSVS
Bp NtrB  ...SEDRLLTRLICDEADRIVTLVDRMEVFGDDRPVARG.....PVNIH
Ac NtrY  QDREIFDQCTDTIIRQVGDIGRMVDEFSSFARMPKP......VVDSQDMS

Ec EnvZ  MEMADLNAVLGEVIAAESGYEREIETAL.YP.....GSIEVKMHPLSIKR
Ec CpxA  SEVLDNAAFEAEQMGRSLTVNFP..PG.PWPLYGNPNALESALENIVRNA
Rm FixL  AALALVG.....SREQGVRTVFEYLPGAEMVLVDRI.QVQQVLINLMRNA
At VirA  ELVTEIAPLLRMALPPNIELSFRF.DQMQSVIEGSPLELQQVLINICKNA
Bp NtrB  SVLDHVKRLAQSGFARNVRFIEDYDPSLPPVLANQ.DQLIQVFLNLVKNA
Ac NtrY  EIIRQTVFLMRVGHP.EVVFDSEVPPAMPARFDRRLVS..QALTNILKNA

Ec EnvZ  AVANMVVNAARYGNGWIKVSSGTEPNRAW.....FQVEDDGPGIA
Ec CpxA  LRYSHT.KIEVGFAVDKD.....GITITVDDDGPGVS
Rm FixL  IEAMRHVDRRELTIRTMPADPGE.....VAVVVEDTGGGIP
At VirA  SQAMTANG.QIDIIISQAFLPVKKILAHGVMPPGDY.VLLSISDNGGGIP
Bp NtrB  AEAVADLGTDAEIQLTTAFRPGVRLSVPGKKSRVSLPLEFCVKDNGSGVP
Ac NtrY  AEAIEAVPPDVRGQGRIRVSANRV.....GED..LVIDIIDNGTGLP

Ec EnvZ  PEQRKHLFQPF..VRGDSARTISGTGLGLAIVQRIVDNHNGMLELGTSER
Ec CpxA  PEDREQIFRPFYRTDEARDRESGGTGLGLAIVETAIQQHRGWVKAEDSPL
Rm FixL  EEVAGQLFKPFVTTKA.....SGMGIGLSISKRIVEAHGGEMTVSKNEA
At VirA  EAVLPHIFEPFFSTRA...RN.GGTGLGLASVHGHISAFAGYIDVSSTV.
Bp NtrB  EDLLPNLFDPFVTTKQ.....TGSGLGLALVAKIVGDHGGIIECESQPR
Ac NtrY  QESRNRLLEPYVTT.....REK.GTGLGLAIVGKIMEEHGGGIELNDAPE

Ec EnvZ  G.GLSIRAWLPVPVTR 424
Ec CpxA  G.GLRLVIWLPLYKRS 461
Rm FixL  GGAT.FRFTLPAYLDE 458
At VirA  GHGTRFDIYLPPSSKE 696
Bp NtrB  KTTFRV...LDADVQR 367
Ac NtrY  GRGAWIRLTLKAEGPK 730
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Fig. 3. Comparison of the C-terminal amino acid sequence of NtrY with those of sensor proteins of two-component regulatory systems. *E. coli* EnvZ reacts to the osmolarity of the medium (Comeau et al. 1985); *E. coli* CpxA senses the presence of F^- cells and in turn activates ArcA (Albin et al. 1986); *R. meliloti* FixL is a putative oxygen sensor (David et al. 1988); *A. tumefaciens* VirA activates VirG in the presence of acetosyringone or hydroxysyringone (Leroux et al. 1987); *B. parasponiae* NtrB is a nitrogen sensor, whose function is regulated by the GlnB(PII)/GlnD system (Nixon et al. 1986; Magasanik 1988). Identical amino acids at homologous positions are highlighted in *black*, chemically similar amino acids are *stippled* and *boxed*. Chemical similarity was defined according to Gribskov and Burgess (1986)

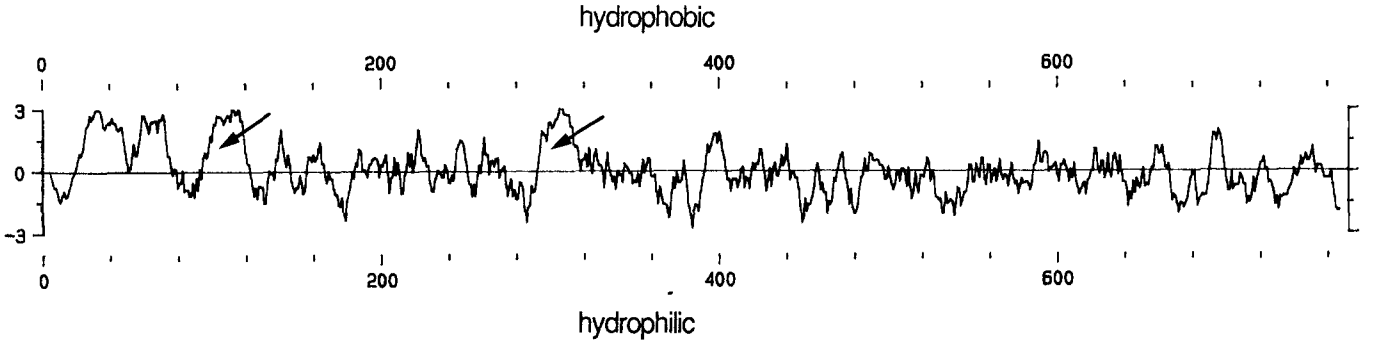

Fig. 4. Hydropathy plot of NtrY. The hydropathy profile of NtrY (according to Kyte and Doolittle 1982) is shown and the putative transmembrane regions (see text) are indicated by *arrows*

plays a role in transposition (Hyde and Tu 1985; data not shown). Tn*5* insertions in this locus have a Nif^+, Fix^+ and Ntr^+ phenotype (I34, I284, I289; Fig. 1; Table 2). Therefore, the significance of these homologies and the possible function of the protein encoded by ORF-1 remain unclear. For the protein encoded by ORF-2 (beginning at position 6549), no significant homology to any other protein could be found. This ORF is also preceded by $-24/-12$ type consensus sequences (e.g. positions 6510–6523; Fig. 2).

Construction of insertion mutations in the ntrC-ntrYX *region of cosmid pLRSC1*

Ten new Tn*5* insertions within or flanking the *ntrC* and *ntrYX* regions were constructed, as described in Materials and methods (I15, I34, I284, I289, YX126, YX1215, YX1219, X2816, I2852; Fig. 1).

Since the DNA sequencing data suggested that *ntrYX* could represent an operon (Fig. 2), Tn*5* insertions in *ntrY* were expected to be polar on *ntrX*. Therefore, a

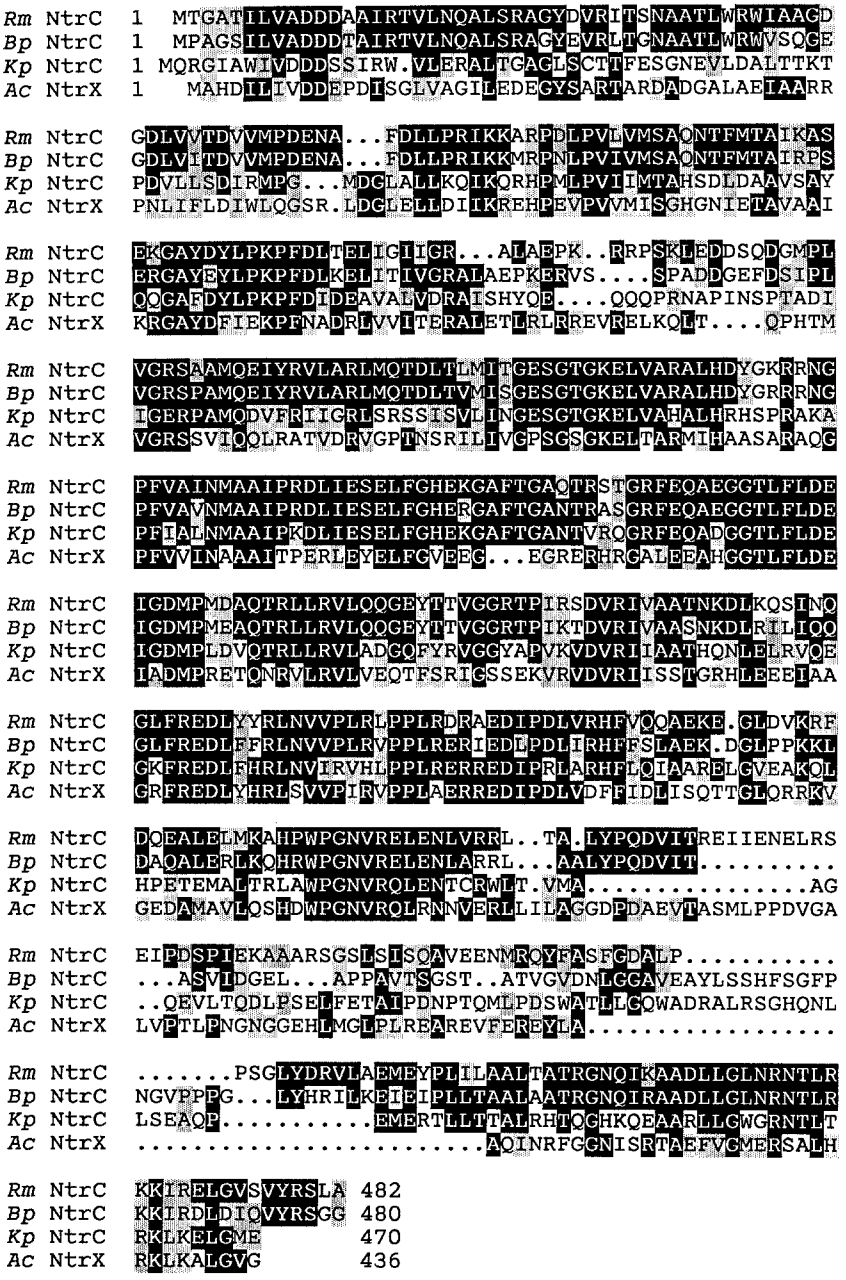

Fig. 5. Amino acid comparison between NtrX and NtrC proteins. *R. meliloti* NtrC was described by Szeto et al. (1987), *B. parasponiae* NtrC by Nixon et al. (1986), *K. pneumoniae* NtrC by Buikema et al. (1985). The amino acids are labeled as described in the legend to Fig. 3

non-polar *ntrY* insertion mutant (Y5; Fig. 1) was constructed (see Materials and methods). In order to construct an *ntrCntrY* double mutant, first a MudIIPR46 (Ratet et al. 1988a) insertion in *ntrC* was made (ORS571C46; Fig. 1). The phenotype of C46 was found to be the same as that of the previously characterized *ntrC*::Tn*5* strains C6 and C7 (Pawlowski et al. 1987). To construct the *ntrCntrY* double mutant (ORS571C46Y5), the Y5 insertion was transferred into strain ORS571C46 by gene replacement. The positions of the transposon insertions in these strains were verified by Southern blotting (data not shown).

Phenotypes of the insertion mutants

All insertion mutants were analyzed for stem and root nodulation capacity on *S. rostrata* (Nod), for free-living (Nif) and symbiotic nitrogen fixation (Fix), as well as for growth on several nitrogen sources (nitrogen assimilation/regulation; Asm/Ntr phenotype). Their phenotypes were compared to those of previously characterized *ntrC*::Tn*5* and *nifA*::Tn*5* mutants (C6, C7; Fig. 1; A5, A7; Pawlowski et al. 1987). The results are summarized in Table 2. ORS571I15, I34, I284, I289 and I2852 showed an essentially Nod$^+$, Nif$^+$ and Fix$^+$ phenotype and exhibited wild-type growth on LSO medium supplemented with glutamine, glutamate, arginine, histidine, leucine, ammonium or nitrate as sole nitrogen (N$^-$) sources (Asm$^+$, Ntr$^+$). These Tn*5* insertions therefore do not appear to be located in genes essential for nodulation, nitrogen fixation and metabolism, or their control regions and are therefore labelled "I" (presumably *I*ntergenic; Fig. 1; Table 2).

Strain ORS571YX126 was found to induce stem and

Table 2. Phenotpyes of ORS571 Tn*5* and MudIIPR46 insertion mutants

Name	Proposed missing gene product	Phenotype					
		Nif[a]	Nod Stem	Nod Root	Fix[b] Stem	Fix Root	Ntr[c]
ORS571A5, A7	NifA	−	+[d]	+[e]	<1	<1	+
ORS571I15		100	+	+	100	100	+
ORS571C6, C7, C46	NtrC	5–15	+[d]	+[e]	<1	del −/+[f]	−/+
ORS571I34, I284, I289	product of ORF-1	100	+	+	100	100	+
ORS571YX126		100	+	+	60–80	60–80	+
ORS571YX1215, YX1219	NtrY, NtrX	50–80	bumps	+[e]	<1	del −/+[f]	+/−
ORS571Y5	NtrY	100	bumps	+[e]	<1	del −/+[f]	−/+
ORS571C46Y5	NtrY, NtrC	5–15	bumps	+[e]	<1	del −/+[f]	−
ORS571X2816	NtrX	30–50	+[d]	+[e]	<1	del −/+[f]	−/+
ORS571I2852	product of ORF-2	~100%	+	+	100	100[g]	+

[a] Nif phenotype is expressed as the percentage of wild-type acetylene reduction activity
[b] Fix phenotype is expressed as the percentage of wild-type acetylene reduction activity of four-week-old stem or six-week-old root nodules
[c] For designation of Ntr phenotype see Table 3
[d] Small, light green nodules (Pawlowski et al. 1987)
[e] Hypernodulated (Pawlowski et al. 1987)
[f] Delayed $Fix^{-/+}$ phenotype (Pawlowski et al. 1987)
[g] Nodulated plants are slightly smaller than those inoculated with ORS571

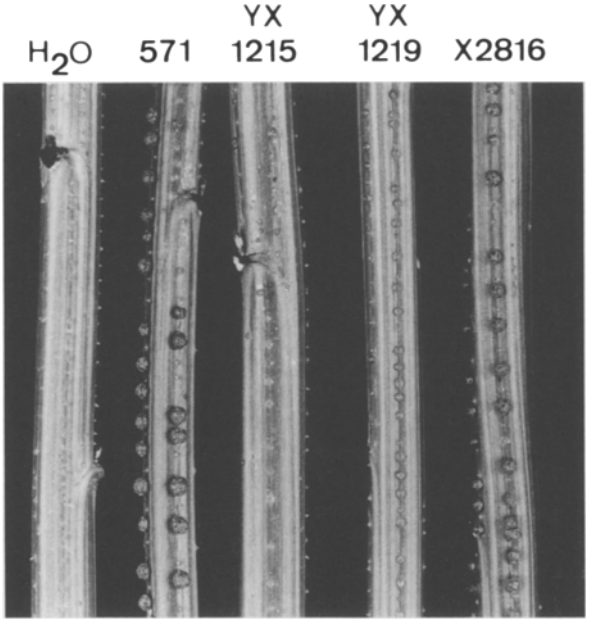

Fig. 6. Stem nodulation of *S. rostrata* by ORS571, YX1215, YX1219 and X2816. Stem segments of *S. rostrata* plants are shown 7 weeks after infection. For details see text

root nodules on *S. rostrata* (Fig. 6) with 60–80% of wild-type Fix activity (Table 2). Strains ORS571YX1215, YX1219, Y5, C46Y5 and X2816 were found to resemble the *ntrC*::Tn*5* mutants C6 and C7, because they exhibited the typical "hypernodulated $Fix^{-/+}$ delayed" phenotype, described in detail by Pawlowski et al. (1987), on the roots of plants grown in test tubes or in Leonard jars. The phenotypes of these strains on the stem of *S. rostrata* plants were more diverse. While strains ORS571YX1215, YX1219, Y5 and C46Y5 induced only Fix^- bumps (swellings of the adventitious root sites) on the stems of mature plants, strain ORS571X2816 induced the formation of Fix^- (light-green) nodules, closely resembling those formed upon infection with other strictly Fix^- strains (see Fig. 6; Pawlowski et al. 1987). Nodule development at the cotyledonary and primary leaf nodes, however, was not affected in ORS571YX1215, Y5 and C46Y5, suggesting that nodule induction at these sites differs from nodulation at adventitious root sites on the stem.

Free-living nitrogen fixation capability of strains ORS571YX126, YX1215, YX1219, Y5, C46Y5 and X2816 varied considerably. Strains YX126 and Y5 showed wild-type free-living acetylene reduction activity. Strains YX1215 and YX1219 showed levels of nitrogenase activity ranging from 50–80% of the wild-type ORS571 strain. Strain X2816 showed 30–50% (Table 2) and C46Y5 showed 5–15% of wild-type activity, thus resembling the *ntrC* mutant strains C6, C7 and C46 (Table 2; Pawlowski et al. 1987).

The nitrogen utilization phenotype of the mutant strains was examined on plates and in liquid cultures (selected cases). One of the nitrogen sources tested was nitrate, since the inability to grow on nitrate as sole nitrogen source represents the most common phenotype of rhizobial *ntr* mutants (Szeto et al. 1987; Ratet et al. 1988b; Ronson et al. 1987; de Bruijn et al. 1990). Since the growth parameters of the liquid cultures were found to affect the nitrogen utilization phenotype of the mutant strains, two independent sets of experiments were carried out. First, 3 ml cultures were grown in 15 ml test tubes and the final optical density at 600 nm (OD_{600}) was measured after 72 h (Table 3). Second, growth curves of 20 ml cultures in well aerated 250 ml flasks were determined, as described (Pawlowski et al. 1987; data not shown). Although slight differences in final OD_{600} values were observed using these two different protocols, the growth characteristics of the strains in LSO-Nitrate medium, combined with the colony morphology (growth rate) on plates with various nitrogen

Table 3. Ntr phenotypes of *ntrC*, *ntrY*, *ntrYntrC*, *ntrYX* and *ntrX* mutant strains

Strain	Growth in liquid cultures[a]			Growth on plates[b]			Ntr phenotype[c]
	Glutamine	Nitrate	No N source	Nitrate	Ammonium	Glutamine	
ORS571	3.09+/−0.29	1.76+/−0.27	0.27+/−0.01	Normal	Normal	Normal	+
ORS571C46	3.06+/−0.18	0.34+/−0.02	0.35+/−0.05	Slimy	Normal	Normal	−/+
ORS571Y5	2.97+/−0.20	0.67+/−0.14	0.28+/−0.02	Slimy	Slimy	Normal	−/+
ORS571C46Y5	2.94+/−0.27	0.27+/−0.02	0.27+/−0.02	Slimy	Slimy	Slimy	−
ORS571X2816	2.49+/−0.50	0.98+/−0.14	0.27+/−0.03	Slimy	Slimy	Slimy[d]	−/+[d]
ORS571YX1219	3.19+/−0.24	1.05+/−0.12	0.29+/−0.02	Slimy	Slimy	Normal[d]	+/−[d]

[a] 3 ml test cultures were inoculated with washed cells from stationary TY cultures and grown for 3 days at 37° C with vigorous shaking. Each number represents the optical density at 600 nm of the culture and constitutes the average from four independent experiments with three cultures each. Standard deviations are given

[b] Normal growth: opaque, white colonies. Slimy growth: translucent colonies producing excess lipopolysaccharides and containing few bacteria

[c] For designation of the Ntr phenotype, growth capacity on nitrate as sole N source on plates and in liquid culture and growth on ammonium as sole N sources on plates were taken into consideration

[d] YX1219 and X2816 could be given the same Ntr designation based on growth on nitrate and ammonium, but X2816 is more strongly impaired in its Ntr phenotype, since it exhibits slimy growth on glutamine as sole N source

sources, allowed the assignment of the different *ntr* mutants to distinct groups, with increasingly severe Ntr phenotypes ($Ntr^{+/-}$, $Ntr^{-/+}$, Ntr^{-}; see Tables 2, 3). Strain YX126 grew like the wild-type strain ORS571 on all N sources tested, except for glutamate (Ntr^{+}). Strains YX1215, YX1219, Y5, X2816 and C46Y5 exhibited poor growth (formed slimy colonies) on LSO plates supplemented with glutamate, ammonium, nitrate, arginine or leucine (see Table 3). Growth in well aerated liquid cultures with nitrate as sole N source was moderately impaired for YX1215 and YX1219, but substantially impaired for Y5 and X2816 ($Ntr^{+/-}$ and $Ntr^{-/+}$, respectively; Tables 2 and 3). This effect was not observed in poorly aerated cultures (data not shown). Strains X2816 and C46Y5 exhibited poor growth also on plates with glutamine (Table 3). ORS571C46Y5 showed no growth in minimal medium with nitrate (Ntr^{-}; Tables 2 and 3). Thus, in ORS571C46Y5 the nitrogen metabolism defects of ORS571C46 and Y5 appear to be combined, suggesting an additive effect of the *ntrC* and *ntrY* mutations.

Complementation studies

To examine whether the *ntrYX* genes form an operon or are transcribed independently, complementation experiments were carried out. Plasmids pLRSC1Δ28, pLRSC1Δ281 (carrying a *ntrY*::Tn*5* insertion) and pLRSC1Δ2816 (carrying the *ntrX*::Tn*5* insertion X2816; Fig. 1) were introduced into the *ntrX*::Tn*5* strain ORS571X2816. Growth of the transconjugants in liquid cultures with nitrate as N source was examined. While pLRSC1Δ28 could complement the $Ntr^{-/+}$ phenotype of ORS571X2816, neither pLRSC1Δ281 or pLRSC1Δ2816 were capable of doing so, suggesting a polar effect of the *ntrY*::Tn*5* insertion on *ntrX* expression and organization of the *ntrYX* loci as an operon.

nifA *expression in* ntrY, ntrX *and* ntrYX *mutant strains*

To examine the effects of different *ntr* mutants on *nifA* expression, a plasmid carrying a *nifA-lac* fusion (pPR54; Ratet et al. 1989) was introduced into strains ORS571YX126, YX1215, YX1219, Y5 and X2816 and integrated into the chromosomal *nifA* locus via a single crossover. The resulting strains were examined for β-galactosidase activity under a variety of physiological conditions. *nifA-lac* expression in YX1215 and YX1219 was found to be the same as in the wild-type strain, whereas *nifA-lac* expression in YX126 was slightly increased as compared to the wild type (data not shown). The results for the other strains are summarized in Table 4. The *ntrX* mutation in X2816 had a similar influence on *nifA* expression as the *ntrC* mutation in C6 (60% of wild-type expression without N source; 10% on leucine), whereas the Y5 mutation only reduced *nifA-lac* expression by approximately 35% relative to the wild type (Table 4), when the cells were grown in the presence of leucine, which has been observed to lead to maximal derepression of the ORS571 *nif/fix* genes (Ratet et al. 1989). As in the case of the *ntrC* strain ORS571C6 (Ratet et al. 1989), repression of *nifA* induction by nitrogen and oxygen was still observed in each mutant strain.

Regulation of ntrC *and* ntrYX *expression*

To analyze the expression of *ntrC* and *ntrYX*, respectively, miniMu-*lac* fusions to these genes were constructed (Fig. 7). The *ntrC-lac* fusion (CmMS4; Fig. 7A) was found to be highly expressed in media containing glutamine (N-repressing) and nitrate (N-derepressing) conditions (Table 5), and there were no significant differences in *ntrC-lac* expression levels in strains YX1219 and X2816, as compared to the wild-type (Table 5). The *ntrYX-lac* fusion (XmMS3; Fig. 7B) was also found to be expressed independently of the N source. However,

Table 4. *nifA-lac* expression in different mutant strains

Strains	β-Galactosidase activity[a]					
	3% O_2[b]			21% O_2[b]		
	$-NH_4^+$	$+NH_4^+$[c]	+Leu[c]	$-NH_4^+$	$+NH_4^+$[c]	+Leu[c]
ORS571A54	100	5	174	8	<1	15
ORS571C6A54	60	2	15–20	<1	<1	<1
ORS571X2816A54	60	2	30	<1	<1	<1
ORS571Y5A54	105	3	114	9	<1	17

[a] β-Galactosidase activity (Miller 1972) is expressed as a percentage of that observed in strain ORS571A54 under normal nitrogen fixation conditions. The percentages shown represent the results of four independent experiments and values did not vary by more than 10%

[b] Cultures were incubated under and air/acetylene mixture (nitrogen fixation conditions; Pawlowski et al. 1987) or under air (21% O_2)

[c] Nitrogen sources [$(NH_4)_2SO_4$; leucine] were added to LSO medium to a final concentration of 0.2%

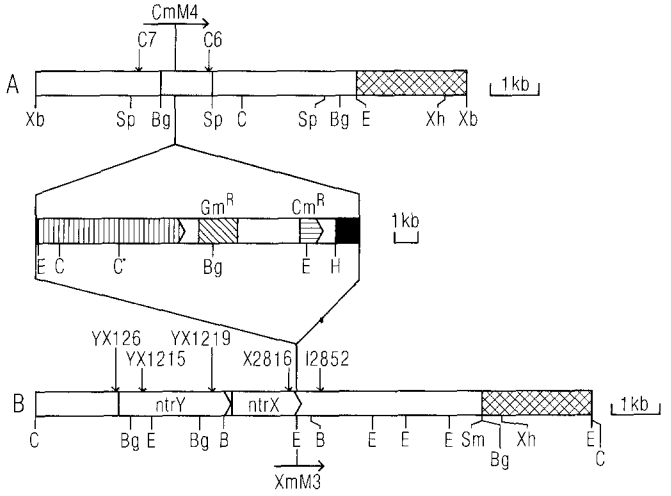

Fig. 7A, B. Construction of the *ntrC*::MudIIPR46 fusion CmMS4 and the *ntrX*::MudIIPR46 fusion XmMS3. A shows the position and orientation of the MudIIPR46 insertion CmM4 in pPR66. The *ntrC* hybridizing region is stippled. B shows the position of the MudIIPR46 insertion XmM3 in pRSX31. The positions of the chromosomal Tn*5* insertions are indicated (*vertical arrows*) as orientation points. The vector sequences (pJRD184) are indicated by *cross-hatching*, the MudIIPR46 ends are labeled in *black*. The extens of the *lac*, Gm^R and Cm^R genes are designated by vertical, diagonal and horizontal hatching respectively. The *horizontal arrows* indicate the orientiation of the MudII insertions (direction of transcription of the *lac* operon). The restriction enzyme abbrevations are described in the legend of Fig. 1

Table 5. *ntrC-lac* and *ntrX-lac* expression under different physiological conditions

Strains	β-Galactosidase activity[a]			
	3% O_2[b]		21% O_2[b]	
	Gln[c]	NO_3^-[c]	Gln[c]	NO_3^-[c]
ORS571CmMS4	560	240	940	310
ORS571X2816CmMS4	620	260	1040	350
ORS571XmMS3	73	68	182	183
ORS571C6XmMS3	135	10	153	9

[a] β-Galactosidase activities are expressed in Miller units (Miller 1972), and represent the average values of three (XmMS3) and two independent experiments (CmMS4), respectively; values for the individual experiments did not vary to more than 13%

[b] Cultures were incubated under an air/acetylene mixture (nitrogen fixation conditions; Pawlowski et al. 1987) or under air (21% O_2)

[c] Nitrogen sources (glutamine, KNO_3) were added to LSO medium at a final concentration of 0.2%

the expression on nitrate was dependent on the presence of an intact *ntrC* gene, since it was reduced to 5–25% of wild-type levels in ORS571C6XmMS3 (Table 5).

The expression of *ntrC* and *ntrX* in the symbiotic state was examined by staining sections of stem nodules induced by ORS571, ORS571CmMS4 and ORS571XmMS3 for β-galactosidase activity. Strain ORS571A54 (Ratet et al. 1989) was used as a positive control. Staining of the symbiotic zone in ORS571A54 and ORS571CmMS4 was observed at similar intensities, whereas in ORS571XmMS3 induced nodules staining was weaker, although blue color appeared at least 12 h earlier than background plant β-galactosidase activity in ORS571-induced nodules (data not shown). Therefore, the *ntrYX* and *ntrC* loci appear to be expressed during symbiosis, which is consistent with the notion that mutations in these genes affect symbiotic nitrogen fixation (Table 2).

Discussion

In this paper we have described the identification and characterization of a novel two-component regulatory system in *A. caulinodans* ORS571 (NtrY/NtrX), involved in the regulation of nitrogen fixation and metabolism genes. The *ntrY/ntrX* genes appear to constitute a bicistronic transcription unit, as observed for most other two-component systems analyzed thus far (see Albright et al. 1989). The *ntrX* gene was initially identified on the basis of homology with the *ntrC* and *nifA* genes of *K. pneumoniae* and found to map approximately 4.5 kb downstream of the ORS571 *ntrC* locus previously

described (Pawlowski et al. 1987; de Bruijn et al. 1988). DNA sequencing data presented here reveal that the *ntrX* gene product shares extensive amino acid homology with NtrC proteins of *R. meliloti* (Szeto et al. 1987), *B. parasponiae* (Nixon et al. 1986) and *K. pneumoniae* (Buikema et al. 1985), suggesting that it represents a (positive) regulatory protein. The pleiotropic phenotype of *ntrX* mutants with respect to nitrogen fixation and metabolism, as well as the high overall homology to *ntrC*, support its designation as a nitrogen regulation (*ntr*) locus. It is interesting that ORS571 harbors (at least) two different NtrC-like proteins, NtrC and NtrX, both of which contribute to *ntr* control, but in distinct ways. Both *ntrC* and *ntrX* mutants display retarded growth on nitrate, a reduction of *nifA* expression under nitrogen fixation conditions and a severely disturbed symbiotic phenotype ($Fix^{-/+}$ delayed on roots; Fix^{-} on stems). However, their free-living nitrogen fixation rates differ, since *ntrC* mutants have 5–15% and *ntrX* up to 50% of wild-type activity. In addition, their growth patterns in medium containing different amino acids as N sources are distinct. The biological significance of having two similar positive regulators in ORS571 involved in nitrogen regulation remains to be elucidated, but it may be a reflection of the flexibility needed in an organism which fixes nitrogen in the free-living and symbiotic states, in stem and in root nodules, under greatly varying physiological conditions.

Generally, the genes encoding regulatory proteins of the NtrC family are closely linked to partner gene encoding the sensor (modulator) of the two-component system (see Albright et al. 1989). In the case of the NtrBC systems in enteric bacteria it has been shown that the C-terminal (conserved) part of NtrB phosphorylates and dephosphorylates the N-terminal (conserved) domain of NtrC in response to the cellular N status, thereby activating or deactivating it (Kustu et al. 1989; Magasanik 1988). It is highly likely that the *ntrY* gene of ORS571, found immediately upstream of *ntrX*, corresponds to such a sensor (modulator) protein, especially since NtrY shares extensive homology with other sensor proteins in its C-terminal domain. Whether in fact NtrY phosphorylates NtrX in response to the cellular N status is not known. The putative transmembrane domains of NtrY would suggest that NtrY may be a membrane protein, which would make it an unusual N-sensor (modulator), since NtrB proteins appear to be cytosolic proteins (Magasanik 1988). Indeed NtrY may be involved in sensing the extracellular N concentration.

It is also interesting to note that the $Ntr^{-/+}$ phenotype of *ntrY* (or *ntrX*) mutants (impaired growth on nitrate) is only observed on plates and in well aerated cultures, suggesting an effect of the cellular oxygen status. However, the N-terminal domain of NtrY shows no significant homology with the putative oxygen sensor FixL of *R. meliloti* (David et al. 1988).

Upstream of the ORS571 *ntrC* gene, a region showing strong homology with the *B. parasponiae ntrB* gene (Nixon et al. 1986) has been found (K. Pawlowski, U. Klosse and F.J. de Bruijn, unpublished observation). This observation suggests that an *ntrBC* operon exists in ORS571, as is the case in all other members of the *Rhizobiaceae* examined up to now. It is not known whether NtrB can modulate NtrX in addition to NtrC and/or whether NtrY can modulate NtrC in addition to NtrX. In *E. coli*, such an interaction between two-component systems has been shown to occur, since the transcriptional activator protein ArcA is modified by two different sensor proteins, ArcB and CpxA (Iuchi et al. 1989a, b). A comparison of the phenotypes of *ntrC* or *ntrX* and *ntrYX* mutants, respectively, shows that Ntr and Nif phenotypes are more severely affected by the removal of one activator protein (*ntrC* or *ntrX*), than by the removal of a complete system (*ntrYX*; see Table 2). This observation suggests that when an entire system is removed, the other can partially substitute to yield an intermediate phenotype, while leaving one activator protein together with two distinct (but related) modulators leads to a more severe perturbation of the system and a more extreme phenotype. Thus there may be cross-talk (Ninfa et al. 1988) between the NtrB/NtrC and NtrY/NtrX regulatory systems.

An interaction between the *ntrYX/ntrBC* systems is suggested by the gene fusion experiments, which show that *ntrYX* expression is affected by a *ntrC* mutation when cells are grown on nitrate. All attempts to construct a *ntrCntrX* double mutant have failed thus far (K. Pawlowski, unpublished observations) and therefore it has not been possible to ascertain if any other *ntrC/ntrX*-like genes are involved in nitrogen control.

Both the *ntrC* and *ntrX* loci appear to be involved in controlling *nifA* gene expression, suggesting that the effects of *ntrC* and *ntrX* mutations on free-living and symbiotic nitrogen fixation may be mediated via the *nifA* promoter (see also Ratet et al. 1989; de Bruijn et al. 1990). A typical NtrA-dependent $-24/-12$ (*ntr*) canonical sequence is present in front of *nifA* (de Bruijn et al. 1988; Ratet et al. 1989) and its importance in *nifA* expression has been suggested (de Bruijn et al. 1990). Whether NtrC and NtrX act in concert with the same sigma factor in activating the *nifA* gene needs to be investigated.

The symbiotic phenotype(s) of *ntrY*, *ntrX* and *ntrYX* mutants are intriguing. *ntrX* mutants resemble *ntrC* mutants in their ability to induce Fix^{-} nodules on the stems and $Fix^{-/+}$ delayed nodules on the roots of *S. rostrata*. *ntrY* and *ntrYX* mutants, however, only induce slight swellings (bumps) on *S. rostrata* stems at the adventitious root sites, while resembling their *ntrC* (*ntrX*) counterparts on the roots. The phenotype of the ORS571YX126 mutant (60–80% nitrogen fixation activity in nodules), while resembling the wild-type strain in other respects, is also puzzling. Restriction mapping of this insertion mutant suggests that it may be located 5′ of the *ntrY* coding sequences and therefore could represent a promoter mutation with a minor effect on *ntrYX* expression. *ntrC(X)*-mediated *nif/fix* gene regulation in the symbiotic state (nodule) is highly unusual in rhizobia (de Bruijn et al. 1990). It could reflect the need for ORS571 to grow at the expense of N_2 during the infection process. Inability to proliferate, especially in the stem infection site, may delay or even prevent nodule

formation/nitrogen fixation (see de Bruijn 1989; Ratet et al. 1989). This is supported by the observation that ORS571 *glt* (GOGAT) and *glnA* (nitrogen assimilation) mutants also have a Fix$^-$ phenotype (Hilgert et al. 1987; Donald et al. 1988; de Bruijn et al. 1988, 1990), in contrast to the corresponding *R. meliloti* mutants (Kondorosi et al. 1977; de Bruijn et al. 1989). However, the capacity to fix nitrogen in the free-living state cannot be the sole determinant of the observed deficiency in stem nodulation, since a non-polar *ntrY* mutant has wild-type Nif activity, but is nevertheless seriously disturbed in its symbiotic properties. Furthermore, several Nif$^-$ Fix$^-$ mutant strains (*nifA*; Donald et al. 1986; Pawlowski et al. 1987; *nifHDK*; Elmerich et al. 1982) are not affected in their stem nodulation ability. Therefore, the stem nodulation deficiency of *ntrY* and *ntrYX* mutant strains must be the result of secondary effects of the *ntr* system, most likely on nitrogen assimilation (metabolism) of bacteria or bacteroids in the (developing) nodule.

By identifying the *ntrYX* two-component system, we have added another level of complexity to the regulatory circuitry controlling the expression of the *A. caulinodans* ORS571 nitrogen fixation and metabolism genes in the free-living and symbiotic states. It is clear that additional ORS571 loci play a role in this process and our present studies are designed to identify them and their target(s) in the promoters of N-regulated genes.

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Note added in proof:

After submission of this paper Kaminski et al. (1991, Mol Microbiol 5:1983–1991) have reported the identification of a *fixK*/*fnr*-like gene in *Azorhizobium caulinodans*, which encodes a positive activator of *nifA* expression. These authors have shown that the *fixLJ* genes control *fixK* expression, presumably in response to the cellular O_2 status and that the *fixK* gene, in turn, controls *nifA* expression. They also report that a *fixL ntrC* double mutant is strictly Nif^-Fix^-, suggesting that the *fixLJ*/*fixK* genes and the *ntrBC*/*ntrYX* system described here are the primary determinants of *Azorhizobium caulinodans*. *nifA* activation under limiting O_2 and nitrogen conditions respectively.

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