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Characterization of Two *Azospirillum brasilense* Sp7 Plasmid Genes Homologous to *Rhizobium meliloti nodPQ*

Claire Vieille and Claudine Elmerich

Unité de Physiologie Cellulaire, CNRS URA 1300, Département des Biotechnologies, Institut Pasteur, 75724 Paris Cedex 15, France.
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Bacteria belonging to the *Azospirillum* genus are nitrogen fixers that colonize the roots of grasses, but do not cause the formation of differentiated structures. Sequences from total DNA of several *Azospirillum* strains are homologous to restriction fragments containing *Rhizobium meliloti* nodulation genes. A 10-kilobase (kb) *Eco*RI fragment from *A. brasilense* Sp7, sharing homology with a 6.8-kb *Eco*RI fragment carrying *nodGEFH* and part of *nodP* of *R. meliloti* 41, was cloned in pUC18 to yield pAB502. The nucleotide sequence of a 3.5-kb *Eco*RI-*Sma*I fragment of the pAB502 insert revealed 60% homology with *R. meliloti nodP* and *nodQ* genes. The *nodP* gene product shares no homology to any known protein sequence. The *Azospirillum nodQ* gene product shares homology with a family of initiation and elongation factors as does the *R. meliloti nodQ* gene product. Since the *nodQ* gene overlaps the *nodP* gene, the two genes might be cotranscribed. *Azospirillum* contains large plasmids, and the *nodPQ* genes were found on the 90-MDa plasmid (p90). A translational *nodP-lacZ* fusion was constructed in the broad host range plasmid pGD926. No β-galactosidase activity was detected in *Escherichia coli*, but the fusion was functional in *Azospirillum* and constitutively expressed. Deletions and mutations of *nodPQ* did not modify growth, nitrogen fixation, or interaction with wheat seedlings.

Additional keywords: plasmid localization of *Azospirillum* genes.

Bacteria of the genus *Azospirillum* are free-living diazotrophs, which associate with the roots of grasses. Five species have been identified in this genus, which has been defined by Tarrand *et al.* (1978): *A. brasilense, A. lipoferum, A. amazonense, A. halopraeferens*, and *A. irakense* (reviewed by Döbereiner and Pedrosa 1987; Khammas *et al.* 1989). The bacteria are gram-negative, heterotrophic, aerobic, curved rods, and they possess a genome of high G+C content (66–71 mol percent). Nitrogen fixation occurs only under microaerobic conditions.

The bacteria have been isolated from the roots of a large number of Gramineae. Plants do not form differentiated structures when *Azospirillum* are present, but it appears that the bacteria can colonize the root cortex. They are commonly observed in the intercellular spaces of the cortex (Umali-Garcia *et al.* 1978). The invasion of these intercellular spaces could be an active process, as suggested by the presence of pectinolytic activity in pure *Azospirillum* culture (Tien *et al.* 1981). After inoculation with *Azospirillum*, the host plant root system proliferates, and the number of lateral roots and root hairs increases (Umali-Garcia *et al.* 1980). This effect, concomitant with an enhanced uptake of minerals and an increase in dry matter (reviewed by Okon 1985), was attributed to phytohormone production (Tien *et al.* 1979). Patriquin *et al.* (1983) described root hair deformations in wheat seedlings. Among them is the "tuning-fork" deformation that appeared only in the presence of *Azospirillum*.

Whatever the mechanism involved, the interaction of the bacteria with its host plant is presumably mediated by bacterial genes. It is becoming apparent that pathogens, symbionts, *Azospirillum*, and other plant-growth-promoting bacteria share similarities with regard to recognition and affinity phenomena, hormone production, pectinolytic activity, and root morphological modifications. In particular, there are similarities between the root hair deformations caused by *Azospirillum* in grasses and *Rhizobium* in legumes. These observations and the report that *Azospirillum* can stimulate nodulation of *R. trifolii* on clover (Plazinski and Rolfe 1985) led us to assume that some of the bacterial genes involved in such interactions might be similar.

In *R. meliloti*, the genes necessary for nodule organogenesis and host specificity are carried by megaplasmids. Twelve genes have been identified, clustered not far from *nifHDK* and *fixABC* on the symbiotic plasmid (pSym) (reviewed by Long 1989). These include *nodDABCIJ*, referred to as the common nodulation (*nod*) genes (Kondorosi *et al.* 1985), though *nodD* is now described as a host-specific activator of the *nod* operons (Spaink *et al.* 1987), and *nodQPGEFH*, referred to as the host-specific nodulation (*hsn*) genes (Kondorosi *et al.* 1985; Cervantes *et al.* 1989; Schwedock and Long 1989).

A 6.8-kilobase (kb) *Eco*RI fragment containing *nodGEFH* of *R. meliloti* 41 (Kondorosi *et al.* 1985; Horvath *et al.* 1986), also called the *hsn* probe, hybridized with several fragments of *A. brasilense* Sp7 total DNA on Southern blots (Fogher *et al.* 1985). Two of the hybridizing fragments, a 10-kb *Eco*RI fragment and a 4-kb *Sal*I fragment, were cloned in pUC18 to yield pAB502 and pAB503,

Address correspondence to C. Elmerich: Unité de Physiologie Cellulaire, CNRS URA 1300, Département des Biotechnologies, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France.

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respectively (Elmerich *et al.* 1987; Galimand *et al.* 1988; Vieille *et al.* 1989). In this study, we report the analysis of pAB502 and the nucleotide sequence of the region homologous to the *hsn* probe, which revealed the presence of two open reading frames (ORFs) corresponding to *nodP* and *nodQ*. The localization of the two genes in the *Azospirillum* genome as well as the activity of a *nodP-lacZ* fusion and the phenotype of *nodPQ* mutants are also reported.

MATERIALS AND METHODS

Strains, bacteriophages, and plasmids. *A. brasilense* wild type was Sp7 (ATCC 29145) (Tarrand *et al.* 1978). *Escherichia coli* strains that were used are as follows: TG1 [Δ(*lac-pro*), *supE, thi, hsdD5*/F′ *traD36, lacI*^{q}, Δ*lacZM15*] (Wain-Hobson *et al.* 1985); S17.1 [*pro, thi, recA* (RP4-2 Tc-Mu Km-Tn*7* Tra$^+$ IncP1)] (Simon *et al.* 1983); and MC1061 [*hsdR2, hsdM*$^+$, *hsdS*$^+$ *araD139,* Δ(*ara-leu*)$_{7697}$, Δ(*lac*)$_{X74}$, *galE15, galK16, rpsL,* (Sm^r), *mcrA, mcrB1*] (Casadaban and Cohen 1980). *Klebsiella pneumoniae* UNF5023 [*hisD2, hsdR1, rpsL4*] (Dixon *et al.* 1977) was used. *Agrobacterium tumefaciens* GMI9023, devoid of pTi and pAt (Rosenberg and Huguet 1984), and *R. meliloti* 2011 (see Cervantes *et al.* 1989) were used. Bacteriophages and plasmids are listed in Table 1 and schematized in Figure 1.

Table 1. Bacteriophages and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Phages		
M13mp9	Derivative of M13	Messing 1983
Plasmids		
pUC18	Ap^r, Lac$^+$	Yanisch-Perron *et al.* 1985
pTZ19R	Ap^r, pUC derivative, F1 replication origin	Pharmacia[b]
pBluescript II KS+ (pBS+)	Ap^r, pUC derivative, F1 replication origin	Stratagene[c]
pBluescript II KS− (pBS−)	Ap^r, pUC derivative, F1 replication origin	Stratagene
pSUP202	Ap^r, Cm^r, Tc^r, Mob, ColE1 replicon	Simon *et al.* 1983
pGD926	IncP, Tc^r, pRK290 derivative, *lacZ* translational fusion vector	Ditta *et al.* 1985
p*kan-lacZ*	Tc^r, RK2 replicon, *kan-lacZ* transcriptional fusion	F. Biville[d]
pEK484	Tc^r, 6.8-kb *hsn* region of *Rhizobium meliloti* 41 cloned in the pACYC184 *Eco*RI site	E. Kondorosi[e]
pHB286	Ap^r, Cm^r, *nod* and *hsn* regions of *R. meliloti* 2011 in pBR328	Batut *et al.* 1985
pMC71A	Cm^r, *Klebsiella pneumoniae nifA* cloned in pACYC184 *Sal*I site	Buchanan-Wollaston *et al.* 1981
pAB502	10-kb *Eco*RI fragment of *Azospirillum brasilense* Sp7 containing *nodPQ*, in pUC18	Elmerich *et al.* 1987

[a] Ap^r, Cm^r, and Tc^r indicate resistance to ampicillin, chloramphenicol, and tetracycline, respectively; kb, kilobase.
[b] Bois d'Arcy, France.
[c] La Jolla, CA.
[d] Institut Pasteur, Paris, France.
[e] Institut des Sciences Végétales (ISV), Gif-sur-Yvette, France.

Media and growth conditions. *Azospirillum* cultures were grown in nutrient broth (Gauthier and Elmerich 1977) or in minimal K medium (Franche and Elmerich 1981; Galimand *et al.* 1989) at 30° C. *E. coli, K. pneumoniae, A. tumefaciens*, and *R. meliloti* strains were grown in Luria-Bertani (LB) medium (Maniatis *et al.* 1982) at 37° C or 30° C. *K. pneumoniae* cultures in nitrogen-free medium (NFM) were incubated at 30° C as previously described (Elmerich *et al.* 1978). Bacteriophage M13 was propagated in *E. coli* TG1 as described by Messing (1983). Antibiotics were used at the following final concentrations (μg/ml): ampicillin, 100; kanamycin (Km), 20; streptomycin (Sm), 250; chloramphenicol, 50; and tetracycline, 5.

Plasmid and phage construction. Transformation, DNA isolation, restriction analysis, and DNA hybridization were performed by conventional techniques (Maniatis *et al.* 1982). Plasmid pAB502 (Fig. 1) contains a 10-kb *Eco*RI fragment cloned in pUC18 (Elmerich *et al.* 1987). Plasmids and phages shown in Figure 1 are pAB502 derivatives constructed by deletion or subcloning using the *Pst*I, *Sal*I, or *Sma*I sites of the pUC18 polylinker as follows: plasmid pAB508 contains a 4.1-kb *Pst*I fragment from pAB502 cloned in pUC18; pAB512 corresponds to a *Sal*I-*Xho*I deletion of pAB508; pAB518 was obtained by cloning a 2.6-kb *Sal*I-*Sma*I fragment of pAB508 in pTZ19R; phages G3 and G6 contain a 1.5-kb *Sma*I fragment from pAB512 cloned in M13mp9, in opposite orientations; pAB524 and pAB525 were obtained by subcloning a 0.9-kb *Sal*I fragment of pAB508 in pBS+ and pBS−, respectively; to obtain pAB519, a 1.5-kb *Nar*I fragment purified from pAB518 was ligated to the *Cla*I site of pBS−; and pAB520 contains a 1.45-kb *Bam*HI-*Xho*I fragment of pAB508 subcloned in pBS− (Fig. 1).

A *nodP-lacZ* fusion was constructed in the plasmid vector pGD926 (Ditta *et al.* 1985). A *Hin*dIII-*Bam*HI fragment of pAB512, which carries *nodP* with a truncated 3′ end and a 1.3-kb upstream region, was subcloned in pGD926 giving pAB527 (Fig. 2). The construction contains an in-frame *nodP-lacZ* translational fusion. Plasmid pAB527 was kept in *E. coli* S17.1 and introduced in *Azospirillum* by conjugation as described by Simon *et al.* (1983).

***nodPQ* mutant construction.** Sp7 derivatives containing mutations in *nodPQ* were isolated after the introduction of mutations carried by plasmids pAB535 (mutant strain 7801), pAB536 (7802), and pAB537 (7803) into the Sp7 genome by homogenotization. Plasmid pAB535 contained a Km cartridge inserted at the unique *Xho*I site of pAB502 (Fig. 3). To construct pAB536, three Tn*5*-Mob insertions in pAB502 were isolated as previously described (Galimand *et al.* 1988) and named pAB502-Tn*5*-Mob2, pAB502-Tn*5*-Mob8, and pAB502-Tn*5*-Mob31 (Fig. 3). Plasmid pAB536 was derived from pAB502-Tn*5*-Mob2 by *Xho*I deletion, which removed most of *nodQ* and the 3′-terminal part of *nodP*. Plasmid pAB537 contained a Km cartridge inserted into pAB502 with a deletion of the two internal *Sal*I fragments (Fig. 3). This deletion covered half of *nodQ, nodP*, and 4.4 kb upstream of *nodP*. The pAB535, pAB536, and pAB537 inserts were subcloned in pSUP202 and then recombined in Sp7 to yield strains 7801, 7802, and 7803, respectively. Tn*5*-Mob insertions 8 and 31 were recombined in an Sm^r derivative of Sp7 to yield strains 7808 and 7831,

respectively. Hybridization analysis with a pAB502 insert probe verified that the mutations had recombined in the Sp7 genome at the desired location (data not shown).

DNA sequencing. Two techniques were used to obtain sequential deletions of the regions to be sequenced. For the 1.5-kb *Xho*I-*Sma*I fragment cloned in M13mp9 (clones G3 and G6), sequential deletions were created using the cyclone biosystem (International Biotechnologies, Inc., New Haven, CT). For the fragments cloned in pBS+ and pBS− (pAB519, pAB520, pAB524, and pAB525), the deletions were created by the exonuclease III digestion procedure of Henikoff (1984). Sequences were determined by the dideoxy chain termination technique of Sanger *et al.* (1977). The sequencing strategy is shown in Figure 1. The data were ordered with the computer program devised by Staden (1983).

Preparation of root exudates. Wheat seeds (variety Fromentin) were surface sterilized under vacuum by treat-

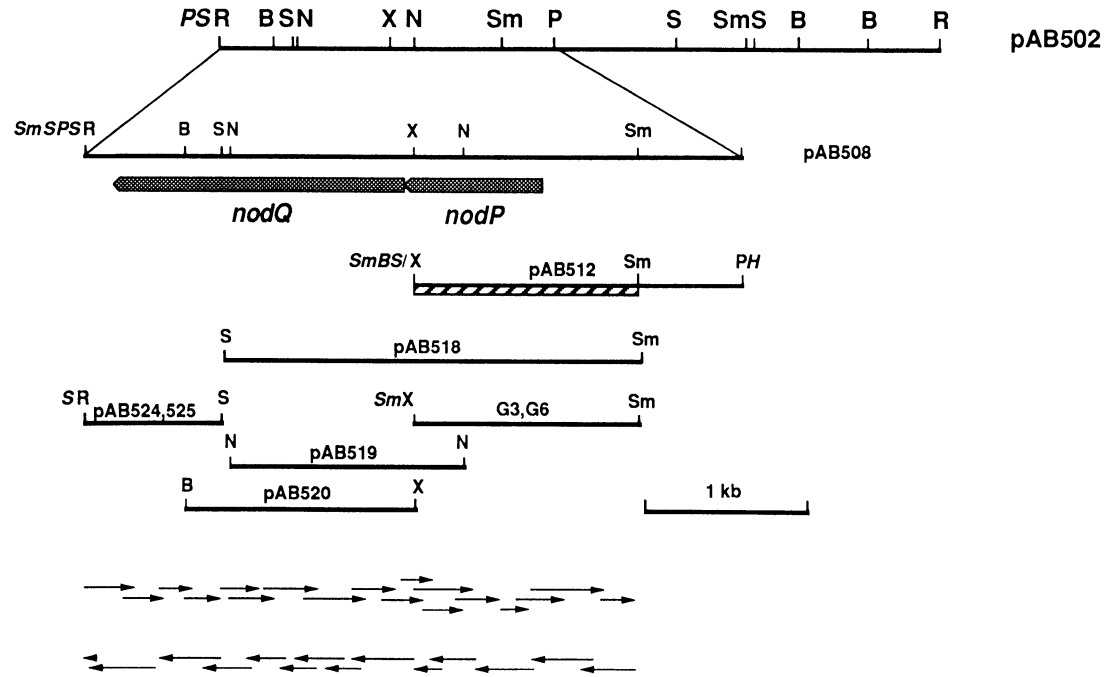

Fig. 1. Physical and genetic maps of pAB502, construction of the plasmids used in this study and sequencing strategy. Restriction sites: B, *Bam*HI; H, *Hin*dIII; N, *Nar*I (only two sites shown); P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; and X, *Xho*I. Plasmid vectors used in the constructions are not shown (see text); vector cloning sites used are indicated in italics. Crosshatched arrows indicate *nodPQ* open reading frames and the direction of transcription. The hatched box represents the fragment used as a probe for the hybridization experiments shown in Figure 8. Lower arrows indicate sequencing strategy.

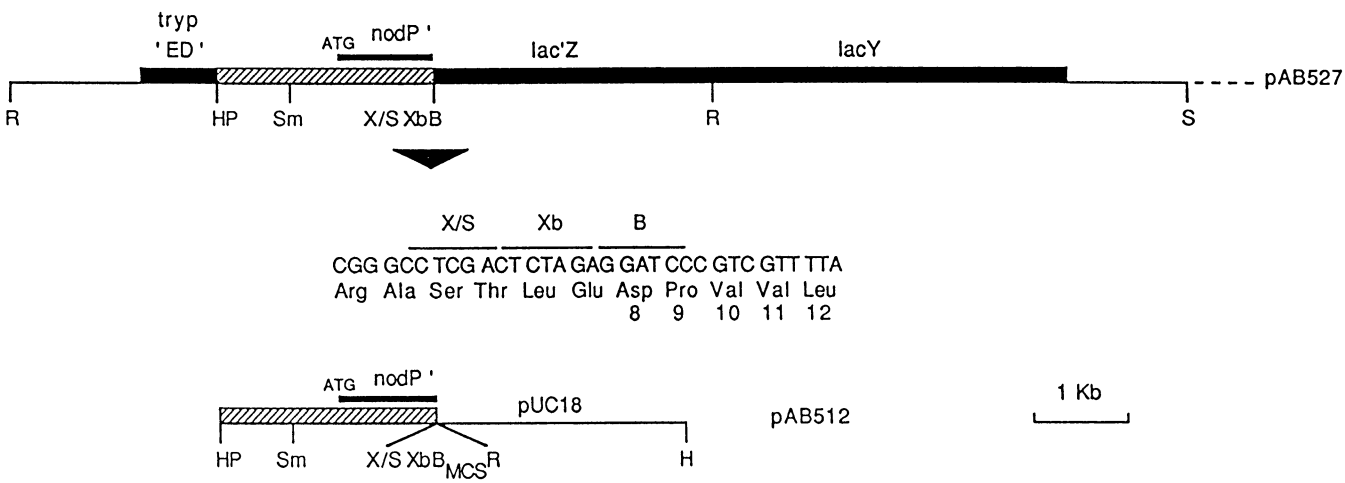

Fig. 2. Contruction of the *nodP-lacZ* translational fusion. Restriction sites are as given in Figure 1, with Xb denoting *Xba*I and MCS indicating the multiple cloning site of pUC18. The hatched boxes represent *Azospirillum brasilense* DNA cloned in pAB512 and pAB527. lac'Z denotes the *Escherichia coli* β-galactosidase gene without the promoter and lacking the first seven codons; lacY, *E. coli* lactose permease gene; tryp'ED', last 530 base pairs (bp) of *E. coli trypE* and the first 270 bp of *trypD* (Ditta *et al.* 1985); and nodP', first 823 bp of *A. brasilense nodP*. The translational fusion between the 275th codon (TCG) of *nodP* and the eighth codon (GAT) of *lacZ* was mediated by three codons of the multiple cloning site of pUC18.

ment for 10 min with 0.1% $HgCl_2$ dissolved in 0.05 N HCl, followed by treatment with 70% ethanol for 10 min and washing in five changes of sterile distilled water, according to Neuer *et al.* (1985). Seeds were kept overnight at 4° C in sterile water. They were then placed on sterile wet filter papers in petri dishes and allowed to germinate for 3 days in the dark at 30° C. Exudates were collected and filtered using a sterile Millipore filter (pore size, 0.22 μm).

Nitrogenase, β-galactosidase, and indoleacetic acid assays. *Azospirillum* nitrogenase activity was assayed under derepressing conditions according to Gauthier and Elmerich (1977). Growth conditions and media used for β-galactosidase assays with *Azospirillum* are described in Table 2. *K. pneumoniae* β-galactosidase activity was assayed after growth in NFM under anaerobic conditions. β-Galactosidase activity in other species was assayed after growth in LB medium. The assays were performed at 28° C, as described by Miller (1972), using 0.5 ml of culture grown under air or 1 ml of culture grown under low oxygen tension (see Table 2). Indoleacetic acid production by *Azospirillum* was determined with the Salkowski reagent (Tang and Bonner 1946) after growth in minimal K medium in the presence or absence of 100 μg/ml of tryptophan.

RESULTS

Homology between pAB502 and the *R. meliloti hsn* region. The physical map of the insert carried by pAB502 is shown in Figure 1. A 1.5-kb *Xho*I-*Sma*I fragment of this insert shares homology with the *hsn* probe of *R. meliloti* 41 (pEK484) (Fig. 1, bacteriophages G3 and G6) (Elmerich *et al.* 1987). The *Xho*I-*Sma*I fragment was used in turn as a probe against restriction fragments of pEK484. Hybridization tests revealed a 1.5-kb *Eco*RI-*Sal*I fragment (data not shown) containing most of *nodG* and 1 kb downstream of *nodG* (Horvath *et al.* 1986). The pAB502 insert was also used as a probe against restriction digests of pHB286, which carries the *hsn-nod* region of *R. meliloti* 2011. The probe hybridized to a 14.5-kb *Hin*dIII-*Eco*RI fragment containing *nodG* and the adjacent 2.2-kb *Eco*RI

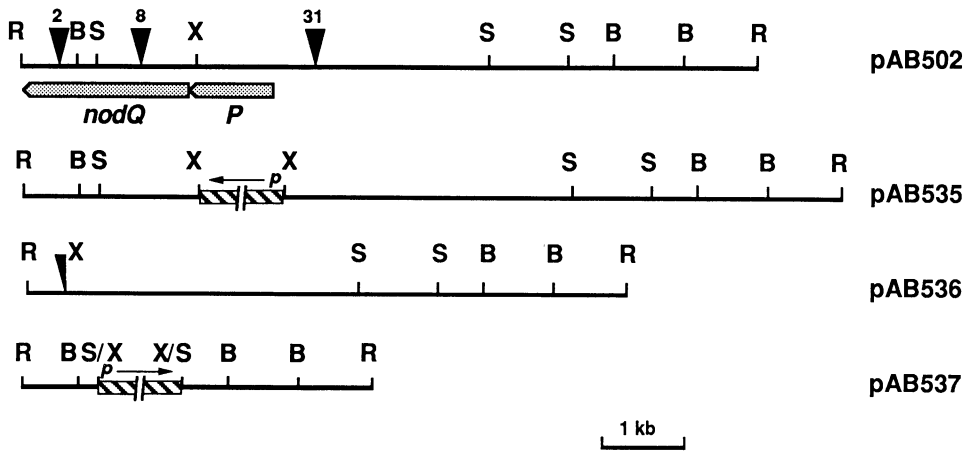

Fig. 3. Construction of *nodP* and *nodQ* mutants. Restriction sites are as given in Figure 1. The arrows indicate the direction of transcription. Arrowheads indicate Tn*5*-Mob insertions; hatched boxes, kanamycin cartridge; and *p*, *kan* promoter.

Table 2. β-Galactosidase activity under different growth conditions

Strain	Medium	Gas phase	Activity[a]	Standard deviation[b]
Sp7	All conditions tested with Sp7(pAB527)		< 1.1	ND
Sp7(pGD926)	All conditions tested with Sp7(pAB527)		< 1.1	ND
Sp7(pLA*kan-lacZ*)	K, NH_4^+ 20 mM	Air	46	ND
Sp7(pAB527)	Nutrient broth	Air	38	±2
	K, NH_4^+ 20 mM	Air	59	±9
	K, glutamate 5 mM	Air	82	ND
	K	N_2 + 1.4% O_2[c]	44	±10
	K, glutamate 5 mM	N_2 + 1.4% O_2[c]	68	±7
	K, NH_4^+ 20 mM	N_2 + 1.4% O_2	45	±8
	50% K, NH_4^+ 10 mM,	Air		
	+ 0% exudates		48	ND
	+ 10% exudates		47	ND
	+ 50% exudates		47	ND
7801(pAB527)	K, NH_4^+ 20 mM	Air	24	±11
7802(pAB527)	K, NH_4^+ 20 mM	Air	63	±1
7803(pAB527)	K, NH_4^+ 20 mM	Air	52	±5
7808(pAB527)	K, NH_4^+ 20 mM	Air	35	±12
7831(pAB527)	K, NH_4^+ 20 mM	Air	50	±13

[a] The number of nmoles of *o*-nitrophenol produced per minute per milligram of protein.
[b] Standard deviation was calculated from three to six determinations from independent experiments. ND, not determined.
[c] Conditions permitting nitrogenase activity.

fragment (Fig. 4). In *R. meliloti* 2011, two genes designated *nodP* and *nodQ*, overlapping the 2.2-kb *Eco*RI fragment (see Fig. 4), have been identified downstream from *nodG* (Cervantes *et al.* 1989; Schwedock and Long 1989). Our data strongly suggested that pAB502 might contain a DNA region homologous to *nodG* and *nodPQ* (see Fig. 4).

Identification of ORFs homologous to *nodP* and *nodQ* in the genome of *A. brasilense* Sp7. The nucleotide sequence of the 3.5-kb *Eco*RI-*Sma*I fragment from pAB508 (see Fig. 1) was determined (Fig. 5). There are 17 ORFs defined by an ATG and a stop codon separated by more than 50 codons. The codon use and the relative position of purines and pyrimidines in the sequence (Shepherd 1981) suggest that only two of them, ORF 1 and ORF 2, are translated. Comparison of these two ORFs with the DNA sequences of *nodG, nodP*, and *nodQ* of *R. meliloti* revealed a strong similarity between ORF 1 and *nodP* (67%) and between ORF 2 and *nodQ* (61%) (data not shown). *nodG* was dissimilar to ORF 1 and ORF 2.

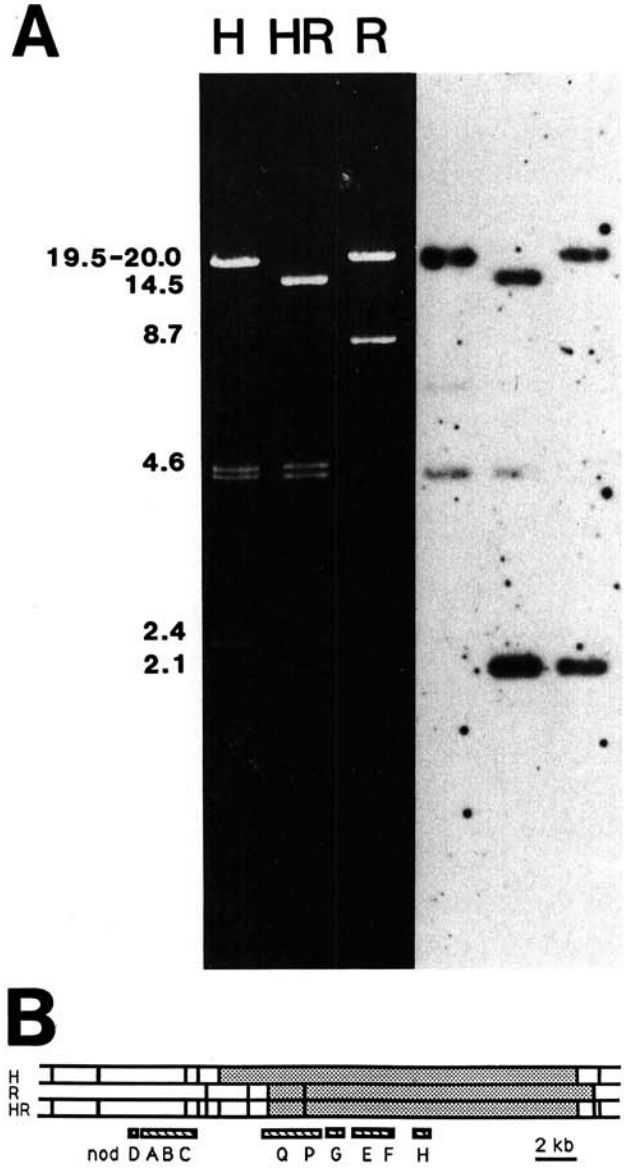

Fig. 4. A, Hybridization of restriction fragments of pHB286 with the pAB502 insert probe. At left, pHB286 digestions. At right, corresponding autoradiogram after hybridization with the pAB502 insert. The gel was exposed to Kodak X-Omat film for 16 hr at −80° C. Lengths of hybridizing fragments are indicated in kilobases (kb).The faint hybridizing bands at 4.6 kb were due to contaminating vector sequences in the probe. **B**, Physical map of pHB286, according to Batut *et al.* (1985); shaded regions indicate hybridizing fragments. Hatched boxes under the map indicate *nod* open reading frames (Cervantes *et al.* 1989). Restriction sites: H, *Hin*dIII, and R, *Eco*RI.

ORF 1. From the putative ATG start codon at position +1 to the TGA stop codon at position +904, ORF 1 encodes a polypeptide of 301 amino acids, with a calculated M_r of 34,098, which is almost identical to that of the calculated *R. meliloti nodP* gene product (34,763). As shown in Figure 6, *A. brasilense* Sp7 and *R. meliloti nodP*-encoded amino acid sequences show 63% identity (76% including conservative changes according to Dayhoff [1978]). The sequence upstream of ORF 1 was analyzed for translation signals, since a second in-frame ATG was found at position −114. This ATG was not preceded by a ribosome binding site (Shine and Dalgarno 1974), and no homology with *R. meliloti nodP* was found in the nucleotide sequence between residues −114 and +1 (Fig. 5). In contrast, the ATG at position +1 is preceded by a GGAGAG ribosome binding site, and a CAAT sequence complementary to the 5′ terminus of the bacterial 16S rRNA is present 15 nucleotides downstream of the ATG position +1 (Fig. 5). This sequence could be involved in the interaction between the mRNA and the 16S rRNA, in addition to the Shine-Dalgarno sequence (Petersen *et al.* 1988).

ORF 2. From the putative ATG start codon at position +906 to the TGA stop codon at position +2766, ORF 2 encodes a polypeptide of 620 residues, with a calculated M_r of 66,965, close to that of the *R. meliloti nodQ* gene product (70,613). ORF 2 shares 57% identity (70% including conservative changes) with the *R. meliloti nodQ* gene product (Fig. 6). The *Azospirillum* ORF 2 ATG start codon overlaps the *nodP* translation termination codon by one nucleotide. This suggests that the translation of the genes is coupled as it is in *R. meliloti* (Cervantes *et al.* 1989). Moreover, as in *R. meliloti*, the GGAAGG sequence at position +891 (Fig. 5) could allow a *de novo* translation initiation of *nodQ*. The AAAC sequence at position +910 (Fig. 5) could also interact with the 16S rRNA (Petersen *et al.* 1988).

The sequence flanking the putative *nodP* coding sequence was determined for 627 nucleotides upstream from the initiation codon. No similarities with *nod*-boxes or with an *E. coli* consensus promoter could be detected. However, a sequence reminiscent of NtrA-dependent promoters (Buck *et al.* 1985) was found at position −226 (see below). Interestingly, we have found two similar sequences upstream of *nodP* of *R. meliloti* (Debellé and Sharma 1986) at positions −75 and −389, respectively. The relevant sequences are as follows:

A. brasilense nodP	(−226)	GTGGAGCCGCTGGTGCG
R. meliloti nodP	(−75)	TTGGGATGCCTTGAGCG
R. meliloti nodP	(−389)	GTGGGCTTGCATTTGCA
NtrA-dependent consensus		CTGGCACNNNNNTTGCA

The noncoding sequence extending downstream from *nodQ* contains a palindrome of 19 base pairs (see Fig. 5),

corresponding to an mRNA hairpin loop with a ΔG of −37.4 kcal/mol (Tinoco *et al.* 1973), which could be a transcription terminator.

The G+C content in the third codon position is 87% in ORF 1 and 89% in ORF 2. Values observed for the other Sp7 genes that have been previously sequenced are as follows: *glnA*, 95%; *hisB*, 93%; and *nifH*, 92% (for references see de Zamaroczy *et al.* 1989). Because the two ORFs are plasmidic and the other genes chromosomal, it is difficult to estimate whether the deviation is significant or not. However, these values are in agreement with those predicted for genes of species with a genomic G+C content

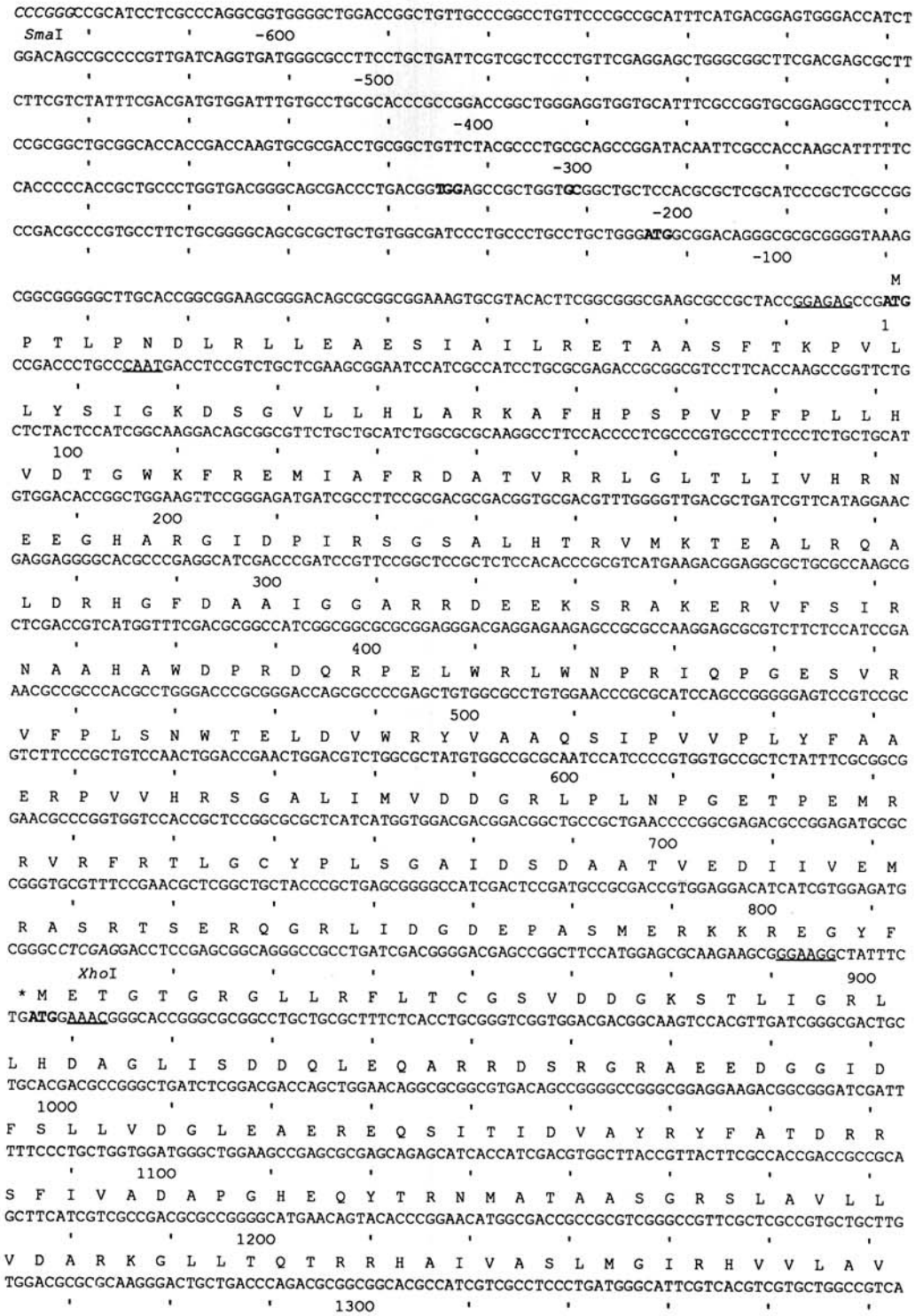

Fig. 5. Nucleotide sequence of the 3.5-kilobase *Eco*RI-*Sma*I fragment of pAB502 containing *nodP* and *nodQ* and the flanking regions. The deduced amino acids are printed above the corresponding codons of the open reading frames. Numbering of the nucleotides starts at the A residue of the putative ATG initiation codon of the *nodP* coding sequence. Relevant restriction sites are indicated in italics. Initiation codons are in bold characters. Potential ribosome binding sites are underlined. Horizontal arrows indicate an inverted repeat. (**Fig. 5** *continued on next page.*)

of about 70% (Muto and Osawa 1987).

A third ORF containing 140 codons was found upstream from *nodP* between positions −512 and −92 (Fig. 5). The G+C content in the third codon position is 81%, thus being lower than in the other ORFs sequenced. Moreover, the ORF is not preceded by a typical Shine-Dalgarno sequence.

***nodQ* gene product shows homology with elongation factors.** The *Azospirillum nodQ* gene product presents significant homology with a family of GTP-binding proteins involved in initiation and elongation during protein synthesis. A similar observation has been reported for the *R. meliloti nodQ* gene product (Cervantes *et al.* 1989; Schwedock and Long 1989). The *Azospirillum nodQ* gene product possesses the three regions of the GTP-binding consensus as proposed by Dever *et al.* (1987): GX_4GK positions 16 to 22, DXXG positions 95 to 98, and NKXD positions 150 to 153 (see Fig. 6). The distances between these regions are in agreement with those proposed by Dever *et al.* (1987) for GTP-binding proteins (see Fig. 7). The sequence GITI is present in *R. meliloti* NodQ between positions 89 and 92 (Fig. 6). In Sp7, this sequence is modified into SITI between positions 74 and 77. However, their immediate environment is identical in the two NodQs. The GITI sequence, only present in elongation factors between the two first conserved regions, seems to be involved in AA-tRNA and/or ribosome binding (Khono *et al.* 1986).

The homology with the elongation factors extends to the regions flanking the four consensus sequences (GX_4GK, DXXG, GITI, and NKXD) and reflects a very strong

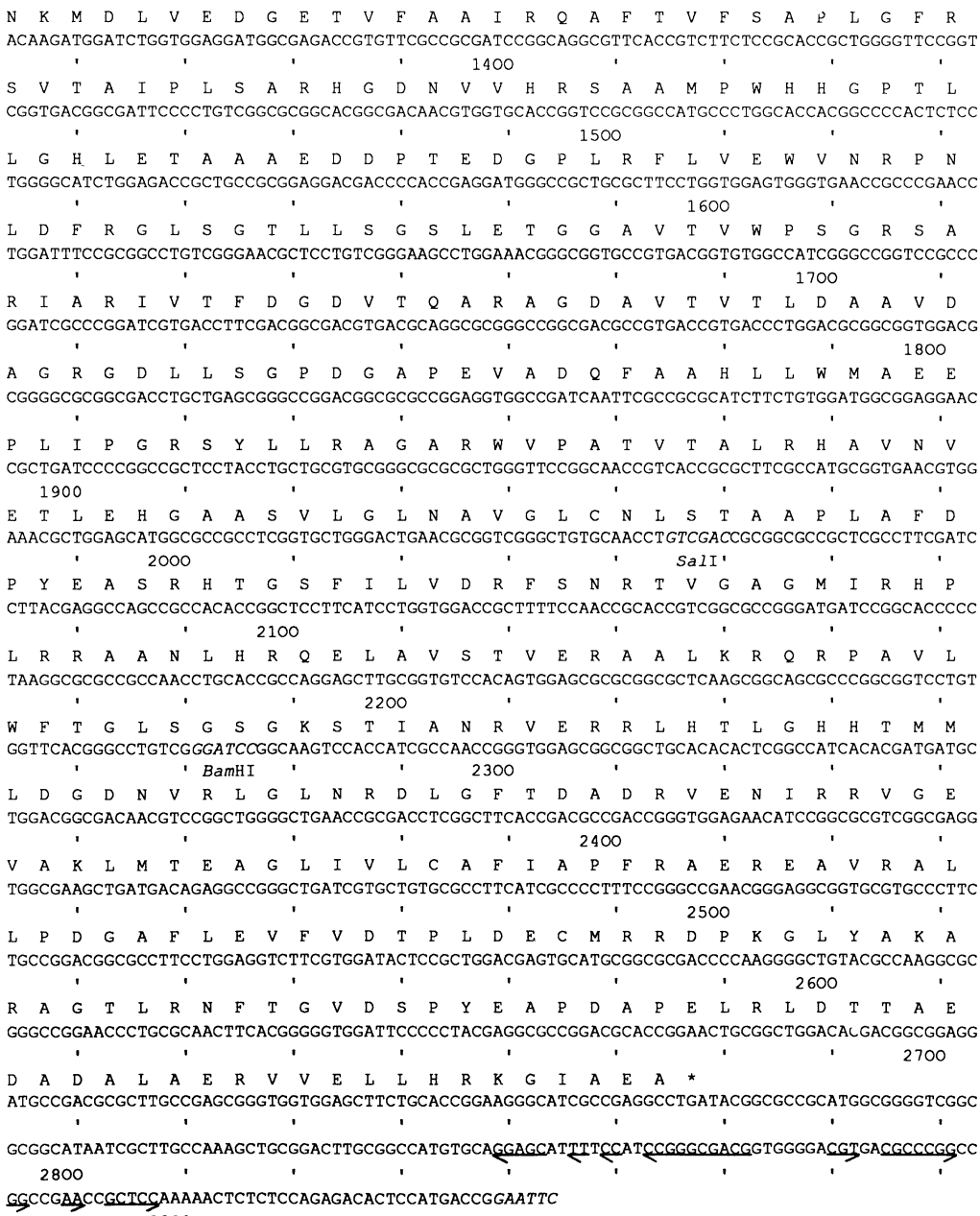

Fig. 5. *continued from previous page.*

similarity between both NodQs in this region. Between positions 9 and 156, they show 67% identical residues and 79% equivalent residues. Though homology with elongation factors is limited to the NH_2-terminal part of the *nodQ* gene products, the COOH-terminal part between positions 448 and 593 is highly conserved between the two NodQ polypeptides (70% identical residues and 78% equivalent residues) (see Fig. 6). The strong conservation of this region is presumably significant to the function of these genes.

No proteins homologous to *A. brasilense* NodP were found in protein data base searches. This is consistent with *R. meliloti* NodP (Cervantes *et al.* 1989).

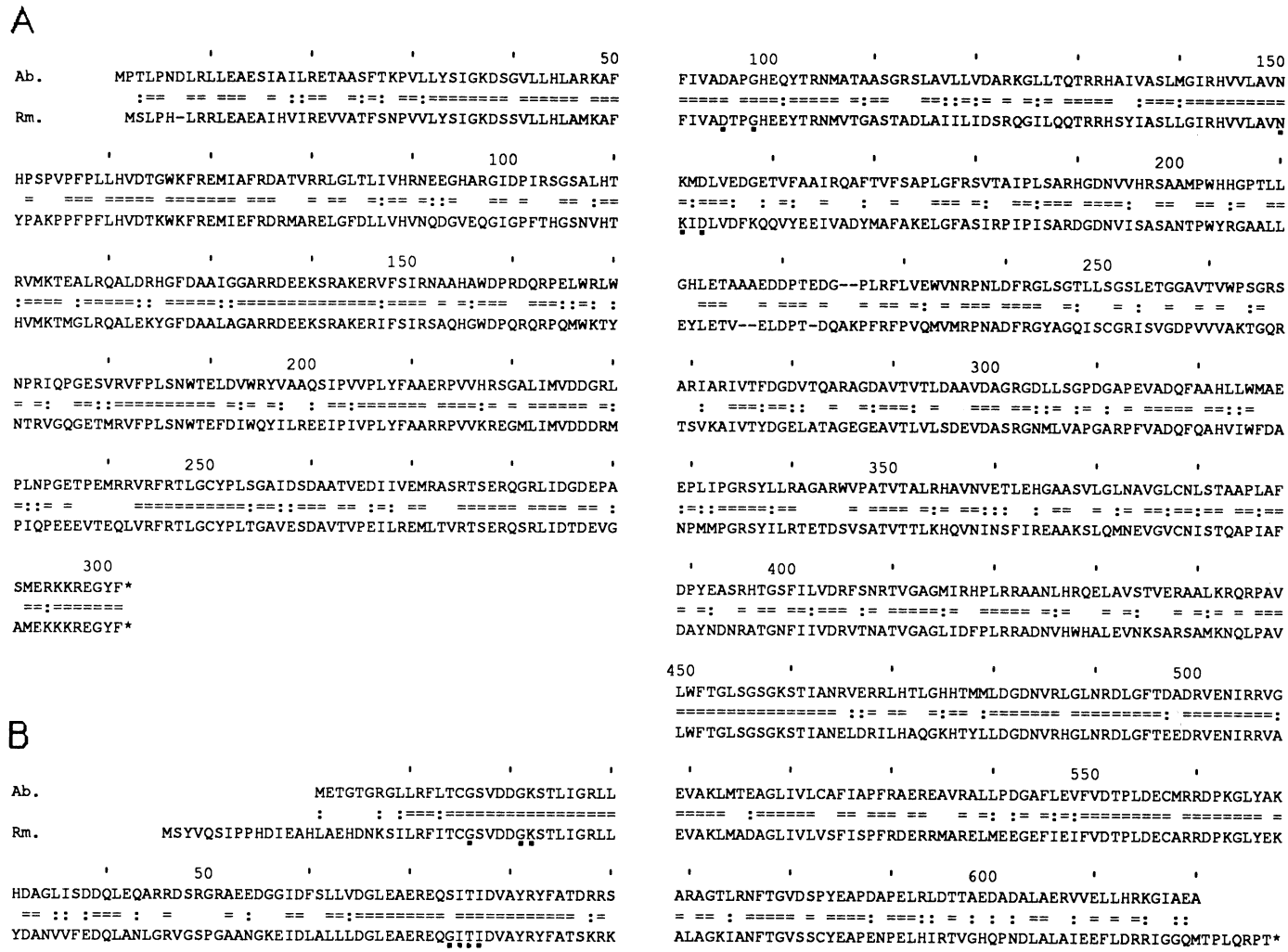

Fig. 6. Comparison of *nodP*-encoded (**A**) and *nodQ*-encoded (**B**) amino acid sequences of *Azospirillum brasilense* (Ab.) and *Rhizobium meliloti* (Rm.) (Schwedock and Long 1989). = indicates identical residues, and : denotes conservative residues, according to Dayhoff (1978). Dots indicate conserved regions as shown in Figure 7.

CONSENSUS	**G**	**X**	**X**	**X**	**X**	**G**	**K**	**(40 to 80)**	**D**	**X**	**X**	**G**	**(40 to 80)**	**N**	**K**	**X**	**D**
EF-TU	**G**	**H**	**V**	**D**	**H**	**G**	**K**	**(56)**	**D**	**C**	**P**	**G**	**H**	**(51)**	**N**	**K**	**C**	**D**
EF-1Alpha	**G**	**H**	**V**	**D**	**S**	**G**	**K**	**(71)**	**D**	**A**	**P**	**G**	**H**	**(58)**	**N**	**K**	**M**	**D**
RAS	**G**	**G**	**G**	**G**	**V**	**G**	**K**	**(41)**	**D**	**T**	**A**	**G**	**Q**	**(55)**	**N**	**K**	**L**	**D**
NodQ,A.b.	**G**	**S**	**V**	**D**	**D**	**G**	**K**	**(72)**	**D**	**A**	**P**	**G**	**H**	**(51)**	**N**	**K**	**M**	**D**
NodQ,R.m.	**G**	**S**	**V**	**D**	**D**	**G**	**K**	**(72)**	**D**	**T**	**P**	**G**	**H**	**(51)**	**N**	**K**	**I**	**D**

Fig. 7. Alignment of the potential GTP-binding sites of *Azospirillum brasilense* and *Rhizobium meliloti nodQ* gene products (Schwedock and Long 1989) with the GTP-binding sites of various proteins (for references, see Dever *et al.* 1987). EF-TU denotes *Escherichia coli* elongation factor EF-TU; EF-1Alpha, human elongation factor EF-1Alpha; RAS, human *ras* oncogene product; NodQ, A.b., *A. brasilense* Sp7 *nodQ* gene product; and NodQ, R.m., *R. meliloti* 1021 *nodQ* gene product. The consensus was processed by Dever *et al.* (1987). Numbers in parentheses represent the number of amino acid residues between the conserved regions.

Localization of *nodPQ* in the *Azospirillum* genome. In *R. meliloti, nodPQ* are localized on a symbiotic plasmid (Cervantes *et al.* 1989; Schwedock and Long 1989). *Azospirillum* contains large plasmids (Franche and Elmerich 1981). To check whether *nodPQ* were plasmid-borne in *Azospirillum*, the insert of pAB502 was used as a hybridization probe against plasmid preparations of several *Azospirillum* strains. In most cases hybridization was detected with a plasmid of 90 MDa termed p90 (Vieille *et al.* 1989). Sp7 p90 was purified according to Onyeocha *et al.* (1990). Restriction digests of the plasmid were hybridized with the 1.5-kb *Xho*I-*Sma*I fragment containing *nodP* (Fig. 1). For each restriction, the probe revealed one plasmid fragment including a 10-kb *Eco*RI fragment and a 5.6-kb *Sal*I fragment (Fig. 8). These lengths are consistent with those predicted from the physical map of pAB502. In addition, the lengths of the restriction fragments revealed with the plasmid preparation and with a preparation of total DNA were the same (data not shown). The physical map of p90 has been established and *nodPQ* has been localized on the map (Onyeocha *et al.* 1990).

The *Azospirillum nodP* gene is expressed in *Azospirillum*. To study the expression of *nodP*, a translational *nodP-lacZ* fusion was constructed (Fig. 2) in the vector pGD926. The resulting plasmid, pAB527, contained most of *nodP* and 1.3 kb of the DNA region upstream from *nodP* (Fig. 2). It was assumed that the *nodP* promoter is contained within the 1.3-kb region.

The basal level of β-galactosidase activity in wild-type *Azospirillum*, containing or not containing pGD926, was just above the background (Table 2). A *kan-lacZ* transcriptional fusion in *A. brasilense* Sp7 was used as a positive activity control. This control proved that the enzyme was produced and could be assayed in this bacterium.

The β-galactosidase activity of strain Sp7 containing pAB527 was measured after growth under various physiological conditions and compared with that obtained with vector pGD926. Physiological conditions assayed included different nitrogen sources, oxygen concentrations, and root exudates. As shown in Table 2, under all conditions tested, the level of β-galactosidase activity in Sp7(pAB527) was of the same order of magnitude as it was for the *kan-lacZ* fusion, and at least 40 times higher than the basal level observed with either strain Sp7 or Sp7(pGD926). The variation in activity between the different growth conditions was less than twofold. In particular, oxygen tension and ammonia concentration had little effect. In the presence of glutamate as nitrogen source, the activity was slightly higher. The level of β-galactosidase activity was constant after growth in the presence or absence of wheat root exudates and at a variety of exudate concentrations. It appears, therefore, that *nodP* expression is neither induced nor repressed by root exudates. Our data strongly suggest that the *nodP* gene is functional in *Azospirillum* and that its expression is constitutive. This is likely to be the case for *nodQ* since the two genes probably belong to the same operon.

Expression of the *nodP-lacZ* fusion in heterologous hosts. The β-galactosidase activity of pAB527 in *E. coli* was indistinguishable from the background level. This sug-

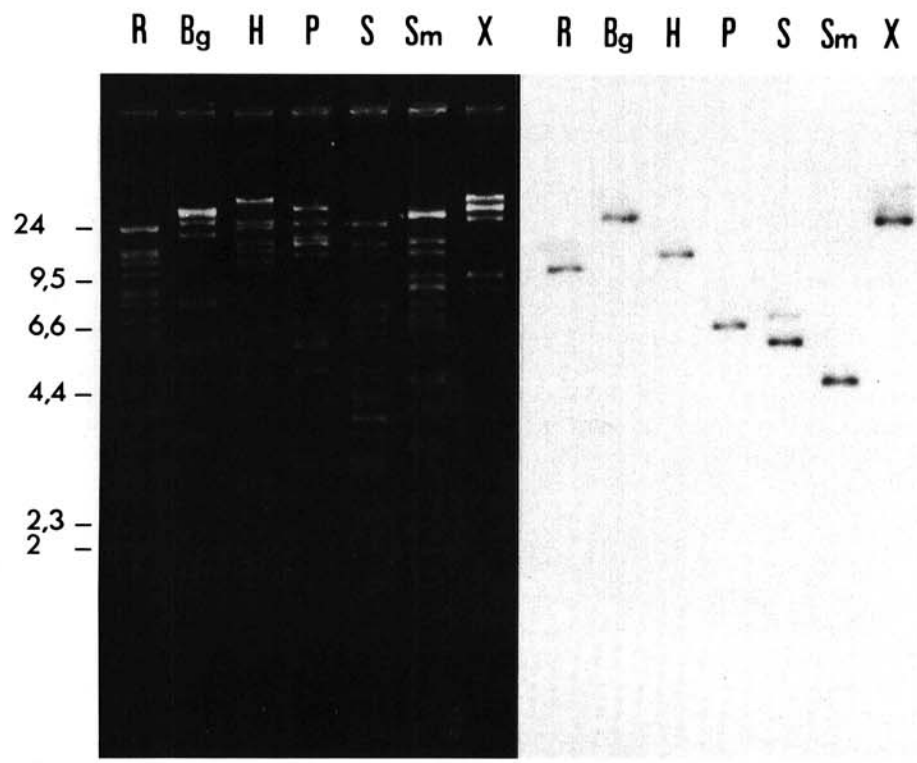

Fig. 8. Hybridization of a *nodP*-containing probe with restriction fragments of the p90 plasmid of *Azospirillum brasilense* Sp7. Details of the purification procedure of p90 and of its physical map have been published elsewhere (Onyeocha *et al.* 1990). At left, p90 digestions. Restriction sites are as given in Figure 1, with Bg denoting *Bgl*II. At right, corresponding autoradiogram after hybridization with the 1.5-kilobase *Xho*I-*Sma*I fragment purified from pAB512 (Fig. 1). The gel was exposed to Kodak X-Omat film for 2 hr at −80° C. Lengths of hybridizing fragments are indicated in kilobases.

gests that no promoter functioning in *E. coli* was located within the 1.3 kb upstream of *nodP* (data not shown). Similarly, no expression was detected in *K. pneumoniae* grown in LB medium. Since a sequence reminiscent of an NtrA-dependent promoter was found upstream of *nodP* and no other putative transcription signal was found, activation of the *nodP-lacZ* fusion by *K. pneumoniae nifA* was assayed. Plasmids pAB527 and pGD936 were introduced by transformation into strain UNF5023, which contains a wild-type *nifA*. β-Galactosidase activity was assayed after growth under conditions of nitrogen fixation (NFM medium in anaerobiosis). No activity was detected. Analogous experiments were performed using pMC71A in which *nifA* is constitutively expressed. This was possible since the pAB527 replicon is compatible with the replicon of pMC71A. No β-galactosidase expression was detected in strain UNF5023 containing pMC71A plus pAB527 (or pGD926) after growth under conditions of nitrogen fixation in the presence or absence of 20 mM ammonia (data not shown).

Plasmids pAB527 and pGD926 were each introduced into *R. meliloti* 2011 and *A. tumefaciens* GMI9023 by conjugation. The β-galactosidase activity detected in both strains containing pAB527 or pGD926 was the same as the background level (data not shown).

Phenotypes of *nodPQ* mutants. Three types of mutants were constructed and are as follows: 1) $NodP^{+}NodQ^{-}$, for example strain 7808 that contains a Tn*5*-Mob in *nodQ*; 2) $NodP^{-}NodQ^{+}$, for example strain 7801 in which *nodQ* is under the control of the *kan* promoter contained in the inserted cartridge; and 3) $NodP^{-}NodQ^{-}$, for example strains 7802 and 7803 that carry part or total deletion of *nodPQ*. The physiological properties of the mutant strains were compared to Sp7 wild type and to strain 7831 that contains a Tn*5*-Mob outside *nodPQ*. No significant difference was observed in the following tests: growth rate at 30° C in nutrient broth or minimal medium, nitrogen fixation under derepression conditions, indoleacetic acid production, and effect on the growth of wheat seedlings. In addition, the effects of NodPQ products on *nodP-lacZ* fusion expression were assessed. Plasmid pAB527 was introduced in the mutants described above. pAB527 transformants of strains 7801 and 7808 showed a lower level of β-galactosidase activity. The explanation for this is unclear. The β-galactosidase activity of *nodPQ* deletion strains was of the same order of magnitude as that of Sp7(pAB527), suggesting that NodPQ have no regulatory effect on their own synthesis.

DISCUSSION

The nucleotide sequence of the DNA region of *A. brasilense* Sp7 homologous to *hsn* of *R. meliloti* established the presence of two ORFs homologous to *R. meliloti nodPQ*. Interestingly, these ORFs are plasmid-borne in *A. brasilense* Sp7 (Fig. 8) and in most of the *Azospirillum* strains containing a plasmid of 90 MDa. This confirms previous experiments using the pAB502 insert as a hybridization probe against plasmid profile preparations (Vieille *et al.* 1989). Construction of a *nodP-lacZ* fusion showed that at least *nodP* and possibly *nodQ* are expressed in *Azospirillum*. The transcriptional organization of the two genes is not known, but they are probably cotranscribed as they are in *R. meliloti*. It cannot be excluded that a third gene, coding for a polypeptide of 140 amino acids and located upstream of *nodP*, is also part of the same transcription unit. This could explain why no transcriptional signal was found in the 627 nucleotides upstream of *nodP*. The absence of expression of pAB527 under the control of *K. pneumoniae nifA* suggests that the TGG-N10-TGC conserved sequence, upstream of *A. brasilense nodP* (and also present upstream of *R. meliloti nodP*), does not correspond to an NtrA-dependent promoter functional in this background. This is also in agreement with the observation that mutation or deletion of *nodPQ* does not affect nitrogen fixation in *Azospirillum*.

In *R. meliloti*, mutations in *nodP* or in *nodQ* led to delayed nodulation of infected alfalfa (Debellé *et al.* 1986; Swanson *et al.* 1987). It has been shown that at least *nodQ* and perhaps *nodP* are involved in host-specific nodulation of alfalfa and particularly in root hair curling (Cervantes *et al.* 1989). In addition, the *nodQ* gene, which shares homology with elongation factors, is involved, together with *nodH*, in the dominance of *hsn* genes from *R. meliloti* over those from *R. leguminosarum* and *R. trifolii* (Cervantes *et al.* 1989; Debellé *et al.* 1988; Faucher *et al.* 1989). According to the model proposed by Faucher *et al.* (1989), the *nodABC* genes are involved in the production of a common precursor signal, subsequently modified by the *nodH* and *nodQ* gene products giving rise to an alfalfa-specific signal.

The strong similarity between the *nodPQ* genes from *R. meliloti* and *Azospirillum* and their constitutive expression in *Azospirillum* are inconsistent with the hypothesis of a casual presence of these genes in *Azospirillum*. In this bacterium, the *nodPQ* genes are not essential for growth in the free-living state, since a deletion of *nodP* and of most of the 5′-terminal part of *nodQ* does not modify the growth rate in minimal medium. It remains possible, however, that both genes are involved in the interaction with the host plants. Experiments with the host, *Digitaria decumbens* Stent, from which strain Sp7 was isolated (Döbereiner and Day 1976), would confirm whether *nodQ* is involved in host specificity as it is in *Rhizobium*. Another possibility is that *nodPQ*, in *Rhizobium* as well as in *Azospirillum*, have as yet unknown functions in addition to involvement in the nodulation process. It should be noted that *nodP* is present in several *Rhizobium* species and perhaps even in *E. coli* (Schwedock and Long 1989), which implies roles other than host specificity. Furthermore, in *R. meliloti, nodP* and *nodQ* are not preceded by a *nod*-box, unlike the other *nod* genes, and there is no clear evidence of cotranscription of *nodPQ* and *nodFEG* (Schwedock and Long 1989).

The *nodPQ* genes of *Azospirillum* and *Rhizobium* appear to be homologous. These genera are not closely related and are associated in different ways with different families of plants. This raises the question of the origin of these genes. The fact that the similarity is greater between the gene products (76 and 70%) than between the nucleotide sequences (67 and 60%) suggests a distant common ancestor with a selective pressure in both bacterial genera for main-

tenance of the activity of the gene products. In this hypothesis, the ancestral genes could have been present in a common ancestor before the interaction of *Rhizobium* with legumes and *Azospirillum* with grasses had evolved.

Other genes, related to genes in *Rhizobium* and *Agrobacterium*, are likely to be present in *Azospirillum*. In particular, the 4-kb *Sal*I fragment carried by pAB503 contains an *R. meliloti nodG* homologue, which is not located on the p90 plasmid (unpublished). In addition, hybridization of *Azospirillum* DNA with DNA fragments containing *nodABC* from *R. meliloti* (Fogher *et al.* 1985) and *chv* from *A. tumefaciens* (Waelkens *et al.* 1987) has been reported, but the corresponding regions of the *Azospirillum* genome have not yet been cloned. Plasmid p90, which carries *nodPQ*, also carries two loci that are involved in exopolysaccharide synthesis. These loci were shown to complement *exoB* and *exoC* mutations in *R. meliloti* (Michiels *et al.* 1989). Moreover, a DNA region hybridizing with *R. meliloti exoC* and not located on p90 has been detected in the *Azospirillum* genome (Onyeocha *et al.* 1990). It therefore appears that *Azospirillum* shares several conserved DNA regions with other soil bacteria which interact with plants. The role of these regions remains to be established.

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