Azorhizobium caulinodans Nitrogen Fixation *(nif/fix)* Gene Regulation: Mutagenesis of the *nifA* −24/−12 Promoter Element, Characterization of a *ntrA(rpoN)* Gene, and Derivation of a Model

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Received 7 October 1992. Accepted 29 December 1992.

Using site-directed mutagenesis, mutations were introduced in the −24/−12 promoter element of the *Azorhizobium caulinodans nifA* gene, and chimeric *nifA-lacZ* reporter gene fusions were constructed. Single base-pair mutations in the conserved −25 or −13 G residues were found to reduce or abolish *nifA* promoter activity, respectively, demonstrating that the −24/−12 promoter element is important for *nifA* gene expression and suggesting the involvement of a σ54 (NtrA/RpoN)-type transcription factor in *nifA* gene regulation. A 2-bp mutation at positions −25 and −16 was found to create a relatively nitrogen-control-independent, highly expressed *nifA* promoter. Using a heterologous *ntrA(rpoN)* gene probe, an *A. caulinodans ntrA(rpoN)*-like gene was cloned and the DNA sequence of this gene and flanking regions was determined. The presence of three open reading frames (ORF1-3) was demonstrated. ORF2 was found to contain regions sharing a high degree of homology with all characterized bacterial *ntrA(rpoN)* genes. ORF1 was found to share homology with ORFs found upstream of other bacterial *ntrA(rpoN)* genes, which have been postulated to encode members of a superfamily of ATP-binding proteins. Transposon Tn*5* insertion mutations were introduced into the cloned *ntrA(rpoN)* gene, and chromosomal *ntrA(rpoN)*::Tn*5* *A. caulinodans* mutants were created. The resulting mutants were found to be unable to fix nitrogen in the free-living state (Nif⁻ in culture) or in stem or root nodules induced on *Sesbania rostrata* (Fix⁻ *in planta*), and to be unable to grow aerobically in the presence of nitrate as sole nitrogen source (Ntr⁻). A *nifH-lacZ* gene fusion was found to be silent in *ntrA(rpoN)*::Tn*5* mutant strains, but a *nifA-lacZ* gene fusion was found to be expressed at a wild-type level, suggesting that the *ntrA(rpoN)* gene identified here controls the expression of some of the *A. caulinodans nif* genes, but not the central *nif* regulatory gene *nifA*. Based on these results, a new model for the regulation of *nif/fix* gene expression in *A. caulinodans* is proposed.

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MPMI, Vol. 6, No. 2, pp. 238-252

Additional keywords: nitrogen regulation, *ntrA, rpoN,* promoter analysis.

The expression of the nitrogen-fixation (*nif/fix*) genes of *Azorhizobium caulinodans* ORS571 (Dreyfus *et al.* 1988; de Bruijn 1989) has been shown to be controlled in a cascade-type fashion by a complex array of regulatory genes, in response to distinct environmental signals. As in the case of other rhizobia and the free-living diazotroph *Klebsiella pneumoniae,* the expression of the structural genes for *A. caulinodans* nitrogenase (*nifHDK*) and other *nif/fix* genes is controlled by the central *nif* regulatory gene *nifA,* both in culture and in nodules induced on *Sesbania rostrata* (*in planta*; Donald *et al.* 1986; Pawlowski *et al.* 1987; de Bruijn *et al.* 1990). Expression of the *nifA* gene, in turn, is controlled by the cellular nitrogen and oxygen status (Ratet *et al.* 1989) and regulated by (at least) three distinct two-component regulatory systems, *ntrBC* (Pawlowski *et al.* 1987, 1991; Ratet *et al.* 1989), *ntrYX* (Pawlowski *et al.* 1991), and *fixLJ* (Kaminski and Elmerich 1991; Kaminski *et al.* 1991), both in culture and *in planta.*

The *A. caulinodans ntrBC* and *ntrYX* pairs of sensor-regulator genes share significant homology with one another and both appear to be involved in the cellular response to the concentration of combined nitrogen, facilitating *nif/fix* gene derepression under nitrogen-(N-) starvation conditions (Pawlowski *et al.* 1991). However, while the *ntrB* gene product closely resembles its cytosolic counterpart in enteric bacteria (NRII; Ninfa and Magasanik 1986), the *ntrY* gene product (NtrY) contains a distinct trans-membrane-like domain and therefore may be involved in extra- versus intracellular N-sensing (Pawlowski *et al.* 1991). The *A. caulinodans fixLJ* gene pair is involved in sensing the oxygen (O_2) concentration and facilitates the derepression of the *nif/fix* genes under microaerobic (O_2-limiting) conditions, through the product of the *fixK* gene (Kaminski and Elmerich 1991; Kaminski *et al.* 1991).

Thus, *A. caulinodans nif/fix* gene expression appears to be controlled by the N-regulation (*ntr*) pathway, commonly found in free-living diazotrophs such as *K. pneumoniae* (see de Bruijn *et al.* 1990; Merrick 1992), as well as the O_2-regulation (*fixLJ*) pathway, found in strictly symbiotic nitrogen-fixing organisms, such as *Rhizobium*

meliloti (David *et al.* 1988; see de Bruijn and Downie 1991). This dual response pathway reflects the unusual chimeric nature of *A. caulinodans* as free-living diazotroph and symbiotic nitrogen-fixing organism (see de Bruijn 1989) and is a consequence of the need of this organism to respond to quite different physiological conditions for nitrogen fixation in culture, as well as *in planta* (see de Bruijn *et al.* 1990; Kaminski *et al.* 1991).

The *A. caulinodans nifA* 5′ upstream region has been found to contain distinct DNA motifs found in the promoter regions of N- and O_2-regulated genes (Nees *et al.* 1988; Ratet *et al.* 1989), including a −24/−12 promoter element ($_{-25}$GG-*N10*-GC$_{-12}$ relative to the transcriptional start site; see Thöny and Hennecke 1989; de Bruijn *et al.* 1990) and a Fnr binding site consensus sequence, found in the promoter regions of genes anaerobically induced via the transcriptional activator Fnr (TTGAT-*N4*-ATCAA; see Spiro and Guest 1990; de Bruijn *et al.* 1990). It is plausible that the Fnr binding site consensus sequence constitutes the target site for the FixK protein, which shares homology with Fnr (Kaminski *et al.* 1991).

The −24/−12 element in the *A. caulinodans nifA* promoter is likely to be involved in mediating *ntr* regulation of *nifA* expression via the *ntrBC* and *ntrYX* systems (Pawlowski *et al.* 1987, 1991; Ratet *et al.* 1989; de Bruijn *et al.* 1990) and to interact with the alternative σ factor NtrA (σ54; also designated as RpoN; see Kustu *et al.* 1989), which has been identified in several rhizobial species (Ronson *et al.* 1987; Stanley *et al.* 1989; van Slooten *et al.* 1990; Kullik *et al.* 1991). To test this hypothesis, we have carried out site-specific mutagenesis of the −24/−12 element in the *A. caulinodans nifA* promoter and characterized a *ntrA(rpoN)*-like gene of *A. caulinodans*. We demonstrate that specific mutations in the −24/−12 element have a drastic effect on *nifA* promoter activity and that *A. caulinodans* contains a *ntrA(rpoN)* gene that is responsible for nitrate assimilation and *nifHDK* expression, but does not appear to affect *nifA* gene expression, suggesting the presence of a second *ntrA(rpoN)* gene with a distinct specificity. Preliminary reports of these results were presented at the 8th International Nitrogen Fixation Congress in Knoxville, TN (de Bruijn *et al.* 1990) and the 13th North American Symbiotic Nitrogen Fixation Conference (Banff, Canada, 1991).

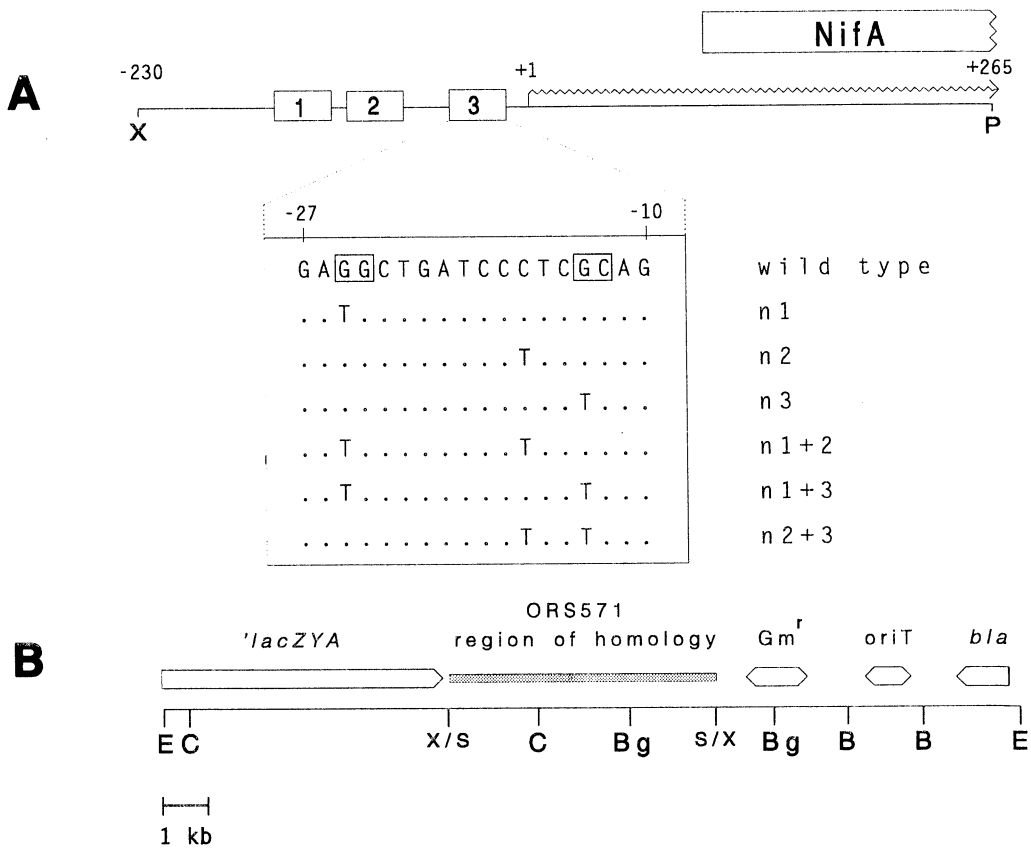

Fig. 1. Structure of the ORS571 *nifA* locus, the integration vector pJS4812, and plasmids carrying mutations in the −24/−12 promoter element. **A,** The start point and direction of transcription of the ORS571 *nifA* gene are indicated by a wavy arrow and are based on data from Ratet *et al.* (1989). The small open boxes designate the Fnr binding-site consensus (box 1), NifA binding site consensus or upstream activating sequence (box 2), and −24/−12 promoter element (box 3), as defined by Ratet *et al.* (1989). The nature of the mutations in the −24/−12 promoter-element are shown in the large box. Restriction enzyme cleavage sites indicated are: X: *Xho*I and P: *Pst*I. **B,** The structure of pJS4812 is shown. The positions of the Gm^r and Ap^r *(bla)* genes, the origin of transfer (oriT) and the truncated lac operon (*'lacZYA*) are indicated by open arrows. The region of DNA homology with the ORS571 chromosome (see text) is indicated by the hatched box. Restriction enzyme cleavage sites indicated are: E: *Eco*RI; S: *Sma*I; X: *Xba*I; B: *Bam*HI; Bg: Bg*l*II; C: *Cla*I.

RESULTS

Effect of point mutations in the −24/−12 promoter element on *A. caulinodans nifA* gene expression.

Specific single and double base-pair mutations were introduced in the −24/−12 element of the *nifA* promoter (see Materials and Methods; Fig. 1A). The mutant *nifA* promoters were fused to the *lacZ* reporter gene, and the resulting chimeric genes were integrated in the *A. caulinodans* chromosome using the integration vector pJS4812 (Materials and Methods; Fig. 1B). The resulting strains were assayed for *nifA* promoter activity (β-galactosidase or β-Gal activity) in cultures grown under different physiological conditions (aerobic or microaerobic; in the presence or absence of combined nitrogen sources; see Materials and Methods). Under nitrogen-fixing conditions (microaerobic in the absence of ammonium [$NH4^+$]), a single base-pair change (G to T; Mutation n1) at position −25 or a G to T change at position −13 (Mutation n3; Figure 1A) resulted in a substantial reduction (n1: threefold) or virtual abolishment (n3: 11-fold reduction) of *nifA* expression (Fig. 2A). The n1+3 double mutation lead to the reduction of *nifA* expression to a background level (20-fold reduction; Fig. 2A). Interestingly, a mutation at position −16 (C to T; Mutation n2; Fig. 1A) resulted in a considerable (threefold) increase of *nifA* promoter activity relative to the wild-type construct (Fig. 2A). A similar phenotype was observed for a n1+2 double mutation (fivefold increase of *nifA* expression), while a n2+3 double mutation was observed to have a wild-type level of *nifA* promoter activity (Fig. 2A).

These results suggested that the n2 and n1+2 mutations may have generated a (partially) constitutive, σ54 independent promoter. To examine this question, the n1+2 mutant promoter-*lacZ* fusion (pJSn1+2) was also introduced into a $NtrA^+(RpoN^+$; DH5α*F'*) and $NtrA^-$ ($RpoN^-$; TH1, Table 1) *E. coli* strains, and β-Gal levels were measured. The n1+2 mutant promoter was found to direct a significant level of reporter gene expression, in the presence or absence of a functional *ntrA(rpoN)* gene (data not shown). The wild-type *nifA* promoter was found to be not expressed in *E. coli* and the n2 mutant to a low level (data not shown).

The effect of repressing concentrations of ammonium on the expression of the wild-type and mutant *nifA* promoters was examined. The wild-type *nifA* promoter was expressed to a ~10-fold lower level at 15 hr and a ~4-fold lower at 40 hr of growth in medium containing ammonium (Fig. 2B). The n3 and n1+3 mutant *nifA* promoters, which already showed very low levels of expression under nitrogen-fixing conditions (-$NH4^+$), were expressed at even lower levels in the presence of ammonium (Fig. 2B). The activity of the n1, n2, n2+3, and n1+2 mutant promoters was repressed to a lesser degree than the wild-type promoter in the presence of ammonium. This was particularly clear at 15 hr after inoculation, at which time the n1 and n2+3 mutant promoters were repressed ~4-5 fold (as compared to 10-fold in the case of the wild-

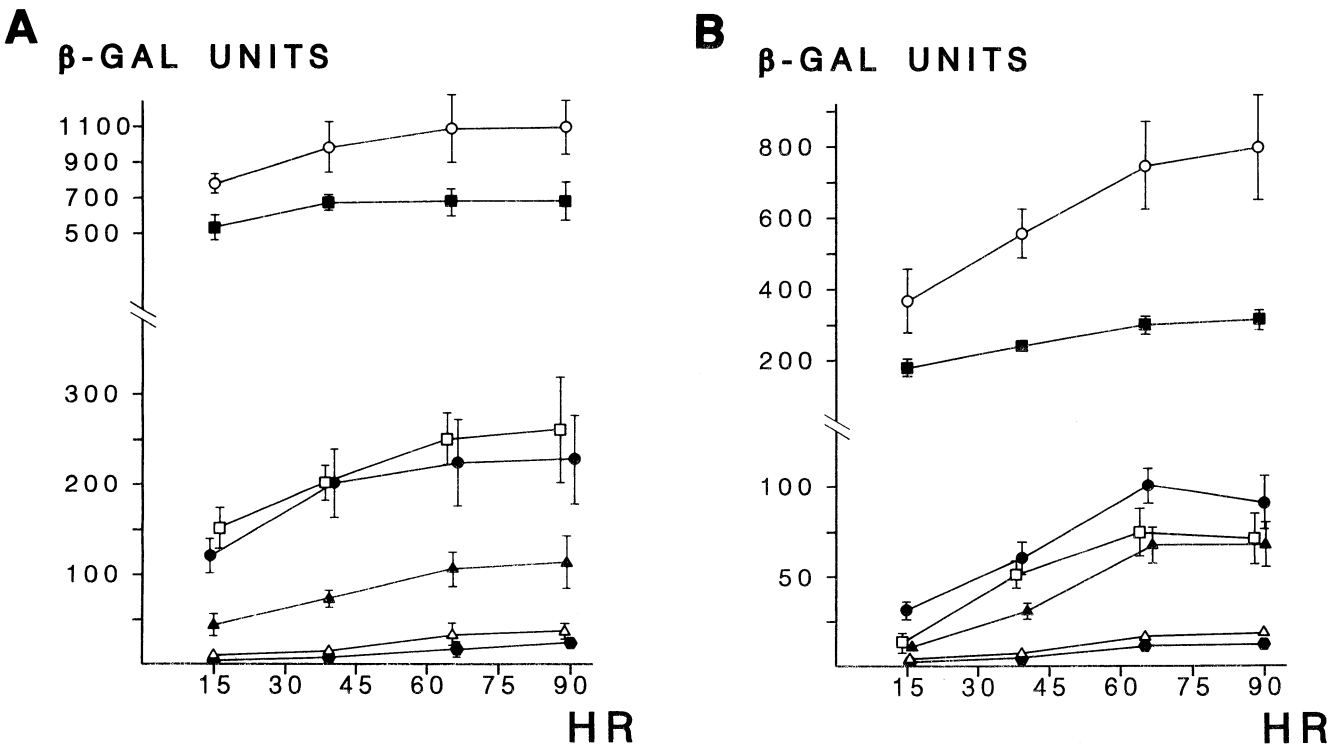

Fig. 2. Expression of wild-type and mutant *nifA* promoters in ORS571 under different physiological conditions. **A,** β-Galactosidase levels of wild-type and mutant *nifA-lacZ* fusions in ORS571 under nitrogen-fixing (derepressing) conditions (LSO medium without N source; 3% O_2). The β-Gal enzyme units (Miller 1972) are shown on the Y-axis and the number of hours after inoculation are shown on the X-axis. **B,** β-Gal enzyme levels of wild-type and mutant *nifA-lacZ* fusions in ORS571 under ammonium-repressing conditions (LSO medium with 0.1% ammonium sulfate; 3% O_2). The open circles denote the strain carrying the nl+2 mutant fusion, the closed boxes the n2 mutant, the open boxes the wild-type promoter, the closed circles the n2+3 mutant, the closed triangles the nl mutant, the open triangles the n3 mutant, and the closed diamonds the n1+3 mutant. The results in A and B represent an average of at least three independent experiments.

type) and the n2 and n1+2 mutant promoters only ~two- to threefold (Fig. 2B). These results suggest that the conserved $_{-25}$GG-N10-GC$_{-12}$ motif in the *nifA* promoter is important for transcriptional activation, as well as ammonium repression, and that by the generation of a double mutation at positions −25 and −16 a highly expressed, relatively nitrogen-regulation-independent promoter can be generated.

The effect of repressing concentrations of O_2 on *nifA* promoter activity was also examined. Incubating the cultures aerobically, in the absence of ammonium, resulted in a severe reduction of expression of the wild-type promoter, the n1, n2, n1+2, and n2+3 mutant promoters, as well as a further reduction of expression of the already poorly expressed n3 and n1+3 mutant promoters (data not shown). In the presence of ammonium under aerobic conditions, the activity of the wild-type, n3, and n1+3 mutant promoters was reduced to background levels (~30-fold reduction), the n1 and n1+2 mutant promoters showed a three- to five-fold reduction of activity at 40 hr after inoculation (relative to the activity under nitrogen-fixing conditions). The n2 and n2+3 mutant promoter showed a 10- to 12-fold reduction in activity (data not shown). The results of these experiments are summarized in Table 2.

Cloning and characterization of an *A. caulinodans ntrA(rpoN)*-like locus.

To identify the *trans*-acting factor(s) involved in *ntr* control via the −24/−12 element in the *nifA* promoter, we searched for the presence of (a) *ntrA(rpoN)*-like gene(s) in *A. caulinodans* using the cloned *R. meliloti ntrA(rpoN)* locus (from plasmid pNtr3.5BE; Ronson *et al.* 1987) as heterologous DNA probe. The 3.5-kb *Bam*HI-*Eco*RI insert of this plasmid was purified, labeled, and used as a hybridization probe for a Southern blot carrying *Eco*RI digested *A. caulinodans* DNA and for a colony bank of *A. caulino-*

Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Source or reference
A. caulinodans		
ORS571	Wild-type, Cb^r, Nif$^+$, Nod$^+$, Fix$^+$	Dreyfus *et al.* 1988
ORS571N136-1c	*ntrA*::Tn*5*, Cb^r, Km^r, Nif$^-$, Nod$^+$, Fix$^-$	This work
ORS571N15-2c	*ntrA*::Tn*5*, Cb^r, Km^r, Nif$^-$, Nod$^+$, Fix$^-$	This work
ORS571N136-3c	*ntrA*::Tn*5*, Cb^r, Km^r, Nif$^-$, Nod$^+$, Fix$^-$	This work
Rhizobium meliloti		
1680	*ntrA1*::Tn*5*, Str^r, Nm^r	Ronson *et al.* 1987
E. coli		
DH5αF′	F′, Δ(lacZYA)	Hanahan 1983
HB101	Sm^r, recA	Boyer and Roulland-Dussoix 1969
MC1061	Sm^r, Δ(lacZYA)	Casadaban *et al.* 1983
TH1	Δ*lacU*169, Δ*glnF*	Hunt and Magasanik 1985
Plasmids		
pNtr3.5BE	Apr^r, pUC8 derivative, *R.m ntrA*	Ronson *et al.* 1987
pUC118/pUC119	Ap^r; used for cloning and sequencing	Vieira and Messing 1987
pRK2013	Km^r, IncN, Mob$^+$, Tra$^+$, helper plasmid in mobilizations	Figurski and Helinski 1979
pRK290	Tc^r, IncP, Mob$^+$, Tra$^-$, broad host range cloning vector	Ditta *et al.* 1980
pWB5	Tc^r, Km^r, pRK290 derivative	W. Buikema and F. M. Ausubel
pPH1JI	Cm^r, Sm^r, Sp^r, Gm^r, IncP, Tra$^+$	Hirch and Beringer 1984
pLAFR1	Tc^r, IncP, Mob$^+$, Tra$^-$, broad host range cosmid cloning vector	Friedman *et al.* 1982
pJRD184	Tc^r, Ap^r	Heusterspreute *et al.* 1985
pLRSC1	Tc^r, ORS571 *ntrBC-ntrYX* region in pLAFR1	Pawlowski *et al.* 1987
pRSA13	Cm^r, ORS571 *nifA* region in pACYC184	Pawlowski *et al.* 1987
pPR3408	Tc^r, Cm^r, Ap^r, ORS571 *nifHD-lacZ* in pRK290	Pawlowski *et al.* 1987
pBS71	Tc^r, ORS571 genomic DNA (*ntrA* region) in pLAFR1	This work
pBS714	Tc^r, 2.7-kb *Sma*I fragment of pBS71 in pJRD184	This work
pNtrA2/pNtrA4	Ap^r, 2.7-kb *Sma*I fragment of pBS714 in pUC119	This work
pNM481	Ap^r, polycloning-sites fused to 8th codon of *lacZ*	Minton 1984
pPR54	Ap^r, Gm^r, ORS571 *nifA*::MudIIPR46′ fusion cloned in pJRD184 for cointegration into the ORS571 chromosome	Ratet *et al.* 1989
pPR57	Ap^r, Gm^r, *Sal*I-*Xho*I deletion derivative of pPR54	P. Ratet
p481	Apr^r, Gm^r, *Eco*RI-*Cla*I fragment from pNM481, containing the polycloning sites fused to 8th codon of *lacZ*, in *Eco*RI-*Cla*I digested pPR57	This work
pJS4812	Ap^r, Gm^r, 5.9-kb *Sma*I fragment of pLRSC1 in *Xba*I site of p481, providing a region of homology for cointegration into the ORS571 chromosome	This work
pJSwp	Ap^r, Gm^r, wild-type ORS571 *nifA* promoter region fused to 8th codon of *lacZ* in pJS4812 for cointegration into the ORS571 chromosome	This work
pJSn1	as pJSwp but G −> T at −24	This work
pJSn2	as pJSwp but C −> T at −16	This work
pJSn3	as pJSwp but G −> T at −13	This work
pJSn1+2	as pJSwp but G −> T at −24 and C −> T at −16	This work
pJSn1+3	as pJSwp but G −> T at −24 and G −> T at −13	This work
pJSn2+3	as pJSwp but C −> T at −16 and G −> T at −13	This work

dans DNA constructed in the vector pLAFR1 (Pawlowski *et al.* 1987). Three hybridizing *Eco*RI fragments of *A. caulinodans* DNA were observed in the Southern blot (~20, 3.6, and 1.5 kb in length) and seven positive colonies were identified in the colony bank (out of 700 colonies screened; data not shown). The latter seven colonies were shown to contain cosmids with overlapping segments of *A. caulinodans* DNA and one cosmid carrying all three (contiguous) hybridizing fragments was selected for further studies (pBS71). This cosmid was introduced into the *R. meliloti ntrA(rpoN)*::Tn*5* mutant 1680 via conjugation and found to be able to complement the Ntr^- phenotype of strain 1680 (Ronson *et al.* 1987), as evidenced by restoration of growth on minimal LSO plates with potassium nitrate (0.2%) as sole nitrogen source (data not shown). Using Southern blotting, the region of homology with the *R. meliloti ntrA(rpoN)* locus was narrowed down to a 2.7-kb *Sma*I fragment of pBS71, and this fragment was subcloned into plasmid pJRD184 (Table 1) to form plasmid pBS714 (Fig. 3).

Creation of transposon Tn*5* insertion mutants in the *A. caulinodans ntrA(rpoN)*-like locus.

Plasmid pBS714 was mutagenized with Tn*5*, as described by de Bruijn and Lupski (1984), and two independent Tn*5* insertions within the 1.5-kb *Eco*RI fragment, showing the highest degree of homology with the *R. meliloti ntrA(rpoN)* probe, were selected (Tn*5*#15 and Tn*5*#136; Fig. 3). These insertions were used for gene-replacement experiments (see de Bruijn 1987), and the position of the Tn*5*'s in the chromosomal *A. caulinodans ntrA(rpoN)*-like locus was verified by Southern blotting (data not shown). The resulting strains were examined for their ability to fix nitrogen in culture and on plates (Nif phenotype), and in stem and root nodules induced on *S. rostrata* (Nod and Fix phenotypes), growth on nitrate or amino acids as sole N source (Ntr phenotype), as well as growth on dicarboxylic acids as sole C source (Dct phenotype). The results are summarized in Table 3. The insertion mutants #15 and #136 were Nif^- (see also below), Nod^+, Fix^-, Ntr^- (assimilatory nitrate reduction deficient), and Dct^-, but grew normally on arginine, histidine, and proline as sole nitrogen sources. This phenotype closely resembled that found for *ntrA (rpoN)*::Tn*5* mutants of *R. meliloti* (Ronson *et al.* 1987) and suggested that pBS714 carried an *A. caulinodans ntrA(rpoN)*-equivalent gene.

Table 2. Expression of wild-type (wp) and mutant (n) *nifA*:*lacZ* fusions in wild-type ORS571 (wt) and *ntrA*(*rpoN*) mutant strains at 40 hr after inoculation[a]

	$-NH_4$ 3% O_2	$-NH_4$ 20% O_2	$+NH_4$ 3% O_3	$+NH_4$ 20% O_2
ntr(rpoN)/wp	++	−	−	−−
wt/wp	++	−−	−	−−
wt/n1	+−	−−	−	−
wt/n2	+++	−	++	+−
wt/n3	−	−−	−−	−−
wt/n1+2	++++	−	+++	++
wt/n1+3	−−	−−	−−	−−
wt/n2+3	++	−−	+−	−

[a] −− : less than 10 units of β-Gal activity; − : 10–50 units; +− : 50–100 units; ++ : 200–500 units; +++ : 500–1,000 units; ++++ : 1000 or more units.

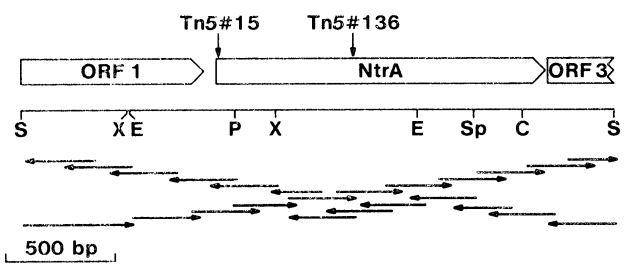

Fig. 3. Physical and genetic map of the ORS571 *ntrA(rpoN)* locus and DNA sequencing strategy employed. The extent and direction of the protein coding regions are shown by open arrows. The positions of the Tn*5* insertions are shown by vertical arrows. The restriction enzyme code used is the same as used in the legend of Figure 1B, except that X: *Xho*I; Additional sites: P: *Pst*I; Sp: *Sph*I. The horizontal arrows show the extent of the fragments sequenced on both strands.

DNA sequence analysis of the *A. caulinodans ntrA(rpoN)*-equivalent locus.

The DNA sequence of the 2.7-kb *Sma*I fragment of pBS714 (Fig. 3) was determined and the results are shown in Fig. 4. This analysis revealed the presence of three open reading frames (ORF1, position 7–849; ORF2, position 916–2457; partial ORF3, position 2470–2674; Figs. 3 and 4). The deduced polypeptide from ORF2 was found to share significant domain homology with NtrA(RpoN) proteins from other bacteria, including *R. meliloti* (see Discussion), and ORF2 was therefore designated as an *A. caulinodans ntrA(rpoN)* locus. Detailed mapping studies revealed that both Tn*5*#15 and #136 were located within ORF2 and thus were designated as *ntrA(rpoN)*::Tn*5* insertions (data not shown; Fig. 3). The ATG at position 916 was designated as the putative NtrA(RpoN) start codon, because of the presence of a Shine-Dalgarno consensus sequence (position 901; GGGAGG versus AGGAGG; Ringquist *et al.* 1992), at a proper distance and because of the results from the sequence comparison studies (data not shown). ORF2 encodes a NtrA(RpoN) polypeptide of 514 amino acids (predicted molecular weight 55,863) and is separated from ORF1 by an intergenic region of

Table 3. Phenotypes of wild-type and *ntrA (rpoN)* mutant strains of *A. caulinodans* ORS571

	Wild-type	*ntrA*::Tn*5*#15 *ntrA*::Tn*5*#136
Nif	+	−
Fix	+	−
Nod	+	+[a]
Ntr (0.1% KNO_3)	+	−
Dct	+[b]	−[b]
Aut	+/−[c]	+/−[c]
Hut	+[d]	+[d]
Put	+[e]	+[e]

[a] Numerous, light green, small nodules (see Pawlowski *et al.* 1987).
[b] Growth on 20 mM L-succinate, L-fumarate, or L-malate as sole C-source.
[c] Growth on 15 mM L-arginine; +/− indicates poor but significant growth.
[d] Growth on 15 mM L-histidine.
[e] Growth on 15 mM L-proline.

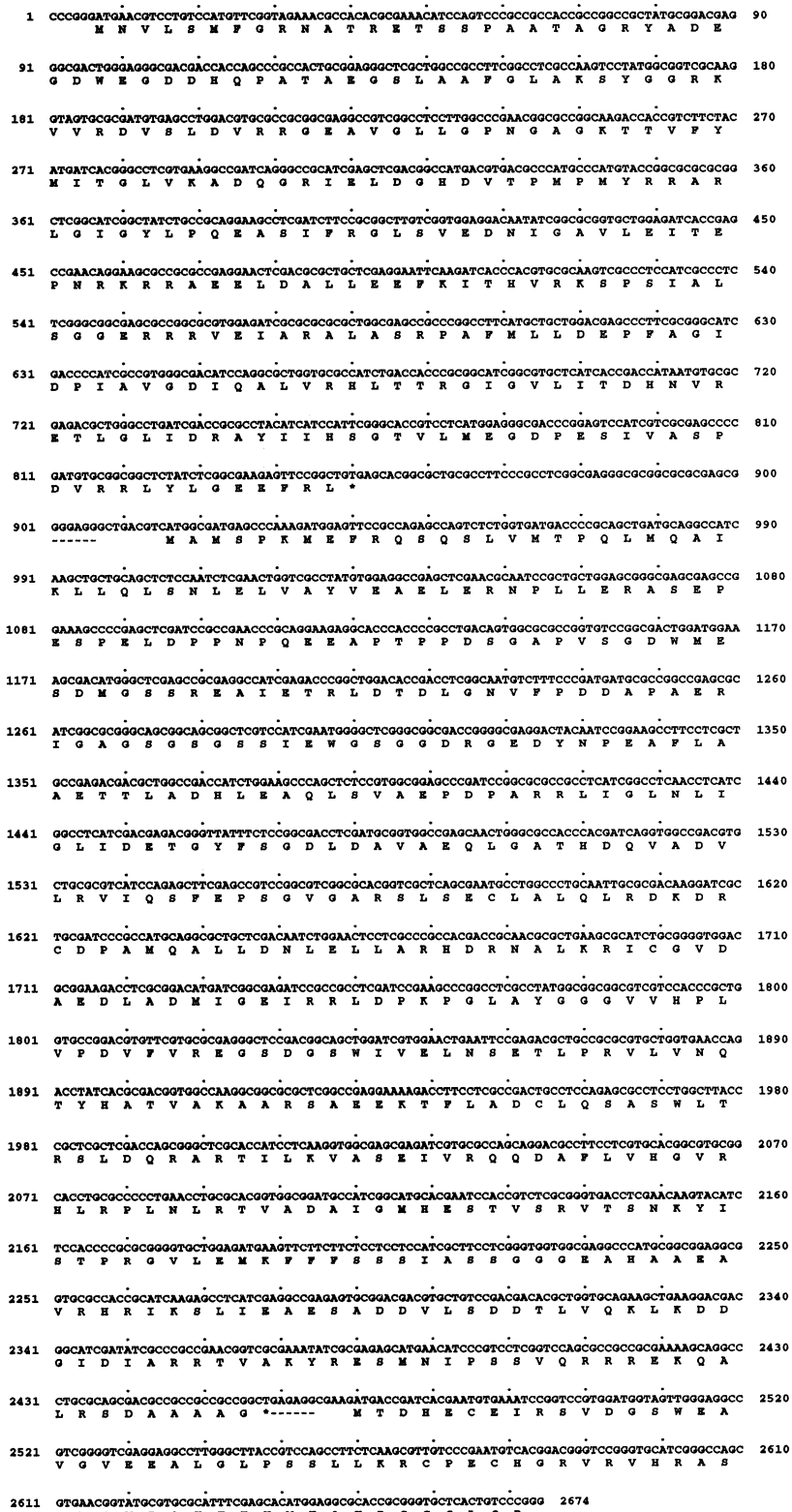

Fig. 4. DNA sequence of an ORS571 *ntrA(rpoN)* locus and predicted protein products. The nucleotide sequence of the 2,674 bp *Sma*I fragment of pBS714 and the deduced amino acid sequence are shown. Stop codons are indicated with asterisks. Putative ribosome binding sites are underlined.

62 bp. DNA sequence analysis of this region failed to identify DNA sequence motifs characteristic of promoters or transcriptional terminators (data not shown), suggesting that the *A. caulinodans ntrA(rpoN)* gene described here may be part of an operon and cotranscribed with ORF1.

The deduced polypeptide corresponding to ORF1 (Figs. 3 and 4) shares significant homology with the polypeptides encoded by the corresponding ORFs upstream of the *ntrA(rpoN)* genes of *R. meliloti* (Ronson *et al.* 1987; Albright *et al.* 1989), *Thiobacillus ferrooxidans* (Berger *et al.* 1990), *Pseudomonas putida* (Inouye *et al.* 1989), *K. pneumoniae* (Merrick and Gibbins 1985), *Rhizobium* sp. NGR234 (Van Slooten *et al.* 1990), and *Salmonella typhimurium* (Popham *et al.* 1991). A limited sequence comparison of these ORFs is shown in Figure 5. The predicted polypeptide encoded by the truncated ORF3, located immediately downstream of the *A. caulinodans ntrA(rpoN)* gene (Figs. 3 and 4) was not found to share significant homology with the products of ORFs found downstream of the *ntrA(rpoN)* gene in other bacteria (data not shown; see Discussion).

Effect of *A. caulinodans ntrA(rpoN)*: Tn*5* mutants on *nifHD-lacZ* and *nifA-lacZ* gene expression.

To examine the effect of the *ntrA(rpoN)*::Tn*5* mutations #15 and #136 on *A. caulinodans nif* gene expression, plasmid pPR3408 (Pawlowski *et al.* 1987), carrying an *A. caulinodans nifHD-lacZ* gene fusion, was introduced into strains ORS571N15-2c and ORS571N136-1c, as well as into the wild-type strain ORS571. The transconjugants were cultured under different physiological conditions (aerobic versus microaerobic; in the presence and absence of ammonium) and the reporter gene activity (β-Gal) was measured. No β-Gal activity was detected in the *ntrA (rpoN)*::Tn*5* mutant strains under any physiological conditions examined, while a high level of β-Gal activity was found in the wild-type strain harboring the *nifHD-lacZ* fusion under nitrogen-fixing conditions (data not shown). Plasmid pJSwp, carrying a fusion of the wild-type *A. caulinodans nifA* promoter to *lacZ* (Table 1), was introduced into the same *ntrA(rpoN)*::Tn*5* strains and integrated into the chromosome (see Materials and Methods). *nifA-lacZ* expression was examined in these strains by measuring β-Gal activity. In addition, the growth curves of the *nifA-lacZ* containing strains were determined under different physiological conditions. The results are shown in Figure 6. The wild-type strain harboring the *nifA-lacZ* fusion grew well in the presence of ammonium under aerobic conditions but could not grow aerobically in the absence of combined nitrogen (ammonium); growth under microaerobic conditions in the presence of ammonium was proficient (but less good than aerobically in the presence of ammonium), while growth under nitrogen-fixing conditions (microaerobically in the absence of ammonium) was intermediate (Fig. 6A). The *ntrA(rpoN)*::Tn*5* mutant strain ORS571N15-2c, harboring the *nifA-lacZ* fusion, grew equally well as the wild-type strain aerobically or microaerobically in the presence of ammonium and did not grow aerobically in the absence of combined nitrogen (ammonium); in contrast to the wild-type, it could not grow under nitrogen-fixing conditions (microaerobically in the absence of combined nitrogen ammonium; Fig. 6B).

The β-Gal enzyme assays on the same cultures under nitrogen-fixing conditions (microaerobically in the absence of ammonium) revealed that, in spite of the fact that *ntrA(rpoN)*::Tn*5* strains could not grow or fix nitrogen, the *nifA* gene was expressed at a wild-type level and fully repressed by ammonium. In fact, *nifA-lacZ* expression appeared to be elevated in the *ntrA(rpoN)*::Tn*5* versus wild-type strains (Fig. 6C).

Search for additional *ntrA(rpoN)*-like loci in *A. caulinodans*.

The results shown above and previous observations (Pawlowski *et al.* 1987, 1991; Ratet *et al.* 1989) revealed that the *A. caulinodans nifA* promoter is *ntr*-controlled, in response to the N status of the cells, and contains a functional $-24/-12$ element, normally responsible for interaction with NtrA(RpoN). However, the *A. caulinodans ntrA(rpoN)* gene described here, while controlling nitrogen fixation and nitrate assimilation, does not appear to be involved in *nifA* promoter activity (Fig. 6C), suggesting the presence of an additional *ntrA(rpoN)*-equivalent gene in this organism, as has been observed in *Bradyrhizobium japonicum* (Kullik *et al.* 1991). To examine this

```
Ac    MNVLSMFGRNATRETSSPAATAGRYADEGDWEGDDHQPATAEGSLAAFGL   50
Ae     MRPAPETPERQTVPMTDTATIADKPSVEASTVLAG.....GSTLVVRHL   44
Rm               MQIPFLHKRKRGKKPSAAAAAARAVDKARYDGTLIARGL   39
Tf                                                MSELLQAQSL   10
CON                                                 L A  L

                                   **********
Ac    AKSYGGRKVVRDVSLDVRRGEAVGLLGPNGAGKTTVFYMITGLVKADQGR  100
Ae    KKRYGSRTVVKDVSLDVKSGEVVGLLGPNGAGKTTSFYMIVGLVALDEGD   94
Rm    TKSYRSRRVVNGVSLVVRRGEAVGLLGPNGAGKTTCFYMITGLVPVDEGS   89
Tf    FKSYRRRVVVRDVSVQVATGEVVGLLGPNGAGKTTTFYMMVGLVRPDRGH   60
CON    KSY  R VV DVSL V  GE VGLLGPNGAGKTT FYMI GLV  D G

Ac    IELDGHDVTPMPMYRRARLGIGYLPQEASIFRGLSVEDNIGAVLEITEPN  150
Ae    IVLDGDHISGLPIHERAPMGLSYLPQEASVFRKLNVEENIRAVLELQVSN  144
Rm    IEINGNDVTTMPMYRRARLGVGYLPQEASIFRGLTVEDNIRAVLEVHDEN  139
Tf    IFLQQRDITALPMHERARMGLGYLPQEPSVFRQMSAADNVLAVLETLPLS  110
CON   I L G D T  PM  RAR G GYLPQEAS FR L VEDNI AVLE    N

                                                *******
Ac    .....RKRRAEELDALLEEFKITHVRKSPSIALSGGERRRVEIARALASR  195
Ae    GKPLPKAEIERRLDSLLDDLQIAHLRNNPALSLSGGERRRVEIARALASS  194
Rm    .....VDRRESKLNDLLGEFSITHLRKSPAIALSGGERRRLEIARALATD  184
Tf    ....PVERQERQ.EQLLSELHLHALRDTKGHSLSGGERRRVEIARALAMS  155
CON          R E  L  LL E  I HLR  P   LSGGERRRVEIARALA

      *******
Ac    PAFMLLDEPFAGIDPIAVGDIQALVRHLTTRGIGVLITDHNVRETLGLID  245
Ae    PRFILLDEPFAGVDPIAVGEIQRIVSFLKARNIGVLITDHNVRETLGICD  244
Rm    PTFMLLDEPFAGVDPISVADIQALVRHLTSRGIGVLITDHNVRETLGLID  234
Tf    PRFILLDEPFAGIDPISVLEIQRLIRDLRARGIGVLITDHNVRETLGICE  205
CON   P F LLDEPFAG DPI V  IQ LVR L  RGIGVLITDHNVRETLG  D

Ac    RAYIIHSGTVLMEGDPESIVASPDVRRLYLGEEFRL*                281
Ae    HAYIISEGTVLAAGQPEEIIANDAVRRVYLGENFRM*                280
Rm    RAYIIHAGEVLTHGRANDIVTNPDVRRLYLGDNFSL*                270
Tf    RAYILHDGKVLTAGSPQEIVDDPMVRQVYLGDQFQI*                241
CON   RAYIIH G VL  G P  IV  P VRR YLG  F
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Fig. 5. Amino acid sequence comparison of the deduced protein products of ORF1s from ORS571, *A. eutrophus, R. meliloti,* and *T. ferroxidans.* The *A. eutrophus* (Ae) sequence was derived from Warrelmann *et al.* (1992), the *R. meliloti* (Rm) sequence from Albright *et al.* (1989) and the *T. ferroxidans* (Tf) sequence from Berger *et al.* (1990). Amino acid residues conserved in at least three out of four proteins are shown in the line labeled "CON." The regions of homology with nucleotide-binding pockets of ATP-requiring enzymes (Walker *et al.* 1982) are indicated by asterisks.

hypothesis, a synthetic oligonucleotide (3′C$^{G}/_{T}$GCGTG-CCAGCGCTT$^{C}/_{T}$AT$^{G}/_{A}$GCGCT5′), corresponding to a highly conserved domain of NtrA(RpoN) proteins (RRT-VAKYRE; data not shown) was prepared and used as a DNA hybridization probe for a Southern blot carrying chromosomal DNA of *A. caulinodans, B. japonicum, R. meliloti,* and *E. coli.* The hybridization results confirmed the presence of two *ntrA(rpoN)* copies in *B. japonicum* (Kullik *et al.* 1991), and suggested the presence of at least two *ntrA(rpoN)*-homologous regions in *A. caulinodans* and possibly as many as four such regions in *R. meliloti* (data not shown).

DISCUSSION

Effect of mutations in the −24/−12 promoter element on ORS571 *nifA* expression.

The DNA sequence of the −24/−12 promoter element of 64 σ54-controlled promoters from 22 different species has been compared and a consensus sequence derived (−27 cTGGCACGgcctTTGCA −11; Morett and Buck 1989; Kustu *et al.* 1989). Three residues of this consensus sequence are completely invariant, namely the GG pair at positions −25/−24 and the G at position −13. The only exception to the latter appears to be the *glnH* promoter of *E. coli* (Claverie-Martin and Magasanik 1991). The −12 position is almost invariant, with the exception of some rhizobial promoters, where it is replaced by an A residue (see Merrick and Chambers 1992 for a discussion and references). In the case of the ORS571 *nifA* promoter, a mutation at position −25 (n1) results in a significant reduction of promoter activity ("down" promoter phenotype). However, the down phenotype is not as severe as found in the case of analogous mutations in the −24 and/or −25 position of the *K. pneumoniae nifH* (Ow *et al.* 1985), the *K. pneumoniae nifL* (Khan *et al.* 1986) and *B. japonicum nifH* (Kaluza *et al.* 1985) promoters or in the promoters of the *Caulobacter crescentus flbG* (Mullin and Newton 1989) and *S. typhimurium argTr* (Schmitz *et al.* 1988) genes. Interestingly, an analogous mutation in the −23 G residue of the *B. japonicum fixRnifA* promoter has no effect on *nifA* expression (Thöny *et al.* 1987).

A mutation in residue −13 of the ORS571 *nifA* promoter (n3) essentially abolishes *nifA* expression. This effect has also been observed for analogous mutations in the −13 and/or −12 position of the *K. pneumoniae nifH* and *nifLA* promoters (Ow *et al.* 1985; Buck *et al.* 1985; Khan *et al.* 1986), the *B. japonicum fixRnifA* promoter (Thöny *et al.* 1987) and the promoters of the *C. crescentus flbG* and the *S. typhimurium argTr* genes (Mullin and Newton 1989; Schmitz *et al.* 1988). These results strongly suggest that the ORS571 *nifA* promoter is σ54 dependent and therefore supports our model that *nifA* activation in this organism requires the presence of a NtrA(RpoN)-like protein (NtrA*; Fig. 7).

A mutation in the −16 position of the ORS571 promoter (n2) results in a substantial increase of *nifA* expression ("up" promoter phenotype) and a severe decrease in repression by ammonia (partially constitutive phenotype). Based on results obtained with mutations (C to T transitions) in the −17 to −15 residues of the *K. pneumoniae nifH* promoter, it has been suggested that the presence of T-rich stretches in this region may result in the formation of a stronger NtrA(RpoN)-RNA polymerase-promoter

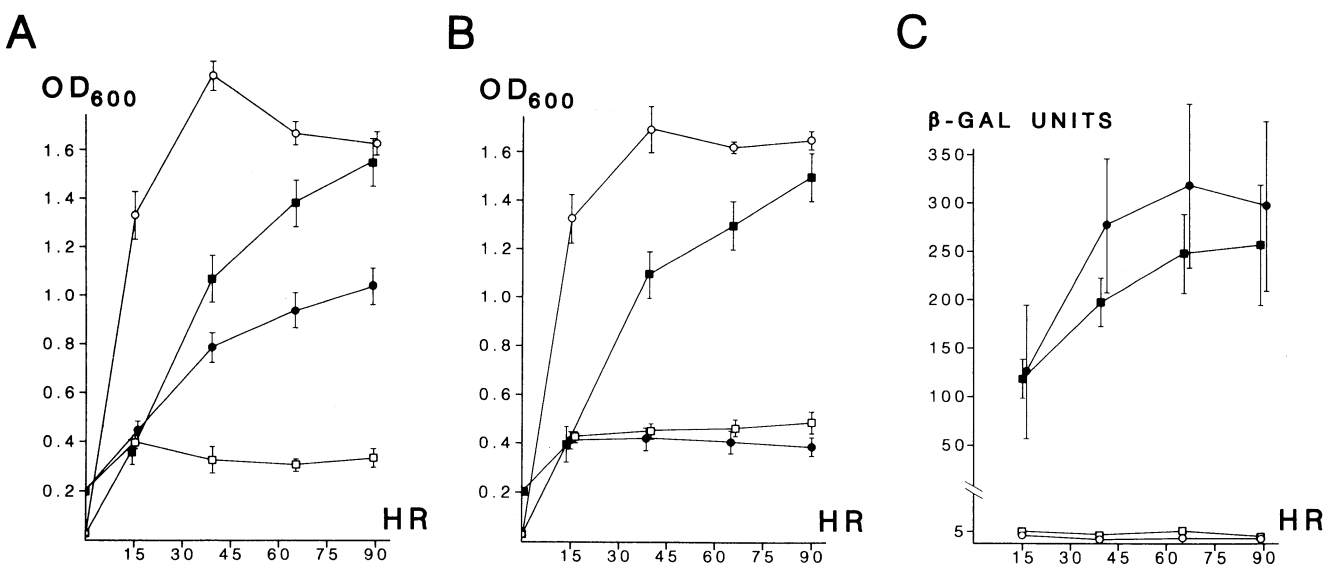

Fig. 6. Growth and β-Gal activity of wild-type and *ntrA(rpoN)*::Tn*5* strains harboring a wild-type *nifA-lacZ* gene fusion under different physiological conditions. **A,** Growth of strain ORS571 harboring the *nifA-lacZ* gene fusion under nitrogen fixing (derepressing) conditions (LSO medium lacking an N source; 3% O_2; solid circles), ammonium repressing conditions (LSO medium supplemented with 0.1% ammonium sulfate; 3% O_2; solid boxes), O_2 repressing conditions (LSO medium lacking an N source; air; open boxes) and aerobically in the presence of 0.1% ammonium sulfate (open circles). The OD_{600} is shown on the Y-axis and the hours after inoculation on the X-axis. The results shown represent an average of more than 10 independent experiments. **B,** Growth of strain ORS571N15-2c *(ntrA[rpoN]*::Tn*5)* harboring the wild-type *nifA-lacZ* gene fusion under the same physiological conditions as described for Figure 6A. The results represent the average of at least five independent experiments. **C,** β-Galactosidase activity of ORS571 wild-type (squares) and *ntrA(rpoN)*::Tn*5* mutant strains (circles) harboring the wild-type *nifA-lacZ* gene fusion under nitrogen-fixing (derepressing) conditions (closed circles/squares) or under ammonium plus oxygen repressing conditions (open circles/squares). The results represent an average of at least three independent experiments.

complex (Buck and Cannon 1989; 1992a; Morett and Buck 1989; Cannon and Buck 1992), possibly due to a more efficient recognition of the target by the NtrA(RpoN)-RNA polymerase (Whitehall *et al.* 1992). The relevance of the −14 to −17 residues for σ54 (NtrA/RpoN) binding has also been demonstrated by Buck and Cannon (1992b), who have suggested that the methyl groups in the DNA major groove of this region are important for binding. This may also explain the up promoter phenotype of the n2 mutation. The extreme up phenotype of the n1+2 double mutation suggests that the −24 part of the NtrA(RpoN)-RNA polymerase recognition site does not play a significant role in the "up" expression pattern observed with the n2 single mutation. However, the diminished up phenotype of the n2+3 double mutation suggests that the other half of the −24 to −12 motif (G residue at position −13) is involved in the n2 expression pattern. Since the increased expression levels of the n2 and n1+2 mutant promoters can no longer be fully repressed by nitrogen or oxygen, it is also possible that the ORS571 *nifA* promoter is subject to both activation under microaerobic, N-limited conditions (involving the $_{-25}$GG$_{-24}$ and the $_{-13}$GC$_{-12}$ residues), as well as repression by aerobic N excess growth conditions (involving the $_{-16}$C residue).

Another, perhaps more simple, explanation may be that the n2 and n1+2 mutations have generated a σ54 (Ntr A/RpoN) independent promoter, since the TTcgCA motif around position −35 now resembles the DNA sequence recognized by a σ70-RNA polymerase. This is supported by the finding that expression of the n2 and n1+2 mutant promoters in *E. coli* appears to be *ntrA(rpoN)* independent.

Phenotype of the OR5571 *ntrA(rpoN)* mutant.

The inability of the ORS571 *ntrA(rpoN)*::Tn*5* mutant to assimilate nitrate or utilize dicarboxylic acids constitutes typical phenotypes of *ntrA(rpoN)* mutants of other (nitrogen fixing) bacteria (see Kustu *et al.* 1989; de Bruijn *et al.* 1990; Merrick 1992 and references cited therein; Kullik *et al.* 1991) and supports our designation of this locus as *ntrA(rpoN)*.

The Nif$^-$/Fix$^-$ phenotype of the ORS571 *ntrA(rpoN)*:: Tn*5* mutant described here resembles that observed with corresponding single *ntrA(rpoN)* mutants of other diazotrophs such as *K. pneumoniae* (de Bruijn and Ausubel 1983; Merrick and Gibbins 1985), *Rhodobacter capsulatus* (Jones and Haselkorn 1989), and *Azotobacter vinelandii* (Toukdarian and Kennedy 1986; Merrick *et al.* 1987), as well as single *ntrA(rpoN)* mutants of symbiotic nitrogen-fixing organisms, such as *R. meliloti* (Ronson *et al.* 1987) and *Rhizobium* sp. NGR234 (Stanley *et al.* 1989), and a double *ntrA(rpoN)* mutant of *B. japonicum* (Kullik *et al.* 1991). In the case of *K. pneumoniae*, both the *nifA* promoter and the promoters of the other *nif* genes require *ntrA(rpoN)* for their expression (see de Bruijn *et al.* 1990; Merrick 1992), while in the other cases cited above *nifA* expression appears to be independent of *ntrA(rpoN)* (see Merrick 1992 and references cited therein; Kullik *et al.* 1991; Preker *et al.* 1992; Foster-Hartnett and Kranz 1992). The expression of the ORS571 *nifA* promoter is also independent of the *ntrA(rpoN)* gene described here, in spite of the fact that the *nifA* 5′, upstream region contains a functional −24/−12 promoter element and is controlled by the N status of the cell, *ntrBC* and *ntrYX* (Ratet *et al.* 1989; Pawlowski *et al.* 1991). In this respect, the regulatory circuit controlling ORS571 *nifA* expression resembles that proposed for *R. capsulatus,* where the presence of another (NtrA(RpoN)-like; NtrA*; Fig. 7) alternative σ factor (specifically) involved in *nifA* promoter activity has also been proposed (Hübner *et al.* 1991; Preker *et al.* 1992; Foster-Hartnett and Kranz 1992). Whether one of the additional fragments showing homology with the oligonucleotide corresponding to the conserved NtrA(RpoN) domain encodes the postulated *ntrA** locus is presently under investigation.

Conservation of the *ntrA(rpoN)* locus.

Our analysis of the amino acid sequence deduced from ORF2 (Figs. 3 and 4) further confirms that the gene inac-

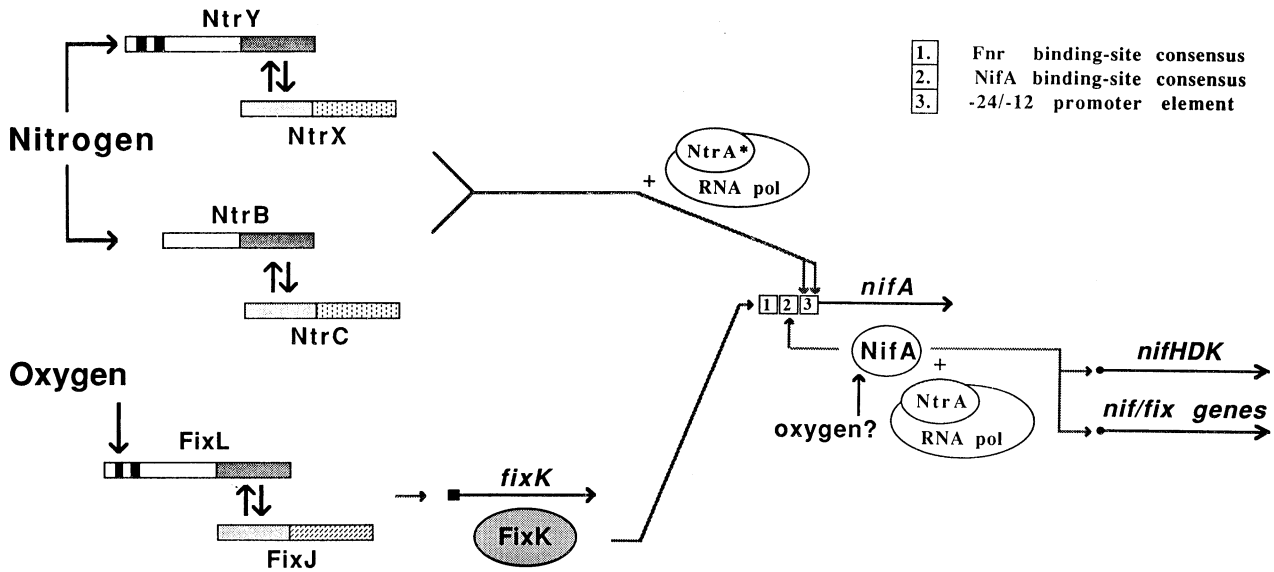

Fig. 7. Model for the regulation of nitrogen-fixation genes in ORS571. For details, see text.

tivated by the Tn*5* insertion #136 encodes an alternative σ factor of the σ54 family, as defined by Hirschmann *et al.* (1985), Merrick *et al.* (1987), and Kustu *et al.* (1989), and therefore can be designated as *ntrA(rpoN)*. The alignment of the ORS571 NtrA(RpoN) amino acid sequence with the sequences of NtrA(RpoN) proteins from 12 different bacterial species (data not shown) reveals that the ORS571 NtrA(RpoN) protein is most closely related to the NtrA (RpoN) proteins from *B. japonicum* (Kullik *et al.* 1991). ORS571 NtrA(RpoN) contains the three major regions defined by Merrick *et al.* (1987). Region I (Met-1 to Gln-50) represents a strongly conserved domain, rich in leucine and glutamine residues. This domain has been postulated to be involved in contacting the −12 region of the promoter, to facilitate the interaction between σ54 and activator proteins, and to play a role in positioning the σ54-RNA polymerase complex near the DNA region to be melted upon activation, since deletions in this domain are unable to progress from a closed complex to a transcriptionally active-open complex (Sasse-Dwight and Gralla 1990; Merrick 1992). However, recently Merrick and Chambers (1992) have proposed that the helix-turn-helix motif in domain III (see below) plays a role in interaction with the −12 region and have suggested an alternative explanation for the results by Sasse-Dwight and Gralla (1990).

Region II (Arg-51 to Thr-148) of the ORS571 NtrA (RpoN) protein shows no significant homology to other NtrA(RpoN) proteins, which is consistent with the fact that this is the least conserved region in these proteins. This region contains multiple negatively charged residues, which have been postulated to play a role in melting DNA in the promoter, since mutating these residues in the *E. coli* NtrA(RpoN) protein (residues 51–77) results in an inability to form open complexes, while leaving the DNA binding activity of the σ54-RNA polymerase complex relatively unaffected (Sasse-Dwight and Gralla 1990). However, the absence of this region in the NtrA(RpoN) proteins of *Rhodobacter spaeroides, R. capsulatus,* and *Bacillus subtilis* (Meijer and Tabita 1992; Alias *et al.* 1989; Jones and Haselkorn 1989; Debarbouille *et al.* 1991) suggests that this region is not essential in all bacteria.

Within region III, four conserved subdomains have been identified (Merrick *et al.* 1987; van Slooten *et al.* 1990). Subdomain IIIA (Leu-188 to Pro-214) shares homology with other σ factors, such as RpoD and SpoIIAC (Gribskov and Burgess 1986; Helmann and Chamberlin 1988) and has been proposed to be involved in interacting with the core RNA polymerase (see Merrick *et al.* 1987). It may also play a role in protein-DNA interactions, since a deletion of the analogous *E. coli* NtrA(RpoN) protein reduces contact formation at the −12 region of the promoter (Sasse-Dwight and Gralla 1990). Subdomain IIIB (Trp-355 to His-382) shares homology with an amino acid sequence near the N terminus of the β-subunit of *E. coli* RNA polymerase (RpoC) and may play a role in protein-protein interactions (Merrick *et al.* 1987). Subdomain IIIC (Asn-391 to Ser-411) contains the α-helix (residues 391-399)-β-turn (400 and 401)-α-helix (402–411) motif, characteristic of DNA-binding proteins (Dodd and Egan 1990; Coppard and Merrick 1991), which has been shown to be involved in recognition of the −13/−12 residues (Merrick and Chambers 1992). The adjacent amino acid sequences (residues 412–429) are also completely conserved in NtrA(RpoN) proteins. Subdomain IIID (Ala-480 to Arg-488) is also conserved in all NtrA(RpoN) proteins and has been designated as the RpoN-box (van Slooten *et al.* 1990). Its function is unknown, but the oligonucleotide synthesized to screen for the presence of additional *ntrA(rpoN)*-like sequences in rhizobial genomes was derived from this region. It is also interesting to note that rhizobial NtrA(RpoN) proteins carry a 14–25 amino acid "tail" at the C-terminal end of subdomain IIID, the function of which remains to be determined.

In a variety of bacterial species, including ORS571 (Fig. 3), an ORF (designated ORF1) has been found immediately upstream of the *ntrA(rpoN)* gene. The ORS571 ORF1 shares significant homology (Fig. 5) with the corresponding ORFs of *R. meliloti* (Ronson *et al.* 1987; Albright *et al.* 1989), *Rhizobium* sp. NGR234 (van Slooten *et al.* 1990), *S. typhimurium* (Popham *et al.* 1991), *T. ferrooxidans* (Berger *et al.* 1990), *P. putida* (Inouye *et al.* 1989), *K. pneumoniae* (Merrick and Gibbins 1985), and *Alcaligenes eutrophus* (Warrelmann *et al.* 1992). The biological function of the protein encoded by ORF1 and the reason for the direct linkage of ORFI to the *ntrA(rpoN)* gene in these diverse bacteria are unknown. Albright *et al.* (1989) have shown that the predicted ORF1 product shares homology with a superfamily of ATP-binding proteins involved in transport, cell division, nodulation, and DNA repair and have attempted, unsuccessfully, to introduce an insertion mutation in ORF1. These authors concluded that ORF1 may encode an essential housekeeping function. However, *in vitro* transcription studies using σ54-dependent promoters have shown that purified activator protein and σ54-RNA polymerase are sufficient for promoter activity (Hirschman *et al.* 1985; Hunt and Magasanik 1985; Wong *et al.* 1987), suggesting that the protein encoded by ORF1 may not be important for NtrA(RpoN) action. Regardless of its actual function, the amino acid sequence comparison suggests that the N-terminal end of the ORF1 product may not be essential, since it is not conserved between ORS571, *R. meliloti* and *A. eutrophus*, and is missing in *T. ferrooxidans* (Fig. 5).

The partial ORF downstream of the ORS571 *ntrA (rpoN)* locus (ORF3; Fig. 3) does not share any significant homology with corresponding ORFs downstream of the *ntrA(rpoN)* genes of other bacteria (data not shown). This is interesting considering that the corresponding ORFs in *R. meliloti* (Ronson *et al.* 1987), *T. ferrooxidans* (Berger *et al.* 1990), *P. putida* (Inouye *et al.* 1989), *K. pneumoniae* (Merrick and Coppard 1989), *A. vinelandii* (Merrick *et al.* 1987), *Rhizobium* sp. NGR234 (van Slooten *et al.* 1990), *B. japonicum* (Kullik *et al.* 1991), *A. eutrophus* (Warrelmann *et al.* 1992), and *S. typhimurium* (Popham *et al.* 1991) do share significant homologies. No mutations in the ORF3s of these organisms have been reported, and therefore no biological role for the ORF3 product has been established. A homology search of the NBRF/PIR and SWISS.PROT protein banks has revealed interesting similarity matches for a stretch of residues (LKRCPECH GRVR; position 31 to 42) of the deduced ORF3 product, to a region (VRRCPQCHGDML; 83% similarity) of the

hypA gene product of *E. coli,* postulated to be a transcriptional activator of hydrogenase genes (Lutz *et al.* 1991), and the (covalent) heme-binding domain (IMKCSQCHTVEK; 75% similarity) of human heart cytochrome-c (Schroeder 1968).

Nitrogen-fixation gene regulation model for ORS571.

A model for the regulation of the ORS571 nitrogen fixation (*nif/fix*) genes, deduced from the data presented here and previous data, is schematically presented in Figure 7. The ORS571 *nifA* promoter mediates the regulatory response to fluctuating nitrogen and oxygen concentrations (Ratet *et al.* 1989). Under N-limiting conditions, *nifA* expression appears to be controlled by two bicomponent regulator systems, consisting of the regulator proteins NtrC and NtrX, and their sensor partners NtrB and NtrY (Pawlowski *et al.* 1987, 1991). The NtrY protein contains transmembrane domains, suggesting it is membrane-bound and may be sensing the extracellular (periplasmic) concentration of nitrogen, while NtrB may be responding to the intracellular N concentration (Pawlowski *et al.* 1991). The expression of the *ntrYX* operon appears to be affected by *ntrC,* therefore it cannot be excluded that *ntrBC* act through *ntrYX* in regulating the expression of the *nifA* promoter (Pawlowski *et al.* 1991).

The *nifA* promoter contains a −24/−12 promoter element (box 3; Nees *et al.* 1988; Ratet *et al.* 1989), that is essential for *nifA* expression, as demonstrated here, and may be mediating the *ntr* response by *ntrBC* and *ntrYX.* However, the "normal" *ntrA(rpoN)* gene, described here, does not appear to be involved in this process, suggesting the presence of an alternative *ntrA(rpoN)*-like gene (*ntrA**) in ORS571, with a distinct specificity for the *nifA* promoter. The latter could possibly be reflected in the differences in DNA sequence (denoted by bold italics) surrounding the −24/−12 regions of the *nifH* versus *nifA* promoters of ORS571 (*nifH*1 and 2: $^{C}/_{A}$TGGCAC$^{A}/_{G}{}^{C}/_{G}{}^{C}/_{T}$CGTTGC$^{A}/_{T}$, Norel and Elmerich 1987; −24/−12 consensus: cTGGCACGgcctTTGCA, Morett and Buck 1989; *nifA*: G*A*GGC*TG*ATCCCTC*G*CA, Ratet *et al.* 1989).

Oxygen control of *nifA* expression (Ratet *et al.* 1989) is mediated by the *fixLJ* genes, acting through *fixK* (Kaminski *et al.* 1991; Kaminski and Elmerich 1991). FixL is likely to be the oxygen sensor, by analogy with *R. meliloti* (Gilles-Gonzalez *et al.* 1991) and activates FixJ, which controls the expression of the *fixK* gene. In turn FixK, a Fnr-like transcriptional activator (Spiro and Guest 1990), activates the *nifA* promoter (Kaminski *et al.* 1991; Kaminski and Elmerich 1991). The target for the FixLJK system in the *nifA* promoter region is unknown, but it is plausible that the Fnr binding site consensus sequence, identified in the *nifA* promoter region (box 1; Nees *et al.* 1988; Ratet *et al.* 1989) and found to be important for *nifA* expression (J. Stigter and F. J. de Bruijn, unpublished results), may be involved in mediating the oxygen response.

The ORS571 *nifA* gene may also be subject to autoregulation (Ratet *et al.* 1989), which may be mediated through the NifA upstream activation sequence (UAS; box 2), found in the *nifA* promoter region (Nees *et al.* 1988; Ratet *et al.* 1989). As found in all other nitrogen-fixing systems (see de Bruijn *et al.* 1990; Merrick 1992), the ORS571 NifA protein is responsible for activating the other *nif/fix* genes (Pawlowski *et al.* 1987), and this activation requires the product of the *ntrA(rpoN)* gene described here. As observed in other systems (see Merrick 1992), and based on the observed homology (Ratet *et al.* 1989) between the ORS571 NifA protein and the *B. japonicum* NifA domain conferring oxygen sensitivity (Fischer *et al.* 1988), the ORS571 NifA protein itself may also be oxygen sensitive. Our present experiments are designed to provide further evidence in support of this regulatory model, which appears to represent a complicated chimera of regulatory circuits found in free-living and symbiotic nitrogen-fixing organisms and continues to provide incentives to study *A. caulinodans* strain ORS571 as an interesting model to elucidate the molecular basis of *nif/fix* expression in culture versus *in planta.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37° C in LB medium (Miller 1972). ORS571 strains were grown at 37° C in TY (Beringer 1974), YLS (Elmerich *et al.* 1982), or in LSO medium (Elmerich *et al.* 1982), supplemented with 40 mg/L of nicotinic acid and 0.1% of the desired nitrogen sources. Antibiotics were used at the following concentrations: For *E. coli*: ampicillin (Ap; 100 μg/ml), chloramphenicol (Cm; 30 μg/ml), gentamycin (Gm; 5 μg/ml), kanamycin (Km; 20 μg/ml), and tetracycline (Tc; 10 μg/ml); for ORS571: carbenicillin (Cb; 500 μg/ml), Gm (50 μg/ml), Km (200 μg/ml), and Tc (10 μg/ml). Indicator medium for *E. coli* or ORS571 strains containing *lac* gene fusions was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 40 μg/ml).

DNA manipulations.

Plasmid DNA was prepared by the alkaline lysis method described by Sambrook *et al.* (1989). Chromosomal DNA was prepared as described by Meade *et al.* (1982). Conditions used for DNA manipulations and transformations were as described by Sambrook *et al.* (1989). The enzymes used in these analyses were used according to the specifications of the manufacturers (Boehringer, Mannheim; Bethesda Research Laboratories, Gaithersburg, MD; New England Biolabs, Boston, MA).

Southern blotting and colony hybridizations.

Plasmid pNtr3.5EB (Ronson *et al.* 1987) was used as the source for the *R. meliloti ntrA(rpoN)* DNA probe. The 3.5-kb *Eco*RI-*Bam*HI fragment was isolated and radioactively labeled by nick translation. Southern blotting and nick translations were carried out as described (Sambrook *et al.* 1989). Hybridizations were carried out in the presence of 50% formamide at 42° C and the blots washed at 68° C in 2× SSC (1× SSC contains 0.1 5 M NaCl, 15 mM Na citrate, pH 7) buffer, containing 0.1% sodium dodecyl sulfate (SDS). Colony hybridizations were carried out by using Whatman 541 filter paper, as described by Maas (1983).

Transposon Tn*5* mutagenesis of the *ntrA(rpoN)* gene.

Transposon Tn*5* mutagenesis was carried out as described by de Bruijn and Lupski (1984) and de Bruijn (1987).

Conjugation and gene replacement experiments.

Plasmids were mobilized from *E. coli* to ORS571 using the helper plasmid pRK2013, as described by Ditta *et al.* (1980). Gene replacement experiments with Tn*5* mutagenized regions were carried out as described (Ruvkun and Ausubel 1981; de Bruijn 1987). *NtrA(rpoN)*::Tn*5* mutants resulting from a double crossover event were identified by examining the (absence of) proper growth of the transconjugants on LSO medium with nitrate as sole N source. The pPH1JI plasmid was cured from the *ntrA (rpoN)*::Tn*5* strains by introducing pRK290 (selecting for Tc^r transconjugants) and loss of pRK290 was achieved by serial culture in the absence of Tc and selection for Tc^S colonies. The resulting strains were labeled with a "c" (e. g., ORS571 N136-1c; Table 1).

Nodulation and nitrogen fixation assays.

Nodulation and symbiotic- or free-living nitrogen fixation assays were carried out as described by Pawlowski *et al.* (1987).

DNA sequencing.

The dideoxynucleotide chain termination method using [^{35}S]dATP and Sequenase version 2.0 (United States Biochemical, Cleveland, OH) was used for DNA sequence determination. Constructs used for DNA sequencing were derived from pNtrA2 or pNtrA4, which carry a 2.7-kb *Sma*I fragment cloned in both orientations in pUC119 (Table 1). The DNA sequence was obtained from nested deletion derivatives, generated with exonuclease III/mung bean nuclease (Ausubel *et al.* 1989) and from restriction fragments cloned into pUC119 and pUC118. The nucleotide sequence reported here has been submitted to GenBank and has the accession number X69959.

Construction of *lacZ*-translational fusion vector pJS4812.

The 598-bp *Eco*RI/*Cla*I fragment from pNM481 (Minton 1984), containing unique cloning sites fused to the eighth codon of *lacZ,* was inserted into plasmid pPR57 (digested with *Eco*RI/*Cla*I), giving rise to plasmid p481. pPR57 is *Sal*I-*Xho*I deletion derivative of pPR54 (Ratet *et al.* 1989). A 5.9-kb *Sma*I fragment of pLRSC1 (Pawlowski *et al.* 1987) was cloned in the *Xba*I site of p481, to provide a region of homology with the ORS571 genome for homologous recombination, giving rise to plasmid pJS4812 (see Fig. 1B).

Oligonucleotide-directed mutagenesis of the *A. caulinodans nifA* promoter and construction of mutant *nifA* promoter-*lacZ* fusions.

The *Xho*I/*Pst*I fragment from pRSA13 (Pawlowski *et al.* 1987), containing the ORS571 *nifA* 5′, region and the coding region for the 43 N-terminal amino acid residues of NifA (Ratet *et al.* 1989), was isolated. The *Xho*I sticky ends were rendered flush with the Klenow fragment of DNA Polymerase and the resulting fragment was cloned in the *Sma*I/*Pst*I site of pUC118, thereby reconstructing the *Xho*I site. A mixture of primers carrying the wild-type −24/−12 promoter sequence (5′-GCGGCTG$^{C}/_{A}$GA$^{G}/_{A}$GGATCAGC$^{C}/_{A}$TCCTGTCGGTG-3′), and derivatives thereof containing one or two mismatches (see Fig. 1A), were synthesized and used together with the pUC sequencing primer in a primary PCR reaction to amplify mutant promoter fragments (PCRI). The amplified dsDNA fragment, together with the reverse pUC sequencing primer of the vector, was used in a second PCR reaction to generate a complete *Xho*I-*Pst*I fragment containing the point mutations, as described by Kammann *et al.* (1989), flanked by an *Eco*RI and a *Hin*dIII site from the pUC118 polylinker (PCRII). The *Eco*RI/*Hin*dIII fragment of the PCRII product was cloned into pUC119 and its DNA sequence was determined to verify the nature of the mutations (see Fig. 1A). The wild-type and mutant *Eco*RI/*Hin*dIII fragments were cloned into pJS4812, thereby generating a translational fusion of *nifA* to the eighth codon of *lacZ,* under the control of the wild-type or mutant *nifA* promoters. For expression studies in ORS571, these *nifA-lacZ* fusions were integrated into the ORS571 chromosome, via a single recombination event in the ORS571 DNA homologous region carried by the pJS4812 integration vector.

Growth and β-galactosidase assays.

β-Galactosidase activity was measured as described by Miller (1972). ORS571 strains carrying *nif-lacZ* gene fusions were grown with continuous shaking in YLS medium, supplemented with 0.05% L-glutamine, at 37° C. A 0.2-ml aliquot of a saturated YLS culture was reinoculated in 10 ml of LSO medium, supplemented with 0.05% L-glutamine and 0.05% ammonium sulfate. A 1.2-ml aliquot of the LSO culture was reinoculated into 10 ml of LSO medium, and the culture was grown aerobically for 6 hr. The OD_{600} of the culture was measured and adjusted to 0.2 with LSO medium. For ammonium repression studies, a 1-ml aliquot was reinoculated into 9 ml of LSO medium supplemented with 0.1% ammonium sulfate. For microaerobic derepression studies cultures were incubated in the presence of a 97% N_2/3%O_2 gas mixture in a GasPak System (Becton Dickinson and Co., Cockeysville, MD). A 0.2- to 0.6-ml aliquot of the final cultures was mixed in a 1.5-ml cuvet with 0.5–0.8 ml of Z buffer to obtain a final OD_{600} of 0.3–0.6, and the exact OD_{600} was measured. A 0.8-ml aliquot of diluted cells was transferred to a 1.5-ml microcentrifuge test tube, vortexed in the presence of 0.01 ml of 0.1% sodium dodecyl sulfate (SDS), and 0.01 ml of chloroform for 1 min and incubated for 20 min at 30° C. The reaction was initiated by the addition of 0.16 ml of *o*-nitrophenyl-β-galactoside (ONPG; 4 mg/ml). After 5–60 min of incubation at 30° C the reaction was stopped by adding 0.4 ml of 1 M Na_2CO_3. This mixture was centrifuged and the OD_{420} of the supernatant was measured using a Pharmacia Ultrospec III spectophotometer. The β-Gal units shown in the figures were derived as described by Miller (1972).

ACKNOWLEDGMENTS

We would like to thank Katharina Pawlowski for helpful discussions, Chris Fetters and Kurt Stepnitz for help in preparing the figures, and Fred Ausubel for providing the *ntrA(rpoN)* probe and *R. meliloti* strains. Initial experiments were carried out at the Max Planck Institut fur Zuchtungsforschung in Cologne (Germany) and we would like to thank Jeff Schell and the Max Planck Gesellschaft for encouragement and support. Work at the MSU-PRL was supported by a grant to FJdB from the U.S. Department of Energy (DE FG02-90ER20021).

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