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Functional Analysis of the *fixNOQP* Region of *Azorhizobium caulinodans*

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The deduced amino acid sequences of four open reading frames identified upstream of the *fixGHI* region in *Azorhizobium caulinodans* are very similar to the putative terminal oxidase complex coded by the *fixNOQP* operons from *Rhizobium meliloti* and *Bradyrhizobium japonicum*. The expression of the *A. caulinodans fixNOQP* genes, which was maximal under microaerobiosis, was positively regulated by FixK and independent of NifA. In contrast to the Fix$^-$ phenotype of *B. japonicum* and *R. meliloti fixN* mutants, an *A. caulinodans fixNO*-deleted mutant strain retained 50% of the nitrogenase activity of the wild type in the symbiotic state. In addition, the nitrogenase activity was scarcely reduced under free-living conditions. Analysis of membrane fractions of *A. caulinodans* wild-type and mutant strains suggests that the *fixNOQP* region encodes two proteins with covalently bound hemes, tentatively assigned to *fixO* and *fixP*. Spectral analysis showed a large decrease in the *c*-type cytochrome content of the *fixN* mutant compared with the wild type. These results provide evidence for the involvement of FixNOQP proteins in a respiratory process. The partial impairment in nitrogen fixation of the *fixN* mutant in planta may be due to the activity of an alternative terminal oxidase compensating for the loss of the oxidase complex encoded by *fixNOQP*.

Azorhizobium caulinodans ORS571 is an unusual member of the family *Rhizobiaceae*. It is able to grow in the free-living state at the expense of dinitrogen and also to fix nitrogen symbiotically in the stem and root nodules of the tropical legume *Sesbania rostrata* (9–11). A model of the regulation of *nif* and *fix* gene expression in *A. caulinodans* has been proposed (20, 21, 35, 46). Transcription of *nifA* is controlled by both oxygen and ammonia via the *fixLJ*, *fixK*, and *ntrBC*/*ntrYX* gene products. The oxygen-regulatory pathway exhibits features common to those determined previously for *R. meliloti* (2, 7). In both cases, the two component regulatory proteins FixL and FixJ induce *fixK* transcription under oxygen-limiting conditions. However, the Fnr-like protein FixK in *A. caulinodans* positively controls *nifA*, whereas in *R. meliloti*, it is a negative regulator of *nifA* and a positive regulator of *fixN* (2, 21).

Pure cultures of *A. caulinodans* present maximal nitrogenase activity under a dissolved O_2 concentration of 10 μM (14), and the nitrogenase activity is much lower at oxygen levels below 1 μM. In contrast, it fixes nitrogen in root and stem nodules of *S. rostrata* when the O_2 concentration is in the range of 0.02 μM (3, 4). This large difference between the optimal oxygen concentrations for nitrogen fixation in the free-living and symbiotic states suggests that there are independent respiratory systems for the two conditions.

Free-living diazotrophs are able to fix dinitrogen under various oxygen tensions. For example, *Azotobacter vinelandii* fixes nitrogen in air, while *Klebsiella pneumoniae* is an anaerobic nitrogen-fixing organism. However, in both cases, a *d*-type terminal oxidase is involved in nitrogen fixation. This enzyme protects nitrogenase against O_2 denaturation in *A. vinelandii* (22) and is required for nitrogen fixation, in the absence of a fermentable carbon source, in *K. pneumoniae* at a 30 nM O_2 concentration (17, 41). Pure cultures of *A. caulinodans* display a branched respiratory system: an *o*-type terminal oxidase is most probably present in addition to the terminal oxidases *d* and aa_3 recently isolated (23).

Comparison of the cytochrome content of free-living cells and bacteroids of several *Rhizobiaceae* led to the hypothesis that different terminal oxidases were present in the two states. The *fixNOQP* operon, first isolated in *R. meliloti*, is a good candidate to participate in a symbiotic respiratory process (2, 18). Indeed, FixP is similar to soluble cytochromes, and the operon is required in *R. meliloti* for symbiotic nitrogen fixation (2, 18). The *fixNOQP* operon has also been identified in *Rhizobium leguminosarum* bv. viciae (39) and in *Bradyrhizobium japonicum* (36). Biochemical data obtained with *B. japonicum* suggested that the *fixNOQP* genes code for a terminal oxidase essential for symbiotic nitrogen fixation (36).

To determine if this respiratory chain was specifically involved in symbiotic nitrogen fixation, we characterized the *fixNOQP* operon of *A. caulinodans*. The cloning of the *fixNOQP-fixGHI* region of *A. caulinodans* and the characterization of *fixGHI* insertional Tn*5* mutants have been reported elsewhere (30).

(A preliminary account of the characterization of the *fixNOQP* genes was reported at the 9th International Congress on Nitrogen Fixation, Cancun, Mexico [29].)

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, nitrogenase, and β-galactosidase assays. The bacterial strains and plasmids used are listed in Table 1. The media and growth conditions for *A. caulinodans* and *Escherichia coli* were described previously (11). For *A. caulinodans*, the minimal medium is termed LSO when devoid of a fixed nitrogen source and LSN when supplemented with 20 mM ammonia.

Nitrogenase assays with *S. rostrata* root nodules were performed as previously reported (8). Nitrogenase assays with bacteria in the free-living state were performed as follows.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Source or reference
E. coli S17.1	*pro thi hsdR*; RP4 integrated into chromosome; *tet*::Mu *kan*::Tn*7* Tn*1*	40
A. caulinodans		
ORS571	Wild type	10
57601	*fixK* Nif$^-$ Fix$^-$ Km^r	21
ORS571A5	*nifA* Nif$^-$ Fix$^-$ Km^r	35
57605	Δ(*fixO'QP/fixG*) Nif$^{+/-}$ Fix$^{+/-}$ Km^r	30
57611	Δ(*fixNO*) Nif$^{+/-}$ Fix$^{+/-}$ Km^r	This work
57612	Δ(*fixNO*) Nif$^{+/-}$ Fix$^{+/-}$ Km^r	This work
Plasmids		
pLA29-17	RK2 replicon, Tc^r Km^r	1
pTZ18	Cloning vector, Amp^r	Pharmacia
pSUP202	ColE1, Mob$^+$ Tc^r Amp^r Cm^r	40
pUC4-K	Amp^r Km^r, source of Km^r cartridge	Pharmacia
pKOK5	Amp^r Km^r, pSUP202 derivative; source of *lacZ*-Km^r cartridge	24
pRS3000	19-kb fragment of ORS571 containing *fixNOQP* and *fixGHI* in pLA29-17, Tc^r	30
pRS3010	6-kb *Bgl*II fragment from pRS3000 in pSUP202, Amp^r Cm^r	This work
pTN1/pTN2	4.1-kb *Bam*HI-*Bgl*II fragment containing *fixNOQP* in pTZ18, Amp^r	This work
pRS3011	4.1-kb *Bam*HI-*Bgl*II fragment containing *fixNOQP* in pLA29-17, Tc^r	This work
pRS3012	pRS3011 derivative, *fixN-lacZ* fusion, Tc^r Km^r	This work
pRS3013	pRS3011/pKOK5 derivative, Tc^r Km^r	This work

Overnight cultures in LSN medium in air were centrifuged, washed with LSO medium, and resuspended in LSO medium at an optical density at 600 nm (OD_{600}) of 0.3. The cell suspension was incubated for 3 h at 34°C under a 3% O_2–97% Ar atmosphere, before injection of 10% acetylene. Acetylene reduction was then determined at hourly intervals.

The same protocol was used for β-galactosidase assays under conditions of nitrogen fixation. For β-galactosidase assays under conditions of ammonia assimilation, samples of an exponentially growing culture at an OD_{600} of 0.1 in LSN medium in air were incubated for 3 or 4 h under the desired oxygen tension (air or 3% O_2–97% Ar). β-Galactosidase activity was then measured. β-Galactosidase activity was determined by measuring hydrolysis of *o*-nitrophenyl-β-D-galactoside as described by Miller (31). Protein determinations were performed by the method of Bradford (5).

In vivo light difference spectra were performed with bacteria grown in LSN medium devoid of $FeCl_3$ and supplemented with 0.1% yeast extract.

Recombinant DNA techniques, construction of plasmids, and primer extension. Cloning, restriction mapping, transformation, plasmid isolation, Southern blotting, and hybridization were performed by standard protocols (37). Chromosomal DNA from *A. caulinodans* was isolated as described previously (8).

Plasmid pRS3010 carries a 6-kb *Bgl*II fragment purified from pRS3000 and subcloned at the *Bam*HI site of the vector pSUP202. pRS3011 carries a 4-kb *Bam*HI-*Bgl*II fragment purified from pRS3000 and subcloned at the *Bgl*II site of pLA29-17. To construct a transcriptional *lacZ* fusion to the *fixNOQP* promoter, a *lacZ*-Km^r cartridge purified from plasmid pKOK5 was inserted, in both orientations, at the unique *Xho*I site of pRS3011. *fixO* and *lacZ* were in same orientation in pRS3012 and in opposite orientations in pRS3013 (Fig. 1).

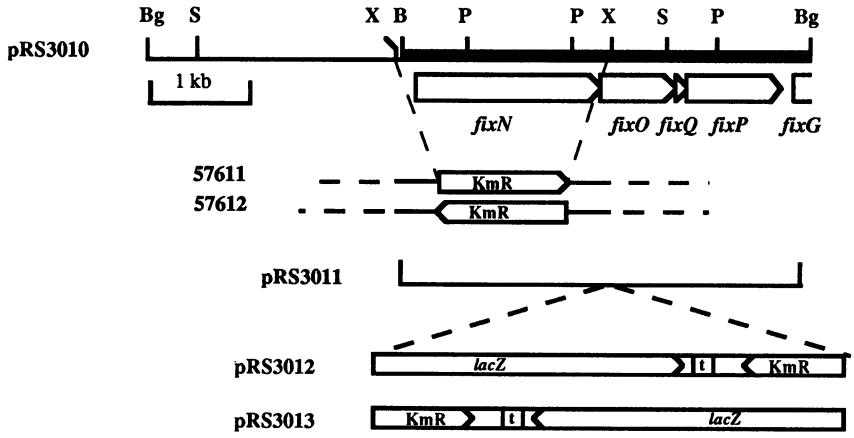

FIG. 1. Genetic and physical map of the *fixNOQP* region of *A. caulinodans*. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I. The bold line in pRS3010 indicates the region that was sequenced. The localization and orientation of the kanamycin resistance (KmR) cartridge used for insertion mutagenesis of the *fixN* operon in the genomes of mutants 57611 and 57612 are given below the map. Plasmids pRS3012 and pRS3013 are pLA29-17 derivatives containing the 4.1-kb *Bam*HI-*Bgl*II fragment of pRS3000 (not shown), in which the *lacZ*-Km^r cartridge of pKOK5 was inserted in both orientations.

RNA was isolated from *A. caulinodans* grown in LSO medium under 97% N_2–3% O_2 as reported previously (21). Primer extension experiments were performed as described before (21) with the following oligonucleotides: (i) GAAAA GAATCCCAGCGCTGCGAACACCAGG and (ii) CGTG TAGGCCTTGGCGCGGCCACGACAATC. The sequence of the oligonucleotide used for the primer extension of *fixK* was reported previously (21).

Plasmid and mutant construction. To construct *fixN* deletion mutants, the 2.1-kb *Xho*I fragment of pRS3010 was deleted and replaced with a *Sal*I fragment containing a kanamycin resistance cartridge purified from pUC4-K (Pharmacia). After transfer by transformation into S17-1 and by conjugation into *A. caulinodans*, recombinant clones were isolated as Km^r (kanamycin resistant) and Cm^s (chloramphenicol sensitive). Recombination at the correct location was checked by hybridization with specific DNA fragments from pRS3010 and with the cartridge as probes. Two strains with the cartridge inserted in opposite orientations, 57611 and 57612, were selected for further analysis (Fig. 1).

DNA sequence analysis. The 4.1-kb *Bam*HI-*Bgl*II fragment of pRS3000 was cloned into pTZ18 in both orientations to yield pTN1 and pTN2. Fragments were deleted by using the Cyclone I kit (IBI) and sequenced with the Taquence kit (USB) and the universal 24-mer reverse sequencing primer (New England Biolabs). Sequence data were compiled and analyzed with the DNA Strider program and by the program of Staden as modified by B. Caudron for the Data General Computer UNIX at the Service d'Informatique Scientifique, Institut Pasteur. Sequence data banks were screened for similarities with the BLAST program of the National Center for Biotechnology Information server.

Membrane preparations. One liter of N_2-fixing cells in the early stationary phase were harvested and washed once with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MgCl_2$ and 1 mM $MgSO_4$. Bacterial pellets were suspended in 10 ml of Tris-HCl buffer containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by two passages through a French pressure cell at 12,000 lb/in^2. DNase I and RNase I were each added to a final concentration of 10 μg/ml, and unbroken cells were removed by three centrifugations at 5,000 × *g* for 10 min. The supernatant was collected and centrifuged at 288,000 × *g* for 3 h. The sedimented membranes were suspended in Tris-HCl buffer (pH 7.5) containing PMSF.

Heme staining. Proteins were loaded in denaturing buffer by the method of Laemmli (26) and separated by polyacrylamide gel electrophoresis in 15%–sodium dodecyl sulfate (SDS) polyacrylamide gels. The resulting gels were stained for covalently bound heme with *o*-dianisidine (13) before being stained with Coomassie blue.

Visible-difference absorbance spectra. In vivo difference spectroscopy was performed with bacterial pellets obtained from stationary-phase cultures (OD_{600}, 3) and chilled on liquid nitrogen (77 K). Spectra were determined with a dual-wavelength scanning spectrophotometer (LERES) (25).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the EMBL data base under accession number X74410.

RESULTS

Identification of *fixNOQP* genes from *A. caulinodans*. A 19-kb DNA fragment of the *A. caulinodans* genome which exhibited similarity to the *fixNOQP* and *fixGHI* operons of *R. meliloti* was cloned previously (30). The region similar to *fixN* was localized in a 6-kb *Bgl*II fragment, which was subcloned into pSUP202 to yield pRS3010 (Fig. 1). The nucleotide sequence of a 4.1-kb *Bam*HI-*Bgl*II DNA fragment covering the *fixN* region (thick line in Fig. 1) was established on both strands. Four open reading frames (ORFs) of 1,656, 741, 156, and 881 bp and an incomplete ORF of 282 bp were identified. Each was preceded by a Shine-Dalgarno-like sequence 5 or 6 bp upstream of the putative ATG (or GTG for ORF4) translation start codon. The deduced amino acid sequences of ORF1, ORF2, ORF3, and ORF4 exhibited 76, 71, 43, and 58% similarity with *B. japonicum* FixN, FixO, FixQ, and FixP, respectively (36), and 64, 66, and 46% similarity with *R. meliloti* FixN, FixO and FixP, respectively (6). ORFs 1 to 4 were designated accordingly.

Alignments of the four polypeptides with those of *B. japonicum* are shown in Fig. 2. The translation product of the fifth ORF was identified previously (30) as a homolog of the N-terminal part of *R. meliloti* FixG (19) and *Rhodobacter capsulatus* RdxA (33). ORFs 1 to 4 were strictly contiguous, which suggested a single operon. A sequence very similar to the consensus sequence of the *E. coli fnr* binding site (TTGAC-N_4-ATCAA) was found at positions −77 to −90 of the initiation codon of ORF1. The presence of this anaerobox suggested FixK-dependent transcription of *fixN*. A total of 107 bp separated the putative start codon of *fixG* and the *fixP* translational stop codon.

Analysis of deduced amino acid sequences of *fixNOQP*. The *fixNOQP* genes encode polypeptides of 551 (61.8 kDa), 246 (27.6 kDa), 51 (5.9 kDa), and 292 (30.9 kDa) amino acids, respectively. The hydropathy profiles show that FixN, FixO, and FixQ have transmembrane segments and are probably membrane proteins (data not shown).

The hydropathy profile of the *fixN* gene product suggests 14 transmembrane segments characteristic of *c*-type and *o*-type terminal oxidase subunits 1 (COI) (for a review, see reference 38), a finding also reported for the *fixN* products in *R. meliloti* and *B. japonicum* (18, 36). Although the subunits 1 of bacterial and mitochondrial heme/copper terminal oxidases are not very similar, they have conserved motifs. In particular, four histidine residues were shown to bind cytochrome *a* and the bimetallic Fe-Cu center in the *o*-type terminal oxidase of *E. coli* (32). By alignment between *B. japonicum* FixN, CoxA, and CoxN, Preisig et al. (36) identified these four histidine residues in the *B. japonicum* FixN sequence. These residues, His-281, His-331, His-332, and His-419, are also conserved in *A. caulinodans* FixN and are boxed in Fig. 2A. In addition, one of the two conserved histidine residues (His-421), which serve as axial ligands for the low-spin heme *b* component of the *o*-type oxidase (27, 32), is conserved in both FixN polypeptides (Fig. 2A) (36).

FixO has an N-terminal membrane domain and a motif, CxyCH and MP residues, at positions 69 to 73 and 141 to 142, respectively (boxed in Fig. 2B), which could correspond to a covalent heme ligand, as proposed by Preisig et al. (36).

The translation product of *fixP* shown in Fig. 2D exhibits two motifs (indicated in boxes: $Gx_3Fx_3Cx_2CHx_nML$ and $Gx_3Fx_3Cx_2CHx_nMP$) that are extremely well conserved among soluble monoheme cytochromes $c_{552/553}$ from photosynthetic bacteria and eucaryotic algae (28, 45). This suggests that *fixP* probably results from a duplication of an ancestral cytochrome $c_{552/553}$ gene and that the gene product contains two covalently bound-heme cytochromes, a feature also observed in *R. meliloti* and *B. japonicum* FixP (18, 36).

Regulation of transcription of *fixNOQP*. The regulation of *fixNOQP* was assayed by monitoring the expression of a transcriptional *lacZ* fusion into the *fixO* coding sequence carried by pRS3012 (see Fig. 1B). Plasmid pRS3013 carries the

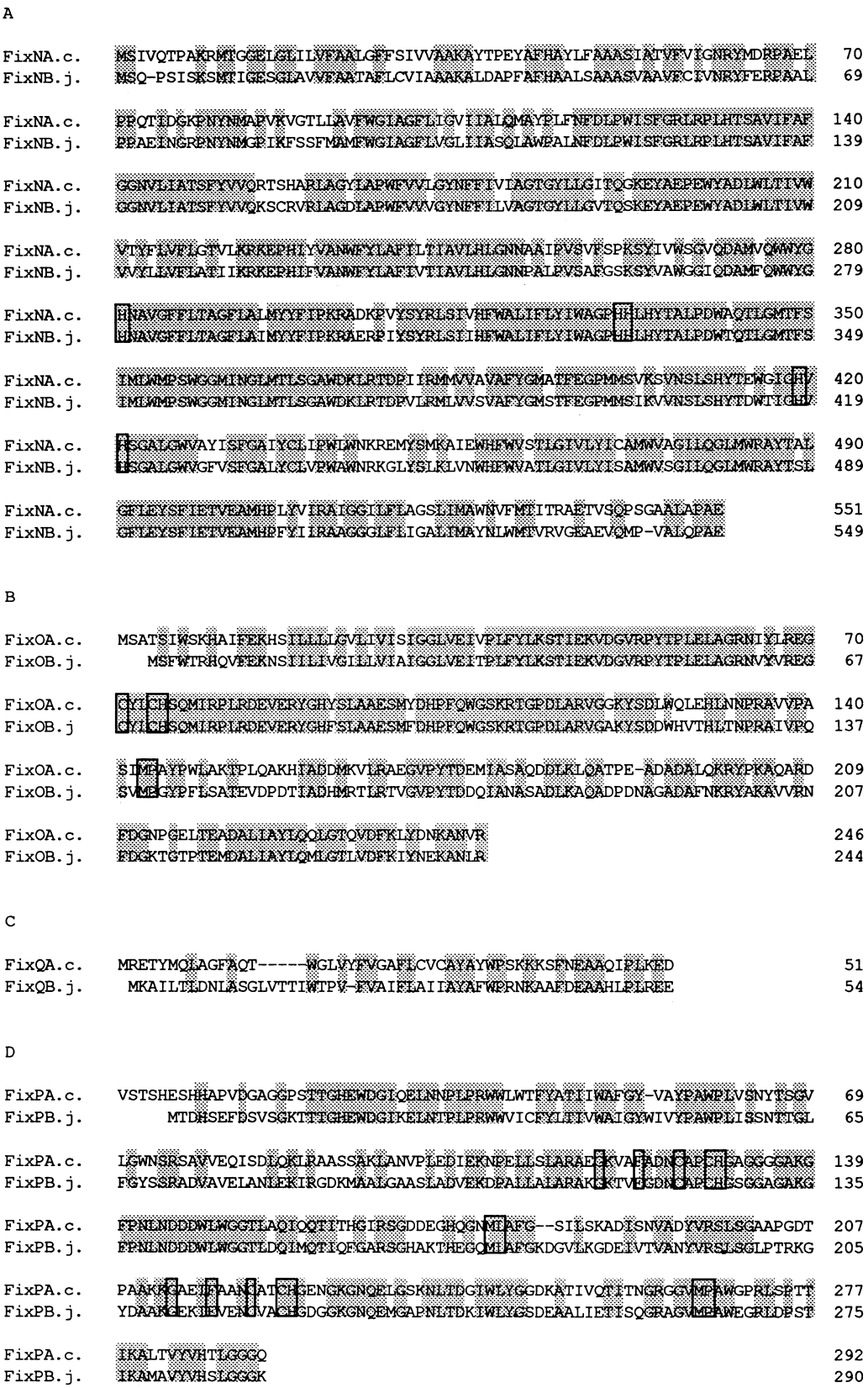

FIG. 2. Comparison of the amino acid sequences of *A. caulinodans* (A. c.) FixN (A), FixO (B), FixQ (C), and FixP (D) with the corresponding polypeptides of *B. japonicum* (B. j.). Identical amino acids are shaded. The conserved histidine residues of terminal oxidase subunit I present in FixN polypeptides and the putative ligand sites of hemes of *c*-type cytochrome in the FixO and FixP polypeptides are boxed.

TABLE 2. β-Galactosidase activity of *pfixN-lacZ* fusion in strain ORS571 and mutants[a]

Strain	β-Galactosidase sp act (U/min/mg of protein)	
	LSN–3% O_2	LSN–21% O_2
ORS571(pRS3012)	4,850 ± 300	1,700 ± 300
57601 (*fixK*)(pRS3012)	900 ± 150	900 ± 150
ORS571A5 (*nifA*)(pRS3012)	4,450 ± 250	1,400 ± 200
ORS571(pRS3013)	500 ± 50	500 ± 50

[a] β-Galactosidase activity is expressed in Miller units. Values are means of three independent assays.

same cartridge cloned in the opposite orientation and was used as a control for the background β-galactosidase activity of the cartridge. Table 2 reports the β-galactosidase activities conferred by pRS3012 on the wild-type, *fixK* (57601), and *nifA* (ORS571A5) strains in minimal medium containing ammonia (LSN). The level of expression of the fusion was very similar in the wild-type and *nifA* mutant strains. Maximal expression was observed under 3% O_2 in minimal medium irrespective of the presence or absence (data not shown) of ammonia. Expression was reduced by a factor of 3 under air. Expression of the fusion was much lower in the *fixK* mutant strain under all physiological conditions tested. However, in general, the plasmid-borne fusions displayed a rather high basal level of activity (e.g., pRS3013).

It was important to confirm that no transcription initiation was observed in a *fixK* mutant background. Primer extension experiments were performed with two different oligonucleotides (described in Materials and Methods) on RNA preparations from the wild type and from a *fixK* mutant strain (57601), either containing pRS3012 or not. The same results were obtained with both oligonucleotides. This enabled us to map the RNA start site 33 nucleotides downstream of the putative anaerobox when the experiment was performed with RNA from the wild-type strain (Fig. 3). The signal was more intense with preparations from ORS571(pRS3012). By contrast, no signal was detected with RNA extracted from the *fixK* mutant strain whether or not it contained pRS3012. It was verified with the same batches of RNA that a signal was detected in both the wild type and *fixK* mutant at the correct location (21) when the primer extension experiment was performed with an oligonucleotide corresponding to *fixK* (data not shown).

These data indicate that transcription of the *fixN* gene originated from a FixK-dependent promoter. NifA does not appear to be involved in the regulation of this promoter.

Involvement of *fixNOQP* in nitrogen fixation. To construct mutations in the *fixN* region, a 2.1-kb *Xho*I fragment of pRS3010 was deleted and replaced with a kanamycin resistance cartridge from pUC4K in both orientations. The deletion, which covers *fixN* and part of *fixO* and was expected to have polar effects on *fixQP*, was introduced into the ORS571 genome by homologous recombination to yield strains 57611 and 57612 (Fig. 1). The 2.1-kb *Xho*I fragment deleted was used as a hybridization probe to confirm that the fragment had been deleted from the mutant strains. This check also showed that there is no reiteration of the *fixN* region in the ORS571 genome. The nitrogenase activities in planta and ex planta of mutant strains 57611 and 57612 were assayed (Table 3). In the symbiotic state, both mutants displayed lower nitrogenase activity (40 to 50%) than the wild type. Under free-living nitrogen-fixing conditions, the nitrogenase activity of the *fixN* mutants was reproducibly only slightly lower than that of the wild type, although the difference was not statistically significant.

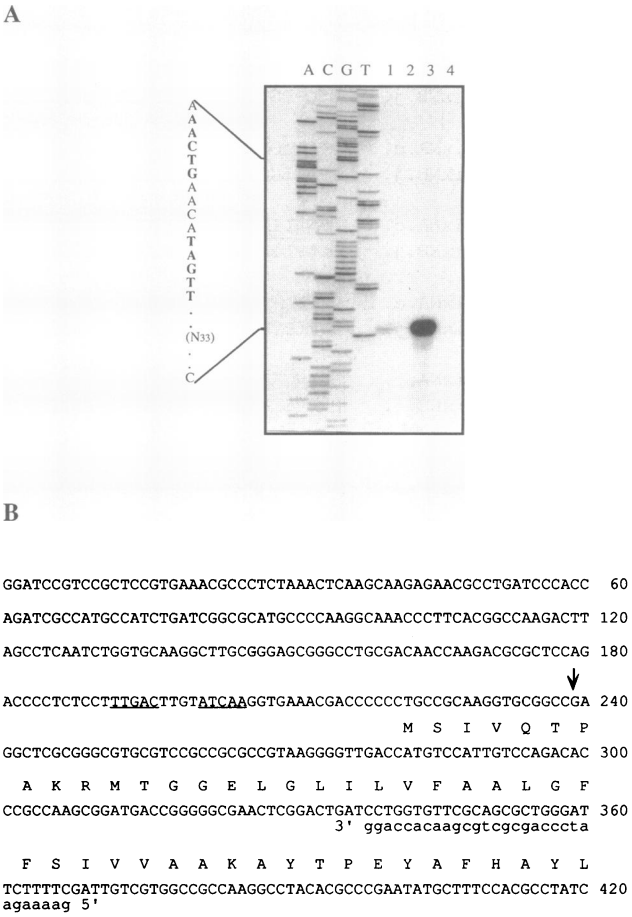

FIG. 3. Mapping of the *fixN* transcriptional start site. (A) Lanes 1 and 3, products of primer extension with RNA from ORS571 cultures containing plasmid pRS3012 (*pfixN-lacZ*) (lane 1) or not (lane 3) grown under nitrogen-fixing conditions. Lanes 2 and 4, RNA from 57601 (*fixK* mutant) containing plasmid pRS3012 (lane 2) or not (lane 4). Lanes ACGT part of the DNA sequence of *fixN* probed with the oligonucleotide indicated in lowercase letters in panel B. (B) Nucleotide sequence of the upstream region of *fixN*. The putative anaerobox (TTGAC-N_4-ATCAA) is underlined. The transcription start site is indicated by an arrow. Amino acids are shown above the DNA sequence.

Analysis of covalently bound heme proteins in ORS571 membranes. Total proteins of the membrane fractions of microaerobically grown cells of *A. caulinodans* wild-type, 57611 and 57612 (*fixN* deletions), 57601 (*fixK*-Km^r), and 57605 (from which the 3′ end of *fixO*, *fixQP*, and *fixGH* has been deleted) strains were separated by electrophoresis in SDS-polyacrylamide gels. Hemoproteins were identified by a heme-specific staining procedure, and the same gels were then stained with Coomassie blue. All mutant strains displayed similar heme-stained bands. A typical result obtained with the wild type and strain 57612 is shown in Fig. 4. Membrane preparations of the wild type contained one major heme-stained band of 34 kDa and two minor heme-stained bands of 32 and 25 kDa. The 32-kDa band was absent from the membrane fractions of strains 57612 (Fig. 4A), 57611, 57605, and 57601 (not shown), whereas the 25-kDa band was present and the 34-kDa band

TABLE 3. Nitrogenase activity comparisons

Strain	Mean nitrogenase activity (nmol of C_2H_4/min/mg) ± SD	
	Free-living[a]	Symbiosis[b]
ORS571	77 ± 20	0.53 ± 0.18
57611	57 ± 13	0.25 ± 0.08
57612	62 ± 11	0.22 ± 0.07

[a] Values are per milligram of protein and are the means of three independent assays.
[b] Values are per milligram (fresh weight) of nodule and are the means for eight plants.

was present but at a lower intensity than in the wild type. To account for the difference in intensity of the 34-kDa band, it is assumed that it was a doublet in the wild type. It thus appeared that all the mutant strains examined have lost a hemoprotein of 32 kDa and another one of 34 kDa. The synthesis of these hemoproteins is under the control of FixK, and they most probably correspond to polypeptides encoded by the *fixNOQP* region. From the nucleotide sequence data reported above, the two hemoproteins most likely correspond to FixO and FixP.

Reduced minus oxidized light absorbance spectra. Reduced minus oxidized spectra obtained for whole cells of wild-type ORS571, strain 57612 (*fixNO*), and strain 57601 (*fixK*) grown to the stationary phase (OD_{600}, 3; see Materials and Methods) are shown in Fig. 5. As covalently bound heme proteins are *c*-type cytochromes with an absorption peak at 550 nm, a decrease in the 550-nm peak in both the *fixN* deletion and *fixK* mutant strains was expected (Fig. 5). In addition, the 558-nm peak that in general corresponds to the *b*-type cytochrome associated with *d*-type terminal oxidase (for a review, see reference 15) was higher in the *fixN* mutant than in the wild type, although it is possible that this peak is simply more clearly apparent because of the decrease in the peak at 555 nm. Interestingly, the peak at 550 nm is lower in the *fixK* mutant than in the *fixN* mutant, although no difference was observed in the pattern of heme-stained membrane proteins.

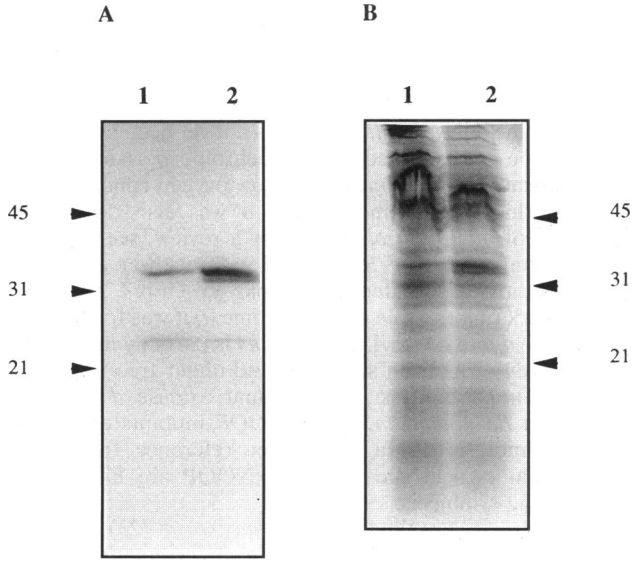

FIG. 4. (A) Heme stain of *A. caulinodans* membrane and soluble proteins from wild-type (lane 2) and mutant 57612 (*fixN*) (lane 1) strains separated by SDS-polyacrylamide gel electrophoresis. The positions of molecular size markers (Bio-Rad) are indicated by arrows in the left margin (in kilodaltons). Approximately 500 μg of total membrane protein was loaded for the two *A. caulinodans* strains. (B) Gel shown in panel A stained with Coomassie blue.

DISCUSSION

A DNA region containing the *fixNOQP* cluster of *A. caulinodans* was cloned by hybridization with *R. meliloti* probes (30). The cloned region is adjacent to *fixGHI* and is not linked to the *fixLJK* genes, and thus the organization is unlike that in *R. meliloti* and *B. japonicum* (30). The nucleotide sequence of a 4-kb fragment contains four ORFs which were assigned to *fixNOQP* and the beginning of a fifth ORF named *fixG* on the basis of their similarity with the corresponding genes of *R. meliloti* and *B. japonicum* (6, 19, 36). Probing total DNA from the *fixNO* deletion mutant strain for hybridization with the corresponding deleted DNA fragment showed that the *fixN* region is not reiterated. A motif resembling an anaerobox was found upstream of the *fixN* transcription start site of *A. caulinodans*, as in *R. meliloti* (2) and *B. japonicum* (36), but no such sequence was identified upstream of the *fixG* coding sequence (30). This was an indication that *fixN* was regulated by an Fnr homolog. The transcription start site of the *A. caulinodans fixN* was mapped 33 nucleotides downstream of the putative anaerobox, a distance in agreement with that found for Fnr-regulated genes in *E. coli* (44). Expression of the *fixNOQP* genes of *A. caulinodans* was maximal under microaerobiosis and independent of NifA but positively controlled by FixK (Table 2), a feature also reported for both *R. meliloti* and *B. japonicum* (2, 12).

Analysis of the hydrophobicity profiles suggests that the *fixNOQP* region encodes polypeptides anchored in the membrane, in agreement with conclusions for the corresponding *B. japonicum* polypeptides (36). Membrane protein analysis of wild-type and mutant strains revealed the presence of two covalently bound heme polypeptides of 32 and 34 kDa, which can be tentatively assigned to FixO and FixP for the following reasons. (i) The sizes of the FixN and FixQ polypeptides do not match that of either the 32- or 34-kDa protein, and the FixN sequence is similar to that of the heme/copper terminal oxydase subunit I and cannot correspond to a *c*-type cytochrome. (ii) FixO and FixP contain a consensus heme-binding site, and their molecular masses (28 kDa plus one heme and 30.9 kDa plus two hemes, respectively) are close to those of the polypeptides identified. (iii) The two polypeptides are absent in the *fixN* deletion strain, which shows that their synthesis is under the control of the *fixN* promoter. (iv) The two polypeptides are absent in the *fixK* mutant, and it was found that FixK controlled transcription from the *fixN* promoter. (v) The two polypeptides were also absent in the deletion strain 57605, which covers the C-terminal part of FixO, FixQ, and FixP. Moreover, the disappearance of the two polypeptides correlated with a decrease in the cytochrome *c* content both in the *fixNO* deletion and in the *fixK* mutants. These results are in agreement with recent biochemical analysis of the FixNOQP proteins of *B. japonicum*, which concluded that the *fixNOQP* genes might code for a new type of heme/copper terminal oxidase composed of a *b*/copper-binding subunit encoded by *fixN* and cytochromes *c* encoded by *fixO* and *fixP* (36). The electron donor for the FixNOQP terminal oxidase of *B. japonicum* is most probably the bc_1 complex (16, 36, 47). A similar electron transport pathway might exist in *A. caulinodans*.

In vivo difference spectra analysis showed a *c*-type cytochrome peak at 550 nm in strain 57601 (*fixK*) lower than that

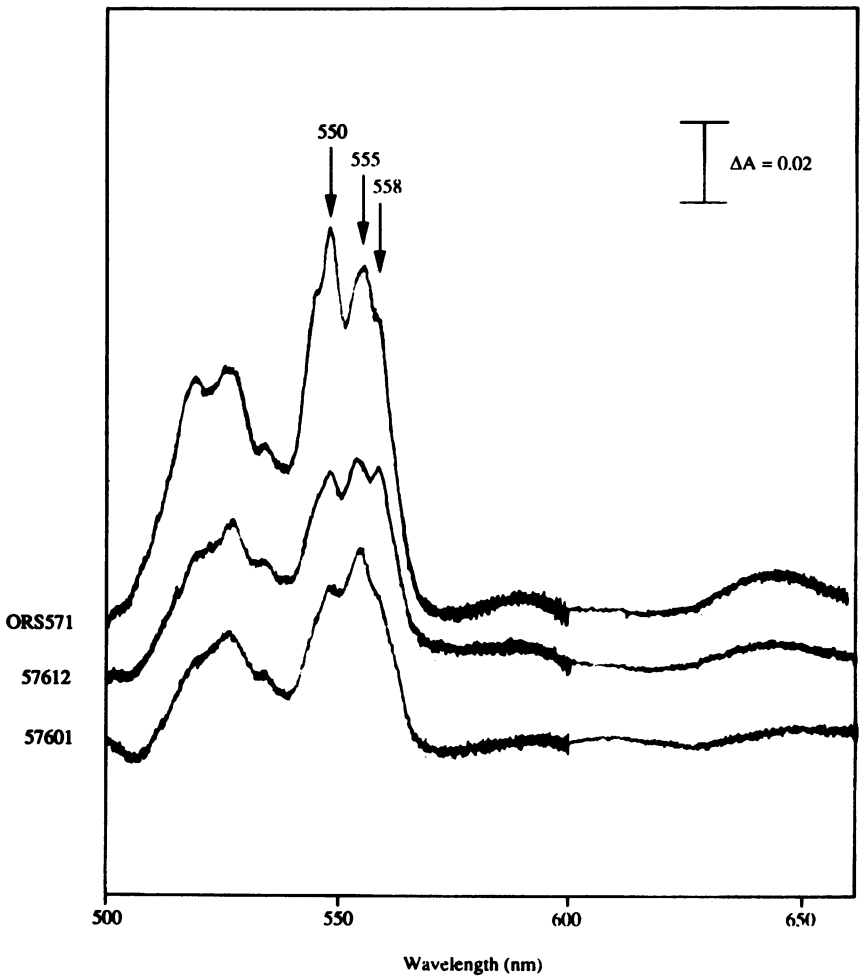

FIG. 5. In vivo difference (reduced minus oxidized) spectra recorded at 77 K for ORS571 (wild type), 57612 (*fixN*), and 57601 (*fixK*). Arrows indicate peaks at 550, 555, and 558 nm discussed in the text.

in the *fixN* mutant. Thus, FixK might be involved in regulation of the synthesis of covalently bound heme proteins other than FixO and FixP, which remain to be identified.

The nitrogenase activity of the *fixNO* deletion strain was comparable to that reported previously for *fixG* and *fixI* mutants (30). The growth rates of free-living wild-type and mutant strains and of strains grown under microaerobiosis with ammonia as the nitrogen source were the same (data not shown). Thus, the slight reduction in nitrogenase activity measured in *fixNO* deletion mutants ex planta does not correlate with a defect in microaerobic growth and might not be significant. In contrast to *R. meliloti* and *B. japonicum*, the *fixNO* deletion strain retained a high nitrogenase activity in planta, suggesting that this region is not essential for nitrogen fixation. However, the decrease in nitrogenase activity, to about 50% of that of the wild type, indicates that this region is required for full nitrogenase activity in the symbiotic state. This also implies the existence of alternative pathways for respiration during symbiosis. In pure culture, *A. caulinodans* exhibits a branched respiratory system which contains different terminal oxidases (cytochromes aa_3, *o*, and *d*) (23) in addition to the putative oxidase coded by *fixNOQP*. Among those, the aa_3-type terminal oxidase was not found to be required for the respiration process either in planta or ex planta (23). The role of cytochrome *o* in *A. caulinodans* has not yet been established. It should be remembered that cytochrome *o* is involved in nitrogen fixation by *A. vinelandii* at low oxygen concentrations (22) and that cytochrome oxidase *o* was also detected in bacteroids of other *Rhizobiaceae* (for a review, see reference 34). A mutant strain of *R. phaseoli* with a lower content of cytochrome *o* was isolated and displayed a Fix^- phenotype (42). Considering the role of cytochrome *d*, it was found that a cytochrome *d* mutant of *A. caulinodans* is partially impaired in nitrogen fixation both in symbiosis and under free-living conditions (23). By contrast, no terminal oxidase *d* has been detected in *B. japonicum*, and *fixNOQP* mutants are strictly Fix^- in symbiosis (for a review, see reference 16). It thus appears that cytochrome *d* and FixNOQP are likely to be active during symbiosis.

In conclusion, terminal oxidases aa_3, *o*, *d* (23), and FixNOQP have now been identified in *A. caulinodans*. Cytochrome aa_3 is probably mainly involved in aerobic respiration (34) and possibly in young nodules, as suggested for *R. phaseoli* (42, 43). Microaerobic respiration pathways are different under free-living and symbiotic conditions. During symbiosis, the two terminal oxidases cytochrome *d* and FixNOQP may have the same role. Under free-living conditions, the FixNOQP oxidase, whose synthesis is derepressed, may nevertheless be active in

the wild type, but its loss in the mutant strains might be compensated for by the presence of the other terminal oxidases, *o* or aa_3, in addition to the terminal oxidase *d*.

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