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Genetics

The expression of *nifA* in *Azorhizobium caulinodans* requires a gene product homologous to *Escherichia coli* HF-I, an RNA-binding protein involved in the replication of phage Qβ RNA

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ABSTRACT **We report the characterization of a mutant of *Azorhizobium caulinodans*, isolated after ethyl methanesulfonate mutagenesis. This Nod$^+$ Nif$^-$ Fix$^-$ mutant is unable to synthesize 10 of 15 polypeptides normally induced under conditions of nitrogen fixation. By using *lacZ* fusions it was shown that *nifA* and *nifA*-regulated genes were not expressed in this strain. The mutation was complemented by a constitutively expressed *nifA* gene or by a 1.1-kb DNA fragment from the wild-type strain, whose nucleotide sequence revealed a single open reading frame of 255 bp coding for an 85-amino acid polypeptide. The deduced amino acid sequence is similar to that of HF-I, an RNA-binding protein of *Escherichia coli*, which is required for replication of bacteriophage Qβ RNA. The similarity can be extended to the function since *hfq*, the structural gene for HF-I, complemented the *A. caulinodans* mutant. The corresponding gene in *A. caulinodans* was termed *nrfA* (for nif regulatory factor). Inactivation of *nrfA* in the mutant was due to a missense mutation resulting in the replacement of a cysteine residue by arginine. A null mutant, constructed by disruption of *nrfA*, exhibited the same phenotype as the missense mutant. Thus, an additional factor can be added to the already complex system of *nifA* regulation in *A. caulinodans*.**

Azorhizobium caulinodans is unique among diazotrophs: it is able to fix nitrogen symbiotically in both root and stem nodules of the tropical legume *Sesbania rostrata* and to grow at the expense of dinitrogen in the free-living state (1). Expression of the *nif* genes, such as *nifHDK*, and of the *fixABCX* genes requires NifA (2, 3) and the product of an *rpoN* gene (4).

The two major environmental signals involved in the regulation of NifA are the nitrogen status and the oxygen status (5). Three different DNA motifs found in the 5′ upstream region of the *A. caulinodans nifA* gene could account for this regulation. These motifs are (*i*) a typical −24/−12 promoter element found in N-regulated genes; (*ii*) an NifA binding site consensus sequence suggesting that NifA regulates its own synthesis; and (*iii*) an Fnr binding site consensus sequence, also called the anaerobox, found in O_2-regulated genes (5, 6). The oxygen control is mediated by the two-component regulatory proteins FixLJ (7). Analogy with *Rhizobium meliloti* (8, 9) suggests that, in oxygen-limiting conditions, phosphorylated FixJ stimulates the expression of the *fixK* gene. FixK, an Fnr-like transcriptional activator, presumably activates *nifA* transcription by binding to the anaerobox (10). Under nitrogen-limiting conditions, *nifA* expression appears to be controlled by two sensor-response regulator systems: NtrBC and NtrYX (3, 11). Stigter *et al.* (4) showed that the product of the *rpoN* gene was not involved in the control of *nifA* transcription and assumed that *A. caulinodans* contains another gene similar to *rpoN* but specific for the *nifA* promoter.

In this study, we report the characterization of a previously isolated Nod$^+$, Nif$^-$, Fix$^-$ regulatory mutant of *A. caulinodans* (12).† This mutant, defective for NifA expression, is impaired in a gene similar to *Escherichia coli hfq*, which codes for an RNA-binding protein required for replication of Qβ RNA by the Qβ replicase. This suggests that *A. caulinodans* displays an additional previously unsuspected type of regulation for NifA.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Growth Conditions, and Nitrogenase and β-Galactosidase Assays. The bacterial strains and plasmids used are listed in Table 1 or schematized in Fig. 1. Media and growth conditions for *A. caulinodans* and *E. coli* were as described (12, 19). Nitrogenase assays with bacteria in the free-living state and with *S. rostrata* roots were performed as reported (12, 19). β-Galactosidase assays were performed as described (10).

Molecular Biology Techniques and Plasmid Construction. Recombinant DNA work used standard protocols (20). PCR was performed as described (7). Isolation of chromosomal DNA from *A. caulinodans* was as described (12). pRS419 originated from a *Bgl* II genomic bank of *A. caulinodans* DNA cloned into pLA2917 (16) (this laboratory, unpublished data). pRS423, pRS423b, and pRS423c/d were obtained by cloning the 2.5-kb *Xho* I fragment from pRS419 into the *Xho* I site of pVK100 (17) (Fig. 1) and pBluescript (Stratagene) and into the *Sal* I site of M13mp19, respectively. The pBluescript construction was subsequently used to construct deletions of the *Xho* I fragment, which were then subcloned into pVK100 to yield pRS427 and pRS432. pRS427 lacks the 1.4-kb *Sph* I fragment of pRS423b. pRS432 has been deleted of 480 nt by exonuclease III (Fig. 1). To construct pRS433, which carries a kanamycin cartridge at the *Sma* I site (Fig. 1), the 2.5-kb insert of pRS423c was first recovered as a *Hin*dIII/*Bam*HI fragment and then subcloned into pBluescript to obtain a derivative containing a unique *Sma* I site in the insert. A purified 1.4-kb *Sma* I kanamycin cartridge from pUC-4-KIXX (Pharmacia LKB) was cloned into this *Sma* I site. Deletion of a *Hin*dIII fragment from the polylinker of the cartridge removed an undesired *Xho* I site. The resulting insert containing the cartridge was finally recovered as an *Xho* I/*Bam*HI fragment and subcloned into the *Xho* I and *Bgl* II sites of pVK100. pRS427b (data not shown) contains the same insert as pRS427 but in pBluescript. It was used for the construction of pRS431. An *Eco*RI fragment was deleted to

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Abbreviations: ORF, open reading frame; EMS, ethyl methanesulfonate.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X76450).

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Ref. or source
A. caulinodans strain		
ORS571	Wild type	1
5751	EMS mutant Nif$^-$, Fix$^-$	12
ORS571A5	*nifA* mutant, Km^r	3
57613	Translational *nifA–lacZ* fusion in ORS571, Gm^r	
57614	Translational *nifA–lacZ* fusion in 5751, Gm^r	
57615	Translational *nrfA–lacZ* fusion in ORS571, Gm^r	
57616	Km^r, *nrfA*::Km	
57617	Transcriptional *nifA–lacZ* fusion in ORS571, Gm^r, Km^r	
57618	Transcriptional *nifA–lacZ* fusion in 5751, Gm^r, Km^r	
E. coli strain		
MG1655	Wild type	*E. coli* Genetic Stock Center
S17-1	*pro, thi, hsdR*, RP4 integrated into the chromosome, *tet*::Mu; *kan*::Tn*7*; Tn*1*	13
Plasmid		
pGS72	IncP, Tra$^-$, Km^r, Tc^r	14
pRS1022	Tc^r (pGS72) 2.2-kb *Xho* I/*Hin*dIII fragment of pLRSA2 containing *nifA*	
pGD926	IncP, Tra$^-$, Tc^r, promoterless *lacZ*	15
pRS2002	*nifH–lacZ* in pGD926	7
pRS2003	*nifA–lacZ* in pGD926	7
pRS2004	*fixK–lacZ* in pGD926	10
pLA2917	IncP, Tra$^-$, Tc^r, Km^r	16
pRS3012	*fixN–lacZ* in pLA2917	This laboratory
pRK415	IncP, Tra$^-$, Tc^r, pRK404 derivative	18
pRK434	Tc^r (pRK415), 0.4-kb *Hin*dIII/*Bam*HI PCR fragment containing *E. coli hfq*	

remove part of the insert, which was then subcloned as a *Kpn* I/*Bam*HI fragment into pRK415 (18). pRS1022 was obtained by cloning the *Xho* I/*Hin*dIII fragment of pLRSA2 (3) (containing the entire *A. caulinodans nifA*) into pGS72 (14).

Insertional Mutation in the *nrfA* Gene of *A. caulinodans*. The 6.6-kb *Pst* I fragment containing *nrfA* was cloned into pKOK4 (21) at the *Pst* I site. The resulting plasmid was digested with *Sma* I and ligated with a purified *Sma* I kanamycin cartridge from pUC-4-KIXX. This construct was used to transform *E. coli* strain S17-1 (13). After conjugation with *A. caulinodans*, strain 57616 was isolated. Double recombination was confirmed by Southern blot.

Construction of *lacZ* Fusions. *nrfA–lacZ fusion.* A 0.7-kb *Hin*dIII/*Sma* I fragment from pRS427b (see above; the *Hin*dIII site originated from the vector polylinker) was cloned in front of the promoterless β-galactosidase gene of pGD926 (15) between the *Hin*dIII and *Bam*HI sites. The in-frame fusion was constructed by filling the 5′ protruding *Bam*HI ends by using DNA polymerase Klenow fragment. The resulting translational *nrfA–lacZ* fusion was verified by nucleotide sequencing. The fusion was then subcloned as a *Hin*dIII/*Sal* I fragment into pKOK4Gm (2) to give pRS2009. This plasmid was used to recombine the *nrfA–lacZ* fusion

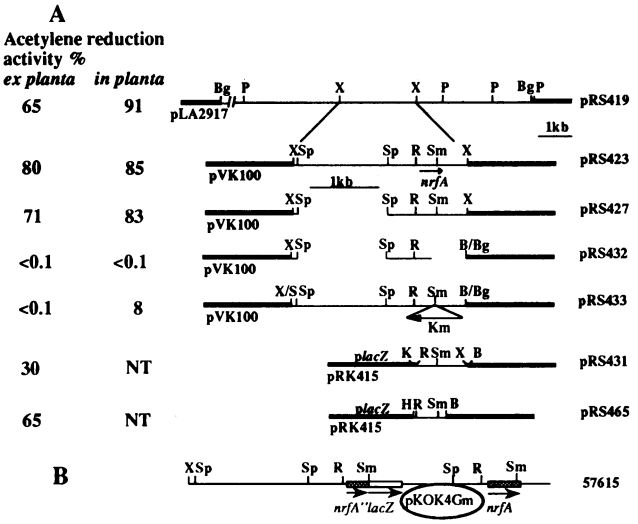

FIG. 1. (*A*) Physical map of the *Bgl* II insert of pRS419 and derivatives and complementation *in planta* and *ex planta* of the mutant 5751. B, *Bam*HI; Bg, *Bgl* II; RI, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I; S, *Sal* I; Sm, *Sma* I; Sp, *Sph* I; X, *Xho* I. Heavy bars are part of the vectors and are not drawn to scale. Nitrogen fixation is expressed as a percentage of wild-type activity. *ex planta* 100% corresponds to 16–20 nmol of C_2H_4 produced per min per mg of protein. *in planta* 100% corresponds to 0.6 nmol of C_2H_4 per min per mg of nodule. NT, not tested. (*B*) Schematic representation of the chromosome-integrated *nrfA–lacZ* fusion 57615 (not to scale).

into the chromosome of *A. caulinodans* to give strain 57615 (Fig. 1). Single recombination was checked by Southern blot.

nifA–lacZ fusion. (*i*) Translational fusion. Two 30-mer oligonucleotides were synthesized (ACGTAAGCTTTTT-TCCGGAAGCTGCGAATA and GATCGGATCCTCATA-GACCCCGATGAGTGC) and used to amplify the sequence in pLRSA2 by PCR. The resulting amplified 0.39-kb fragment containing the promoter region and 174 bp of the coding region of *nifA* was digested with *Hin*dIII and *Bam*HI and cloned into pBluescript. Several independent clones were sequenced to verify that no errors were introduced during PCR. After cloning into pGD926 digested with *Hin*dIII/*Bam*HI, the fragment containing the translational *pnifA–lacZ* fusion was recovered as a *Hin*dIII/*Sal* I fragment, as above, cloned into pKOK4Gm to yield pRS2012, and used to recombine the fusion into the chromosome of *A. caulinodans* to give strain 57613 and into the chromosome of mutant 5751 to yield strain 57614. (*ii*) Transcriptional fusion. The 0.39-kb *Hin*dIII/*Bam*HI fragment was first cloned into pKOK4Gm. The resulting plasmid was digested with *Bam*HI and ligated with a purified *Bam*HI *lacZ-km* cartridge (*lacZ* contains its own ribosome binding site) from pKOK5 (21) to yield pRS2010. pRS2010 was used to recombine the fusion into the chromosome of *A. caulinodans* and of mutant 5751 to yield strains 57617 and 57618, respectively.

DNA Sequence Analysis. pRS423c and -d inserts were deleted using the Cyclone I kit (IBI) and sequenced with the Taquence kit (United States Biochemical). Sequence data were compiled and analyzed by using the program of Staden modified by B. Caudron for the Computer Unix at the Service d'Informatique Scientifique, Institut Pasteur. Similarity searches were performed using FASTA and BLAST programs of the National Center for Biotechnology Information server.

RESULTS

Strain 5751 Is a Regulatory Mutant Impaired in *nifA* Expression. Characterization of the Nod$^+$, Nif$^-$, Fix$^-$ mutant 5751 reported by Denèfle *et al.* (12) suggested that it was impaired in regulation of nitrogen fixation. Indeed, in auto-

radiograms of two-dimensional protein gels, 10 of 15 polypeptides, including the nitrogenase polypeptides, synthesized in the wild type under nitrogen fixation conditions were absent from mutant 5751. To determine which step(s) of the regulatory pathway was impaired in the mutant, the relative expression of *nif–lacZ* and *fix–lacZ* fusions in the wild-type strain ORS571 and in mutant 5751 were compared.

The *pnifH–lacZ* fusion carried by pRS2002 was expressed in the wild type at a maximal rate in nitrogen-free medium (LSO) under 3% O_2/97% Ar, whereas expression was reduced to the basal level under air or in the presence of ammonia (LSN) (Table 2). In the 5751 mutant, the *pnifH–lacZ* fusion was expressed at the basal level under all conditions tested. Similarly, the *pnifA–lacZ* fusion carried by pRS2003 was expressed at the basal level under all physiological conditions tested in the mutant strain, whereas it was expressed at a high level under nitrogen fixation conditions in the wild type. The background level of expression of the *nifA–lacZ* fusion (carried by a low-copy-number plasmid) was only one-fifth of the fully derepressed level. Thus, it was of interest to measure the expression of this fusion when integrated as a single copy into the chromosome. Results obtained with the wild type showed that the basal level of *nifA* expression was much lower when the translational *pnifA–lacZ* fusion was integrated into the chromosome (57613) than when it was carried by a plasmid (ORS571 pRS2003) (Table 2). In strain 57614 (5751 mutant carrying the translational *pnifA–lacZ* fusion integrated into the chromosome), this fusion was expressed at the background level whatever the conditions used. Similar results were obtained with strains 57617 and 57618 (the transcriptional *pnifA–lacZ* fusion integrated into ORS571 and mutant 5751, respectively). Thus, neither *nifH* nor *nifA* was expressed in mutant 5751 in agreement with the Nif^- pleiotropic phenotype of this strain. *nifA* is positively controlled by FixK (10). This led us to determine whether the absence of *nifA* expression in the mutant 5751 was correlated with a defect in *fixK* transcription. In both the wild-type strain and mutant 5751, the *pfixK–lacZ* fusion (pRS2004) was expressed at a maximal rate in LSO or LSN medium under 3% O_2/97% Ar but was reduced by a factor of 4 in LSO or LSN under air. A similar pattern of expression was found with the *pfixN–lacZ* fusion carried by pRS3012, which is under the control of FixK. This shows that the synthesis of neither FixK nor FixNOQP is affected in the mutant 5751 (Table 2).

Complementation of Mutant 5751. A cosmid library of *Bgl* II wild-type DNA fragments cloned into pLA2917 was introduced into strain 5751 by conjugation. Conjugants were selected for growth on medium lacking combined nitrogen under 3% O_2/97% N_2. One transconjugant, which grew in nitrogen-free medium, was isolated. It contained a cosmid, designated pRS419, with a 16.5-kb *Bgl* II insert. The physical map of the fragment is shown in Fig. 1. Genetic complementation of mutant 5751 with various subclones of pRS419 revealed that the 2.5-kb *Xho* I fragment of pRS423 was sufficient to restore nitrogen fixation both *ex planta* and *in planta* (Fig. 1).

Derivatives of pRS423 carrying various deletions and insertions of a kanamycin cartridge were constructed and used for complementation to localize the mutated gene more precisely. Deletion of the internal 1.4-kb *Sph* I fragment (pRS427) had no effect on nitrogen fixation. This restricted the complementing locus to a 1.1-kb *Sph* I/*Xho* I fragment. Insertion of a kanamycin cartridge at the *Sma* I site (pRS433) and/or deletion of this *Sma* I site (pRS432) abolished complementation. The smallest DNA fragment allowing complementation was obtained with pRS431, which contained the 0.7-kb *Eco*RI/*Xho* I fragment cloned into the vector pRK415.

Identification of the *nif* Regulatory Locus. The nucleotide sequence of the 1.1-kb *Sph* I/*Xho* I fragment was determined. It contained a single open reading frame (ORF). This ORF is 255 nt long and codes for a polypeptide of 85 amino acids (9.6 kDa). It showed the characteristic *A. caulinodans* codon usage and third position GC bias (i.e., 83.6%). In addition, the location of this ORF was in agreement with the complementation experiments reported above. pRS465 contains a 0.46-kb fragment (including this ORF) amplified by PCR using the oligonucleotides CCCAAGCTTATGTTGCAGGGCAAAA-TAATCTTGCGC and CCGGATCCGTCCACGCCCTTCA-

Table 2. β-Galactosidase activity of *A. caulinodans* wild-type strain ORS571 and mutant strain 5751 carrying plasmid-borne and chromosomal-integrated *lacZ* fusions

	β-Galactosidase activity			
	LSO		LSN	
Strain/plasmid	3% O_2	Air	3% O_2	Air
Plasmid-borne fusions				
ORS571 pRS2002 (*nifH*)	3940 ± 1250	340 ± 67	275 ± 78	288 ± 57
5751 pRS2002 (*nifH*)	280 ± 86	290 ± 43	340 ± 38	352 ± 38
ORS571 pRS2003 (*nifA*)	1043 ± 84	220 ± 65	289 ± 46	224 ± 20
5751 pRS2003 (*nifA*)	198 ± 64	183 ± 49	232 ± 47	229 ± 36
ORS571 pRS2004 (*fixK*)	4925 ± 1660	1145 ± 360	5085 ± 2620	1025 ± 300
5751 pRS2004 (*fixK*)	5400 ± 1040	1300 ± 600	4270 ± 1100	1090 ± 320
ORS571 pRS3012 (*fixN*)	2322 ± 1362	705 ± 300	2507 ± 856	507 ± 119
5751 pRS3012 (*fixN*)	2734 ± 895	712 ± 234	3488 ± 1013	868 ± 277
Chromosomal-integrated fusions				
57613 (ORS571–*nifAa*)	700 ± 194	50 ± 7	34 ± 8	8 ± 3
57614 (5751–*nifAa*)	17 ± 8	13 ± 5	11 ± 2	11 ± 2
57617 (ORS571–*nifAb*)	714 ± 90	NT	NT	91 ± 17
57618 (5751–*nifAb*)	148 ± 30	NT	NT	119 ± 22
57615 (ORS571–*nrfA*)	3966 ± 1130	4797 ± 1953	2600 ± 988	2216 ± 384

β-Galactosidase activity is expressed in Miller units (1 Miller unit = 1 μmol of *o*-nitrophenyl β-galactoside hydrolyzed per min per mg of protein). pRS2002 carries a *nifH–lacZ* fusion, pRS2003 a *nifA–lacZ* fusion, pRS2004 a *fixK–lacZ* fusion, and pRS3012 a *fixN–lacZ* fusion from *A. caulinodans* strain ORS571. Chromosomal-integrated fusions were obtained after a single recombination event and carry a wild-type copy of *nifA* (57613/57614) or of *nrfA* (57615). *nifAa* and *nifAb* correspond to translational and transcriptional *nifA–lacZ* fusions, respectively. LSO, minimal medium devoid of ammonia; LSN, minimal medium containing 20 mM ammonia. Data are means of at least four independent experiments. NT, not tested.

GTCCACCCCCCCGCCG. pRS465 restored nitrogen fixation to mutant 5751 (Fig. 1). The higher level of nitrogen fixation obtained with pRS465 compared with pRS431 might be due to the different 5′ regions present in these two plasmids. The gene was called *nrfA* (for nif regulatory factor).

Expression of a *nrfA–lacZ* Fusion. To determine the expression pattern of *nrfA*, a translational fusion with *lacZ* was constructed and integrated into the chromosome of *A. caulinodans*. *nrfA* is relatively highly expressed for a regulatory gene (strain 57615 in Table 2). The expression does not seem to be regulated by the two major environmental signals, ammonia and oxygen, that control nitrogen fixation, although the expression of the fusion was reduced by a factor of 2 in the presence of ammonia.

Mutant 5751 Contains a Mutation in *nrfA*. We verified that the mutation in mutant 5751, which was isolated after ethyl methanesulfonate (EMS) mutagenesis, was indeed located in *nrfA*. The same oligonucleotides as described above were used to amplify a 0.46-kb sequence from DNA isolated from mutant 5751, including the entire *nrfA* and its proximal flanking sequences. DNA sequence analysis of the PCR product indicated that strain 5751 contained a single mutation at position 633 (thymine to cytosine), which resulted in replacement of the cysteine residue at position 45 of the amino acid sequence by an arginine residue (Fig. 2). This experiment was repeated to ensure that this mutation was not an artefact of PCR amplification (four independent clones from two independent amplifications). Furthermore, PCR products from the wild-type strain and mutant 5751 were subcloned into the vector pRK415. The wild-type DNA carried by pRS465 but not the mutated gene in pRK415 complemented the mutant 5751.

Insertional Mutagenesis of *nrfA*. To demonstrate that the absence of a functional NrfA polypeptide was responsible for the phenotype of strain 5751, directed mutagenesis of *nrfA* was performed. A kanamycin cartridge from pUC-4-KIXX was inserted into the *Sma* I site in *nrfA* and introduced into the chromosome of the wild-type strain. A double crossover event resulting in insertion of the kanamycin cartridge oriented in the opposite direction relative to *nrfA* was selected. This mutant (57616) exhibited the same phenotype as strain 5751—i.e., it grew as well as the wild-type strain in LSN medium but did not fix nitrogen in LSO medium under 3% O_2/97% Ar (Fig. 3).

HF-I from *E. coli* Is Homologous and Functionally Equivalent to NrfA. The predicted amino acid sequence of NrfA was compared with the Swiss-Prot protein sequence data bank (release 25.0). This search identified a significant similarity, ≈50% in a stretch of 67 amino acids, with the HF-I protein of *E. coli* (Fig. 2). To determine whether HF-I (encoded by the *hfq* gene) was functionally equivalent to NrfA, the *hfq* gene from *E. coli* was cloned into the vector pRK415. The oligonucleotides CCCGAAGCTTGTGTACAATTGAGACGTA-

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NrfA A.c  MAAERTQNLQDTFLNHVRKSKTPLTIFLVNGVK
              ::| |||:||| :|: : |::|:|||||:|
HF-I E.c    MAKGQSLQDPFLNALRRERVPVSIYLVNGIK
                  10        20        30

                     R
                     ↑
NrfA A.c LQGVVTWFDNFCVLLRRDGHSQLVYKHAISTIM
         |||  :  ||:| :|| ::: ||:|||||||::
HF-I E.c LQGQIESFDQFVILL-KNTVSQMVYKHAISTVV
               40        50        60

NrfA A.c PGHPVQLFDPTDEVASEKA
         |::||          ::
HF-I E.c PSRPVSHHSNNAGGGTSSNYHHGSSAQNTSAQQDSEETE
           70        80
```

FIG. 2. Amino acid comparison of *E. coli* (E.c) HF-I and *A. caulinodans* (A.c) NrfA. Identical amino acids are indicated by vertical bars. Dots represent similar residues. Numbering corresponds to NrfA amino acid sequence. The Cys-45 to Arg mutation, in mutant 5751, is indicated by an arrow. The FASTA program of the Genetics Computer Group package was used.

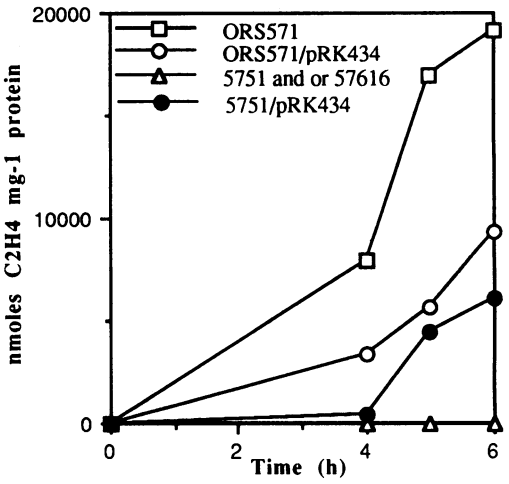

FIG. 3. Kinetics of nitrogenase derepression in the wild-type strain and in *nrfA* mutants. Activity is expressed in nmol of C_2H_4 per min per mg of protein. Strains: ORS571, wild type; 5751, *nrfA* mutant obtained by EMS mutagenesis; 57616, *nrfA* mutant obtained by insertion of a kanamycin cartridge; ORS571/pRK434; and 5751/pRK434. pRK434 contains the *E. coli hfq* gene.

TC and GTGTGGATCCAAACAGCCCGAAACCTTATTC were used to amplify a 0.4-kb fragment of the chromosomal DNA isolated from *E. coli* strain K-12 MG1655. This fragment includes the entire *hfq* gene and its proximal flanking sequences. Four derivatives of pRK415 containing an insert of correct size were introduced into strain 5751. Two of them complemented the mutation (shown in Fig. 3 for pRK434), whereas the two others did not. DNA sequence analysis of the four inserts revealed that the two plasmids complementing strain 5751 carried the wild-type *hfq* gene, whereas the two others contained a frameshift mutation caused by the insertion of a cytosine at position 872 of the published sequence of *hfq* (22). These results indicated that HF-I is functionally equivalent to NrfA for *nif* gene expression in *A. caulinodans*.

Complementation of an *A. caulinodans nrfA* Mutant by a Constitutively Expressed *nifA* Gene. If *nifA* were the only target of NrfA in the nitrogen-fixation process, a constitutively expressed *nifA* would complement the *nrfA* mutant. To test this hypothesis, we constructed plasmid pRS1022, in

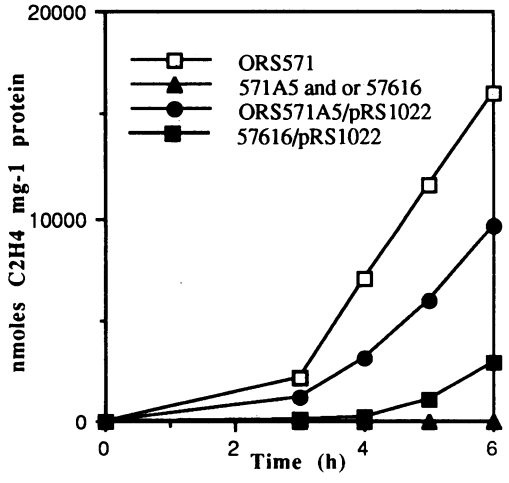

FIG. 4. Kinetics of nitrogenase derepression in the wild-type strain, *nrfA* mutant, and *nifA* mutant. Activity is expressed in nmol of C_2H_4 per min per mg of protein. Strains: ORS571, wild type; 571A51, *nifA* mutant; 57616, *nrfA* mutant obtained by insertion of a kanamycin cartridge; 571A5/pRS1022; and 57616/pRS1022. pRS1022 contains *nifA* expressed under the control of the promoter of the kanamycin-resistance gene from the vector.

which *nifA* was placed under the control of the promoter of the kanamycin-resistance gene of the vector. pRS1022 restored ≈30% of the nitrogenase activity compared with an *nifA* mutant containing the same plasmid (Fig. 4). This result indicates that *nifA* expression is regulated by NrfA. However, as the level of complementation was only 30%, it is possible that *nifA* is not the only target of NrfA for maximal expression of nitrogenase activity.

DISCUSSION

Previous work has shown that the regulation of *nifA* expression in *Azorhizobium* is more complex than in *Rhizobium* and *Bradyrhizobium* (4, 10). It is under the control of the Fnr-like activator FixK; two two-component regulatory systems, NtrBC and NtrXY, which respond to ammonia; and a third, FixLJ, which responds to oxygen. Single mutants of each pathway retain partial nitrogenase activity, whereas double mutants are strictly Nif^- Fix^-, suggesting that NtrBC/NtrYX and FixLJK are the primary determinants of *nifA* activation. However, *nifA* expression is independent of *rpoN* (4), and the σ factor required for transcription of *nifA* is unknown.

Analysis of the *A. caulinodans* mutant 5751 showed that the Nif^- phenotype of the mutant was due to a point mutation in a previously undescribed gene, called *nrfA*, that prevented expression of *nifA*. The requirement of an intact *nrfA* gene for nitrogen fixation was confirmed by the Nif^- phenotype, due to the lack of *nifA* expression, of a null mutant in which the gene was disrupted. This accounts for the pleiotropic phenotype of mutant 5751 in which 10 of 15 polypeptides synthesized under conditions of nitrogen fixation were not detectable. These polypeptides probably correspond to products of *nif* or *fix* genes previously described in *A. caulinodans* and that are either known or presumed to be regulated by the NifA protein (2, 23–26). The deduced amino acid sequence of *nrfA* exhibited a significant similarity with the *E. coli* HF-I protein, a factor required for Qβ RNA synthesis (27, 28). In addition, NrfA and HF-I are functionally equivalent; mutant 5751 was complemented by a plasmid carrying *hfq*, the structural gene for HF-I.

Protein HF-I is a hexamer of 12- to 13-kDa subunits that has been purified from Qβ-infected and uninfected *E. coli* (28). It is an RNA-binding protein specific for single-stranded RNA; it does not bind to single-stranded or double-stranded DNA or with double-stranded reovirus RNA (28). However, binding of HF-I is not limited to Qβ RNA since it also binds to *E. coli* rRNA and to RNA from R23 and f2 phages (28). HF-I is specifically required for *in vitro* synthesis of Qβ RNA by the Qβ replicase, in particular for the plus-strand-directed synthesis of the minus strand of the RNA (27). It is not required with any other template of the enzyme. Recently, it was suggested that the role of HF-I was to induce a conformational change in Qβ RNA that would favor the initiation of replication (29). In a recent report, it has been proposed that HF-I is one of the proteins essential for *E. coli* growth (30).

The lack of expression of β-galactosidase activity in strain 5751 carrying *nifA–lacZ* fusions and partial complementation of the strain by a constitutively expressed *nifA* gene reflect a defect in *nifA* transcription. However, if NrfA, like HF-I, binds only to single-stranded RNA, it is unlikely that NrfA directly interacts with the promoter region of *nifA*. By analogy with HF-I, NrfA may be active at the posttranscriptional level. With the *nifA–lacZ* fusions used, the 5′ extremities of the presumed mRNAs contain a fragment of *nifA* mRNA to which NrfA could bind. NrfA might be involved, for example, in the presentation of *nifA* mRNA to ribosomes in a form appropriate to translation. The predicted *nifA* mRNA contains four AUG initiation codons preceded by potential ribosome binding sites (5, 6). It is also possible that NrfA is required for translation of another mRNA whose product is required for *nifA* transcription. This might be, for example, the unknown σ factor.

The presence of NrfA in *A. caulinodans* prompted us to look for *hfq*-like genes in other nitrogen-fixing organisms. By Southern blot analysis, DNA fragments hybridizing with an *A. caulinodans nrfA* and with an *E. coli hfq* probe were detected in *R. meliloti*, *Bradyrhizobium japonicum*, and *Klebsiella pneumoniae* genomes (data not reported). It is therefore possible that NrfA (HF-I) homologues exist in other diazotrophs where perhaps mutations of the corresponding gene are pleiotropic or abolish essential functions. From this point of view, it is worth noting that no *E. coli* mutant in the *hfq* gene has been isolated so far. In addition, it is possible that NrfA controls genes other than NifA that may be required for normal growth in LSO medium but not in LSN medium.

The discovery of NrfA in *A. caulinodans* adds supplementary complexity to the regulation of *nifA* expression in this bacterium. Further work should lead to the identification of the target of NrfA and to the characterization of the mechanism of action of this class of regulatory proteins.

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