Identification of DNA Regions Homologous to Nitrogen Fixation Genes *nifE*, *nifUS* and *fixABC* in *Azospirillum brasilense* Sp7

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A 30 kb DNA region from *Azospirillum brasilense* Sp7, containing the nitrogenase structural genes (*nifHDK*), has been cloned. The presence of *nif* genes, in the 20 kb located next to *nifHDK*, was explored by Tn5 mutagenesis after subcloning various restriction fragments in the broad-host-range suicide vehicle pSUP202. Over 25 mutations due to Tn5 random insertions were obtained in the 20 kb and each recombined into the genome of strain Sp7. Four new *nif* loci were identified, located at about 4, 9, 12 and 18 kb downstream from *nifK* respectively. Hybridization with heterologous *nif* probes from *Klebsiella pneumoniae*, *Bradyrhizobium japonicum* and *Azorhizobium caulinodans* was performed to characterize the new *nif* regions. The region proximal to *nifK* appears to contain *nifE* and the region distal to *nifK* contains genes homologous to *nifUS* and *fixABC*. *nif* gene(s) from the fourth locus were not identified. Mutants in this locus, which were devoid of nitrogenase activity when tested under nitrogen-free conditions, displayed a high nitrogenase activity when glutamate was added to the growth medium. This phenomenon was also observed with mutants of the *fixABC* homology region, but to a lesser extent. Homology between strain Sp7 total DNA and a *nifB*-containing probe from *B. japonicum* was detected, although the hybridizing region was not part of the *nif* cluster described above.

INTRODUCTION

The nitrogen fixation (*nif*) genes of *Klebsiella pneumoniae* are clustered on a 22 kb DNA fragment and are organized in a regulon containing eight transcription units, *nifJ*, *nifHDKY*, *nifENX*, *nifUSV*, *nifM*, *nifF*, *nifLA* and *nifBQ* (reviewed by Beynon *et al.*, 1983; Dixon, 1984; Elmerich, 1984). The nucleotide sequence of the *nif* cluster revealed the presence of three additional open reading frames (ORFs), *nifT*, between *nifK* and *nifY*, and *nifW* and *nifZ* between *nifV* and *nifM* (Paul & Merrick, 1987; Arnold *et al.*, 1988; Merrick, 1988). Except for *nifL* and *J*, the same genes and ORFs were found in *Azotobacter* (Jacobson *et al.*, 1989; Merrick, 1988). Nitrogenase protein 1 is encoded by *nifDK* and protein 2 is encoded by *nifH*. *nifQ* is involved in molybdenum transport. Five genes are involved in processing of protein 1 – *nifB*, *nifV*, *nifN*, *nifE* and *nifH* – while *nifM* plays a role in the processing of protein 2. Electron transport to the nitrogenase is mediated by the products of *nifF* and *nifJ*. The roles of *nifT*, *nifY*, *nifX*, *nifU*, *nifS*, *nifW* and *nifZ* are not yet established. Regulation is mediated through *nifLA* (reviewed by Dixon *et al.*, 1987; Merrick, 1988).

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Abbreviation: ORF, open reading frame.

The analysis of the genetics of nitrogen fixation in *Azospirillum* was initiated with *A. brasilense* Sp7 (ATCC 29145). Isolation of *Azospirillum* Nif⁻ mutants using chemical mutagens or UV radiation was difficult, because of the residual growth of the mutants on nitrogen-free medium (Jara *et al.*, 1983). Nevertheless a few mutants were characterized. Strain 7571, derived from Sp7, appeared to be impaired in nitrogenase protein 1 activity (Jara *et al.*, 1983; Nair *et al.*, 1983). Pedrosa & Yates (1984) reported the isolation of nitrogenase mutants and of *nif* regulatory mutants, one of them corresponding to a *nifA*-like mutation. The other Nif⁻ mutants described have been isolated either as glutamine auxotrophs (Gauthier & Elmerich, 1977) or as Asm⁻ mutants impaired in glutamate synthase biosynthesis (Bani *et al.*, 1980). Using random Tn5 mutagenesis in strain Sp7, Vanstockem *et al.* (1987) identified a *nif* locus carried on a 4.2 kb *EcoRI* fragment. Singh & Klingmüller (1986) have cloned three *EcoRI* fragments of 15, 16 and 17 kb containing *nif* loci from strain ATCC 29710. However, the *nif* genes were not identified. DNA hybridization tests using *K. pneumoniae* *nif* probes revealed that *Azospirillum* contained regions which hybridize to *nifHDK* (Quiviger *et al.*, 1982) and *nifA* (Nair *et al.*, 1983). A 6.7 kb *EcoRI* fragment containing the region that hybridized to *nifHDK* was cloned from strain Sp7 (Quiviger *et al.*, 1982). The same fragment was also recently cloned from a bacteriophage λ library of strain Sp7 (De Arujo *et al.*, 1988). Hybridization with *fix* genes from *Bradyrhizobium japonicum* revealed homology with *fixA* in several *Azospirillum* strains, belonging to the species *A. lipoferum* and *A. brasilense*, including *A. brasilense* Sp7 (Fogher *et al.*, 1985).

Due to the difficulty of isolating and characterizing *nif* mutants obtained after chemical or random Tn5 mutagenesis, we decided to perform Tn5 site-directed mutagenesis on cloned fragments of *Azospirillum* DNA. Mutagenesis was performed using pSUP202 as a suicide vehicle (Simon *et al.*, 1983). This vector, a derivative of pBR325, contains the mobilization (*mob* region) site of P-type incompatibility plasmids and it cannot replicate in *Azospirillum*. Insertions in *nifH*, *nifD* and *nifK* homology regions led to a Nif⁻ phenotype (Perroud *et al.*, 1985). Complementation analysis suggested that *nifHDK* were transcribed as a single operon, though two insertions, in mutants 7313 and 7339, located between *nifH* and *nifD* led to a Nif leaky phenotype, suggesting a possible reinitiation between *nifH* and *nifDK* (Perroud *et al.*, 1985).

Here we report the identification of new *nif* loci downstream of *nifK* in *Azospirillum brasilense* corresponding to *nifE*, *nifUS*, and *fixABC* genes of other species. Part of this work has been presented in symposia proceedings (Elmerich *et al.*, 1985, 1987, 1988).

METHODS

Strains, plasmids, media, and growth conditions. *Azospirillum brasilense* wild-type was strain Sp7 (ATCC 29145) (Tarrand *et al.*, 1978). *Escherichia coli* strains were: S17.1 [*pro thi recA* (RP4-2 Tc-Mu Km-Tn7 Tra⁺ IncP1)] (Simon *et al.*, 1983) and C600 (*trp his recA rif*). *Azorhizobium caulinodans* was strain ORS571 (Dreyfus *et al.*, 1983). Plasmids are listed in Table 1 and schematized in Fig. 1. *E. coli* was grown in LB medium (Maniatis *et al.*, 1982). *Azospirillum* cultures were grown in either nutrient broth (rich medium) or K medium, (minimal medium) as previously described (Frache & Elmerich, 1981), except that the carbon source used was sodium lactate (5 g l⁻¹) instead of malate. The nitrogen source was 20 mM-ammonium chloride, 5 mM-sodium glutamate or N₂.

Assay for nitrogen fixation. Nitrogen fixation was assayed in K medium, devoid of ammonia and in the presence or absence of 5 mM-glutamate. Inocula (0.1 ml) from an overnight preculture in rich medium were placed in 70 ml flasks containing 10 ml K medium, with or without glutamate. Flasks were filled with N₂ and the O₂ content was adjusted to 1.3% (v/v). After 16 h incubation, at 30 °C with shaking, 5 ml acetylene was added and the ethylene content was measured by gas chromatography as previously described (Frache & Elmerich, 1981), after 30, 60, 90 and 120 min. Under these conditions, the final OD₆₀₀ of a culture of the wild-type was 0.35 in the absence of glutamate and 0.5 in the presence of glutamate.

In routine assays, two drops of an overnight culture in rich medium were inoculated into 10 ml bijou bottles containing 7 ml K medium supplemented with glutamate. The bottles were capped and incubated at 30 °C, with shaking. After 16 h 1 ml acetylene was added and ethylene production was measured 3 or 4 h later. The method was adequate for preliminary screening, as the Nif defective phenotypes of the mutants were not too leaky.

Plasmid construction and molecular biology techniques. Plasmid construction, transformation, DNA isolation, restriction analysis and DNA hybridization were performed by conventional techniques (Maniatis *et al.*, 1982). Cloning of pAB3, pAB10 and pAB16 has previously been reported (Perroud *et al.*, 1985; Elmerich *et al.*, 1987). Subclones of pAB10 and pAB16 were obtained in pSUP202 to yield pAB12 (not shown) and pAB19, respectively

Table 1. *Plasmids*

Plasmid	Vector	Relevant properties	Source or reference
pSUP202	pBR325	Ap ^R Cm ^R Tc ^R Mob ⁺	Simon <i>et al.</i> (1983)
pSUP2021	pBR325	Ap ^R Cm ^R Km ^R Mob ⁺	Simon <i>et al.</i> (1983)
pRK2013	ColE1	Km ^R Tra ⁺	Figurski & Helinski (1979)
pVK100	pRK290	Km ^R Tc ^R Mob ⁺ <i>cos</i>	Knauf & Nester (1982)
pUC18	–	Ap ^R <i>lacZ</i>	Yanisch-Perron <i>et al.</i> (1985)
pBR322-Tn5	–	Ap ^R Tc ^R Km ^R	Denèfle <i>et al.</i> (1987)
pAB3	pSUP202	Ap ^R Tc ^R ; contains a 6.7 kb <i>Eco</i> RI fragment from <i>A. brasilense</i> Sp7, carrying <i>nifHDK</i>	Perroud <i>et al.</i> (1985); see Fig. 1
pAB10	pUC8	Ap ^R Km ^R ; contains <i>nif</i> DNA from <i>A. brasilense</i> Sp7	Elmerich <i>et al.</i> (1987); see Fig. 1
pAB16	pUC8	Ap ^R Km ^R ; contains <i>nif</i> DNA from <i>A. brasilense</i> Sp7	Elmerich <i>et al.</i> (1987); see Fig. 1
pCRA37	pMB9	Tc ^R ; contains four <i>Eco</i> RI fragments from <i>K. pneumoniae</i> M5al covering <i>hisDG</i> and <i>nifXUSVWZMFLABQ</i> ^(a)	Cannon <i>et al.</i> (1977); see Fig. 4
pPC937	pACYC177	Ap ^R ; contains a 4.8 kb <i>Eco</i> RI– <i>Xho</i> I fragment from <i>K. pneumoniae</i> M5al carrying <i>nifXUSVW</i> and part of <i>nifZ</i> ^{*(a)}	Norel <i>et al.</i> (1985)
pGR113	pACYC184	Tc ^R ; contains a 2.2 kb <i>Eco</i> RI fragment from <i>K. pneumoniae</i> M5al carrying part of <i>nifE</i> and most of <i>nifN</i> ^{*(b)}	Riedel <i>et al.</i> (1983)
pRS46	pSUP202	Ap ^R Cm ^R ; contains a 13 kb <i>Hind</i> III fragment from <i>A. caulinodans</i> ORS571 carrying <i>nifHDK</i> and <i>nifE</i>	Denèfle <i>et al.</i> (1987); see Fig. 4
pRS54	pHE3	Tc ^R ; contains a 9.5 kb <i>Pst</i> I fragment from <i>A. caulinodans</i> ORS571 carrying <i>nifH2</i> and <i>fixABC</i>	Norel & Elmerich (1987); see Fig. 4
pRS55	pLA2917	Same insert as pRS54	Kaminski <i>et al.</i> (1988)
pRJ7128	pACYC184	Ap ^R ; contains a 3.4 kb <i>Sma</i> I fragment from <i>B. japonicum</i> I110 carrying <i>nifA</i> and <i>fixA</i>	Fisher <i>et al.</i> (1986)
pRJ7011	pACYC177	Ap ^R ; contains a 3.6 kb <i>Pst</i> I– <i>Hind</i> III fragment from <i>B. japonicum</i> I110 carrying <i>nifB</i>	Ebeling <i>et al.</i> (1987)

* According to physical mapping and DNA nucleotide sequence from: (a) Beynon *et al.* (1983, 1987) and Paul & Merrick (1987); (b) Beynon *et al.* (1983) and Setterquist *et al.* (1988).

(Fig. 1). Plasmid pAB14 was obtained by deletion of a *Bgl*II fragment from pAB12. To construct pAB131, a *Xho*I–*Hind*III fragment from pAB10 was cloned in pVK100, to yield pAB13 (not shown), then a *Bgl*II deletion was performed. Plasmids pAB21, pAB23 and pAB24 are subclones of pAB19 in pUC18, and pAB22 is a subclone of pAB19 in pSUP202. The DNA insert in pAB19 was used as a probe to screen, by colony hybridization, a gene bank of *Hind*III fragments of strain Sp7 constructed in pVK100 (Bozouklian *et al.*, 1986). Two plasmid clones, pAB48 and pAB49, each containing the same 20 kb *Hind*III fragment but in opposite orientations, were isolated (Fig. 1). The plasmid vector pVK100 contains a single *Xho*I site, thus a *Xho*I deletion of pAB48 led to pAB481 and a *Xho*I deletion of pAB49 led to pAB483. Plasmid pVK100 contains no *Pst*I sites. A *Pst*I deletion of pAB48 led to pAB482. To construct pAB485, a 10 kb *Hind*III fragment purified from pAB10 was ligated at the unique *Hind*III site of pAB483 and after *in vitro* packaging in λ heads, Km^R clones were selected.

Tn5 mutagenesis. Tn5 mutagenesis of pAB3, pAB14 and pAB19 was performed in *E. coli* S17.1-Tn5 as previously described (Perroud *et al.*, 1985; Bozouklian *et al.*, 1986). Plasmids carrying Tn5 insertions were subsequently introduced into strain Sp7, using pRK2013 as a mobilizing agent as described by Perroud *et al.* (1985). *Azospirillum* recombinants were selected on minimal medium containing 20 μ g kanamycin ml⁻¹. For each plasmid containing an insertion, recombination in strain Sp7 was performed twice and nitrogen fixation was determined by the routine assay with at least three independent recombinant colonies. Random mutagenesis of strain Sp7 was performed with pSUP2021, a derivative of pSUP202 containing Tn5 (Simon *et al.*, 1983).

Physical mapping of Tn5 insertions. Localization of the Tn5 elements after marker exchange in the Sp7 genome was performed by hybridization with a pBR322-Tn5 probe and with a *nif* probe as described by Denéfle *et al.* (1987). The *nif* probes were purified from pAB14 or pAB19. For each cross, total DNA of the Km^R mutants was extracted from three independent recombinants whatever the Nif phenotype observed. DNA was digested by *Pst*I and, after Southern blotting, hybridized with the probes.

RESULTS

Physical map of the DNA region adjacent to the nifHDK cluster

The physical maps of pAB10, pAB16 and their subclones are shown in Fig. 1. The correct construction of plasmids pAB14, pAB19, pAB21, pAB22, pAB23, pAB24 and pAB481 was verified by hybridization with total DNA from strain Sp7 digested with *Bgl*II, *Eco*RI, *Hind*III, *Pst*I and *Sal*I. In each case, the physical map of the subclones corresponded to that deduced from those of the initial clones (data not shown). It is unlikely that *nifHDK* and genes located in the newly cloned regions are reiterated.

Random and site-directed mutagenesis

The Tn5 insertions in pAB3, pAB14 and pAB19 (Fig. 1) were mapped in the cloned fragments and were subsequently recombined in the genome of strain Sp7. The frequency of Km^R mutants obtained by marker exchange ranged between 10⁻⁵ and 10⁻⁶. The frequency of Km^R mutants, obtained by random mutagenesis using pSUP2021, ranged between 10⁻⁷ and 10⁻⁸. Although this value was lower than that of marker exchange, it was important to check that the Km^R mutants obtained by site-directed mutagenesis resulted from recombination of the transposon at the target location.

The locations of the Tn5 insertions were checked by hybridization to verify that they had recombined at the predicted place in strain Sp7 genome (see Fig. 1). Fig. 2(a) shows an example of the physical mapping of mutants in regions II, III and IV. The *nif* probe used was pAB19.

In the wild-type, the probe revealed four *Pst*I fragments of 11, 5.6, 4.7 and 3 kb (Fig. 2a, lane 1). Recombination of Tn5 led to the disappearance of one of the *Pst*I fragments and to the appearance of two new fragments which were revealed both by the *nif* and the Tn5 probes. For example, the Tn5 insertion in mutant 1935 (Fig. 1, region III) led to the disappearance of the 11 kb *Pst*I fragment and to the appearance of 9 kb and 2.8 kb *Pst*I fragments hybridizing to both probes (Fig. 2a, lanes 2 and 14). The Tn5 probe revealed three additional *Pst*I fragments of 2.5, 1.1 and 0.9 kb, corresponding to the internal fragments of the transposon (see Fig. 2b).

Characterization of the nif loci

Mutants with altered Nif phenotypes were obtained in five regions (I to V, Fig. 1); the nitrogenase activity of these mutants determined in the presence or absence of glutamate is shown in Table 2. Hybridizations with heterologous probes were performed to identify the genes which had been impaired in regions II, III, IV and V. Restriction fragments covering each of the Sp7 *nif* loci were used as probes in hybridization experiments against total DNA from *K. pneumoniae* and *Azorhizobium caulinodans* and against plasmid clones from the same strains and from *B. japonicum*. Reverse experiments were performed using plasmid clones from the three strains as probes against total DNA and plasmid clones of strain Sp7. The conclusions from these experiments were that region II corresponded to *nifE*, region IV to *nifUS* and region V to *fixABC*. Region III could not be identified.

Region I. Analysis of the region containing the *nifHDK* cluster has previously been reported (Perroud *et al.*, 1985). The three genes are probably organized as a single transcription unit.

Region II. Another *nif* locus was characterized at about 4 kb downstream from *nifK* in region I. Six mutants mapping in region II, strains 1403, 1469, 1462, 1447, 1439 and 1420, displayed no nitrogenase activity (Table 2). No complementation was observed after introduction of pAB131,

Fig. 1. The major *nif* cluster of *Azospirillum brasilense* Sp7: physical map, plasmid construction and localization of Tn5 insertions. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; P, *Pst*I; R, *Eco*RI; S, *Sal*I (only two sites shown); Sm, *Sma*I (only one site shown); X, *Xho*I. The physical maps of plasmid vectors are not shown. Plasmid vectors, see Methods. Horizontal arrow, direction of transcription; question mark, unidentified *nif* locus; open boxes, Km^r cartridges or part thereof; dotted line, deletion; hatched boxes, DNA fragments used as probes for the hybridizations shown in Fig. 3; I to V, DNA regions described in the text; vertical arrows, Tn5 insertions. *pAB131 does not contain the *Hind*III–*Bgl*II fragment part of the Km^r cartridge. Nitrogenase activity of the mutants in regions II, III, IV and V is reported in Table 2. Activity of the mutants in region I was previously reported (Perroud *et al.*, 1985): mutants 7336, 7310, 7303, 7324 and 7347 were Nif⁺, mutants 7313 and 7339 were leaky (Table 2) and other mutants were totally devoid of nitrogenase activity.

while pAB485 restored a Nif⁺ phenotype. This suggested that these mutations mapped in a cistron or a transcriptional unit that overlapped the *Hind*III site of pAB131. It is likely that this region, which covers at least 2.5 kb, contains more than one gene. In preliminary experiments, a 5.8 kb *Eco*RI–*Hind*III fragment purified from pAB10 was used as a probe against *K. pneumoniae* and *Azorhizobium caulinodans* *nif* clones digested with various restriction enzymes. A signal was

Fig. 2. (a) Physical mapping of the Tn5 insertions after recombination into strain Sp7 genome. Total DNA of each mutant was extracted and digested by *Pst*I before hybridization with the probe. The probe was the DNA insert of pAB19 (lanes 1 to 4), 1, Sp7; 2, 1935; 3, 1927; 4, 1928; or pBR322-Tn5 (lanes 5 to 20), 5, 1413; 6, 1438; 7, 1451; 8, 1450; 9, 1453; 10, 1403; 11, 1447; 12, 1420; 13, 1934; 14, 1935; 15, 1993; 16, 1936; 17, 1927; 18, 1928; 19, 1948; 20, 1986. Size is indicated in the left margin in kb.

(b) Physical map of Tn5 indicating the size in kb of the *Pst*I (P) restriction fragments. The two distal *Pst*I sites are located at 0.55 kb of the extremities of the transposon. IS50, inverted repeats; NPTII/Km, localization of the neomycin phosphotransferase type II (NPTII) gene conferring resistance to kanamycin and neomycin (Rothstein *et al.*, 1980).

Table 2. Nitrogenase activity of the *Km^R* mutants obtained after marker exchange

Specific activity of the wild-type was close to 50 nmol C₂H₂ produced min⁻¹ (mg protein)⁻¹ in N-free medium and 30 nmol in glutamate-containing medium. It was checked, in all cases, that no nitrogenase activity was detectable in cells grown in medium containing 20 mM-ammonia. Values are the mean $\pm$ SD of triplicate experiments.

	Nitrogenase activity (%)		Nitrogenase activity (%)	
	N-free medium	Plus glutamate	N-free medium	Plus glutamate
Sp7	100 $\pm$ 21	100 $\pm$ 27		
<i>Region I</i>			<i>Region III</i>	
7309 nifH	<0.3	<0.3	1934	0.5 $\pm$ 0.3
7313	7 $\pm$ 2	20 $\pm$ 7	1935	0.8 $\pm$ 0.5
7339	4 $\pm$ 1.5	8 $\pm$ 1.5	<i>Region IV</i>	
7317 nifK	<0.3	<0.3	1993	2 $\pm$ 1.6
<i>Region II</i>			1936	0.7 $\pm$ 0.5
1413	12 $\pm$ 4	22 $\pm$ 15	1927	1.5 $\pm$ 1
1438	60 $\pm$ 18	100 $\pm$ 13	1928	0.9 $\pm$ 0.6
1451	38 $\pm$ 16	14 $\pm$ 5	1948	1.4 $\pm$ 0.8
1450	95 $\pm$ 13	NT	1986	70 $\pm$ 17
1453	90 $\pm$ 17	NT	<i>Region V</i>	
1429	95 $\pm$ 14	NT	1904	1.6 $\pm$ 0.9
1403, 1469	<0.3	<0.3	1938	2 $\pm$ 1.2
1462, 1447	<0.3	<0.3	1941	1.6 $\pm$ 0.8
1439, 1420	<0.3	<0.3	1930	1.8 $\pm$ 1

detected with pGR113 and pRS46, which both carry *nifE* (data not shown). A 0.8 kb *PvuI* fragment purified from pRS46 and containing *Azorhizobium caulinodans nifE* (Norel *et al.*, 1985) was then used as a probe against pAB10; this led to the localization of the region of hybridization in the 2.3 kb *HindIII*–*SmaI* fragment where the six *nif*-Tn5 mutants were located (data not shown). This fragment (Fig. 1) was used in turn as a probe against restriction fragments of pRS46. As shown in Fig. 3(a), it revealed a 2.8 kb *BglII* fragment (lane 1) and, after digestion with *BglII* plus *PvuI*, a 1.2 kb *BglII*–*PvuI* and a 0.8 kb *PvuI* fragment (lane 2) corresponding to *nifE* (Norel *et al.*, 1985; Denèfle *et al.*, 1987; see Fig. 4a). It could not be established whether *nifN* and *nifX*, which belong to the same operon as *nifE* in *K. pneumoniae* and *Azotobacter vinelandii* (Jacobson *et al.*, 1989; Merrick, 1988), were adjacent to the *nifE* homology region in *Azospirillum*.

Strains 1413 and 1451 carried insertions in region II that led to a reduced nitrogenase activity. In partial diploids, containing pAB131, nitrogenase activity was significantly increased (data not shown). It is possible therefore that these two mutations affect other as yet unidentified genes required for the maximal expression of nitrogenase.

Region III. Strains 1934 and 1935 displayed a very low nitrogenase activity in N-free medium. However, in the presence of glutamate, activity was restored to near wild-type levels (Table 2). This phenotype is different from that of mutants in the nitrogenase structural genes and in the *nifE* homology region, which displayed no nitrogenase activity even when glutamate was added to the growth medium (Table 2). It was confirmed that mutant strains 1934 and 1935 grew normally on media containing 2 or 20 mM-ammonia and did not require glutamate or glutamine for growth. Addition of glutamate, at time zero of the acetylene reduction assay (see Methods), to a culture grown under N-free conditions, did not restore nitrogenase activity. The two mutants are likely to belong to a different transcription unit from the mutants in regions II and IV, since nitrogen fixation in the former, but not the latter, was fully restored by pAB482 (data not shown).

Region IV. Five mutants, strains 1993, 1936, 1927, 1928 and 1948, displayed nitrogenase activity ranging between 1 and 2% of that of the control (Table 2). Nitrogen fixation was restored by pAB48, pAB483, pAB485 but not by pAB481 or pAB482 (data not shown).

The *PstI* fragment, purified from pAB22 or pAB483 (Fig. 1), when used as a probe against total DNA of *K. pneumoniae* or *Azorhizobium caulinodans*, did not reveal any DNA fragment (data not shown). However, the probe did hybridize with restriction digests of pCRA37 and pPC937, which both carry the *nifXUSV* region of *K. pneumoniae*. Fig. 3(b) shows the results of the hybridization of pCRA37 with the *PstI* fragment purified from pAB483. The probe hybridized to a 2.5 kb *BamHI* fragment and a 2.8 kb *EcoRI*–*SmaI* fragment, suggesting the presence of genes homologous to *nifUS* region (Beynon *et al.*, 1987; see Fig. 4b). However, it was not possible to determine if the homology was due to the presence of both genes or to only one of them. Moreover, the presence of a region homologous to *nifV* or *nifM* was not detected.

Region V. Insertion mutants 1904, 1938, 1941 and 1930 in region V displayed a phenotype reminiscent of that of mutants in region III. They retained a rather high residual nitrogenase activity when grown in the presence of glutamate, but assays in the absence of glutamate revealed a much lower nitrogenase activity (Table 2). By genetic complementation in nitrogen-free medium, pAB48 restored a significant nitrogenase activity to the four mutants, whereas pAB481 and pAB483 had no effect (data not shown). This suggests that the four insertions are contained in the same transcription unit.

Two *fixA* probes were used, a *B. japonicum* probe corresponding to a 1.5 kb *PstI*–*SmaI* DNA fragment from pRJ7128, and an *Azorhizobium caulinodans* probe corresponding to a 1.4 kb *XhoI* fragment from pRS54. The two probes revealed the same fragments in *Azospirillum* total DNA (*BglII*, 18 kb; *EcoRI*, 24 kb; *HindIII*, 20 kb; *PstI*, 3 kb; *SalI*, 12 kb; data not shown). In colony hybridization experiments, the probes identified clones containing pAB48 and pAB49, though the signal was weak. This suggested that *fixA* may span pAB23. As shown in Fig. 3(c), when the

Fig. 3. Identification of *nif* genes by hybridization with heterologous probes. (a) Homology to *nifE*: restriction pattern of pRS46 digested with *Bgl*II (1) and *Bgl*II plus *Pvu*II (2) and corresponding autoradiogram after hybridization with the 2.3 kb *Hind*III-*Sma*I fragment purified from pAB14 (Fig. 1). Sizes are indicated in kb. The schematic representation of the extent of the homology is shown in Fig. 4(a). (b) Homology to *nifUS*: restriction pattern of pCRA37 digested with *Bam*HI (1) and *Eco*RI plus *Sma*I (2) and corresponding autoradiogram after hybridization with the 5.6 kb *Pst*I fragment purified from pAB483 (Fig. 1). The schematic representation of the extent of the homology is shown in Fig. 4(b). (c) Homology to *fixABC*: restriction pattern of pRS54 digested with *Sph*I (1) and *Xho*I (2) and corresponding autoradiogram after hybridization with the 3 kb *Pst*I fragment from pAB23 (Fig. 1). The schematic representation of the extent of the homology is shown in Fig. 4(c). (d) Homology to *fixABC*: restriction pattern of pAB19 digested with *Pst*I plus *Bam*HI (1), *Sal*I plus *Xho*I (2) and *Sal*I plus *Bgl*II (3) and corresponding autoradiogram after hybridization with the 1.4 kb from pRS54 containing *fixA* from *Azorhizobium caulinodans*. The schematic representation of the extent of the homology is shown in Fig. 4(d). (e) Homology with a *nifB*-containing probe: autoradiogram of strain Sp7 total DNA digested with *Pst*I (1), *Hind*III (2), *Sal*I (3), *Eco*RI (4) after hybridization with a 1.9 kb *Sal*I fragment containing *nifB* from *B. japonicum*.

Fig. 4. Localization on pRS46, pCRA37, pRS54 and pAB19 of the hybridizing fragments revealed in Fig. 3. Restriction sites: as Fig. 1, plus Pv, *Pvu*II; Sp, *Sph*I. Vectors are not shown. Arrows indicate the direction of transcription. Hatched boxes indicate the DNA fragments revealed by the *nif* probes.

3 kb *Pst*I fragment of pAB23 (Fig. 1) was used as a probe against restriction digests of pRS54, it revealed a 3.8 kb *Sph*I fragment which covers *fixABC* (lane 1), a 1.4 kb *Xho*I fragment containing *fixA* and a 3 kb *Xho*I fragment containing *fixBC* (Kaminski *et al.*, 1988; Fig. 4c). The two *Xho*I fragments of pRS54 were used in turn against a restriction digest of pAB19. Fig. 3(d) shows the result obtained with *fixA*. It revealed a 3 kb *Pst*I fragment (lane 1), a 3.7 kb *Sal*I-*Xho*I fragment and a 4 kb *Sal*I-*Bgl*II fragment (Fig. 4d), suggesting that a *fixA* homologous region is entirely contained within the 2.6 kb *Pst*I-*Xho*I fragment. The same fragments plus the adjacent *Xho*I-*Bam*HI fragment were revealed by the 3 kb *Xho*I fragment of pRS54 containing *fixBC* (data not shown), suggesting that *fixBC* overlapped the *Xho*I site. Since the 1.4 kb containing *Azorhizobium caulinodans fixA* carries part of *nifO*, a newly characterized *nif* gene (Kaminski *et al.*, 1988), hybridization was performed with a *nifO* specific probe. No homology was detected (data not shown).

Homology with *nifB*

Homology was detected between total DNA of Sp7 and a 1.9 kb *Sal*I fragment purified from pRJ7011. This plasmid contains *nifB* from *B. japonicum*. According to the nucleotide sequence of *B. japonicum nifB* (Noti *et al.*, 1986), the probe contains a 1387 bp from *nifB* ORF and the DNA region upstream of *nifB*. It does not contain *frxA*, a newly identified gene encoding a ferredoxin-like protein, located downstream of *nifB* (Ebeling *et al.*, 1988). As shown in Fig. 3(e), the *nifB* probe revealed a 4 kb *Pst*I, a 14 kb *Hind*III, a 3 kb *Sal*I and a 5 kb *Eco*RI fragment. It is possible that the hybridization was due to the DNA upstream of *nifB* in *B. japonicum*. Analysis of the size of the restriction fragments revealed indicates that the *nifB* homologous region is not part of the 30 kb *nif* cluster described here and it is unlikely therefore that *nifB* is located in or immediately adjacent to this cluster.

DISCUSSION

The data reported here are consistent with the existence of a cluster containing several nitrogen fixation genes in *Azospirillum brasilense* Sp7. The *nif* loci in this cluster were identified after Tn5 site-directed mutagenesis and genetic complementation. Although the identities of the *nif* genes from the various loci are yet to be confirmed by DNA sequencing, the DNA hybridization analysis performed indicates the presence of *nifE* in region II, of *nifU* or *nifS* or possibly both in region IV, and of *fixABC* in region V. In addition, another *nif* locus (region III) was identified between the *nifE* and *nifUS* homology regions. It is worth noting that the gene(s) contained in this region may not be essential for nitrogen fixation, in particular when glutamate is present in the medium. This was also observed, but to a lesser extent, for mutants in the *fixABC* homology region.

The role of glutamate in the regulation of *nif* gene expression in these particular mutants remains to be elucidated. Hartmann *et al.* (1988) have shown that under microaerobiosis, glutamate, which is a poor nitrogen source for *Azospirillum brasilense*, does not repress nitrogenase activity in this species, whereas in *Azospirillum lipoferum* and *Azospirillum amazonense*, glutamate is utilized more efficiently and represses nitrogenase activity. Pedrosa & Yates (1984) reported that *Azospirillum brasilense* was able to fix nitrogen in air when glutamate was added to the culture medium. It was proposed by Hartmann *et al.* (1988) that, under these conditions, glutamate increases growth, and thereby reduces the oxygen concentration to a level compatible with nitrogenase derepression and functioning. The effect of glutamate on *nif* mutants has been previously reported by Pedrosa & Yates (1984), who isolated Nif⁻ mutants on nitrogen-free solid medium, after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. They observed that many of the mutants did not display nitrogenase activity when tested in nitrogen-free semi-solid medium, but recovered wild-type nitrogenase activity when grown in air in the presence of glutamate. It is not known if the mutants described by Pedrosa & Yates (1984) are analogous to the mutants in region III or in the *fixABC* homology region described here. In this work, we did not test the effect of oxygen and glutamate simultaneously, and nitrogenase determination was performed at a low oxygen tension. Thus, further physiological and genetic studies of the mutants should help to clarify the data reported here. In addition, it would be interesting to check whether gene(s) analogous to that/those of region III are present in other aerophilic or microaerophilic nitrogen fixers, in particular in *Azospirillum lipoferum* and *Azospirillum amazonense*, which do not derepress nitrogenase activity when glutamate is present.

Homology to a *nifB*-containing probe was detected and the analysis of the data obtained suggests that *Azospirillum nifB* is not part of the 30 kb *nif* cluster described here. The same comment is true for the homology detected previously with a *K. pneumoniae nifA* probe (Naïr *et al.*, 1983). In the case of *nifA*, several fragments were revealed. These fragments have not yet been cloned. Homology with a *K. pneumoniae nifJ* probe was also reported for *Azospirillum lipoferum* Br17 (Naïr *et al.*, 1983) but no signal was detected with *Azospirillum brasilense* Sp7. The region containing *A. lipoferum nifJ* was recently cloned from strain Br17, and it was shown to be located upstream of the *nifHDK* cluster, between *nifHDK* and *draGT* (Ludden *et al.*, 1988). The localization of the corresponding genes in *Azospirillum brasilense* has not yet been determined.

In *K. pneumoniae*, all the *nif* genes are contiguous in one DNA region (Merrick, 1988). In *Azotobacter vinelandii* and *Azotobacter chroococcum* the *nif* genes are more scattered and organized in a major cluster containing *nifHDKTY*, *nifENX*, *nifUSV*, *nifWZM* and *nifF*, and another cluster containing *nifA* and *nifBQ* (Jacobson *et al.*, 1989; Joerger & Bishop, 1988; Merrick, 1988). Homology to *fixABC* was also observed in *Azotobacter* (Gubler & Hennecke, 1986; Evans *et al.*, 1988). The homology region is not located in the major cluster nor in the *nifAB* cluster (Evans *et al.*, 1988). *K. pneumoniae* does not contain genes homologous to *fixABC* (Gubler & Hennecke, 1986; Earl *et al.*, 1987). Thus, *nif* gene organization in *Azospirillum brasilense* Sp7 is closer to that in *Azotobacter* than to that in *K. pneumoniae*. From data reported here, it appears that in *Azospirillum brasilense* Sp7 most of the *nif* genes are part of one cluster. As in *Azotobacter*, this major cluster does not contain *nifB* or *nifA*, but in contrast to *Azotobacter*, homology to *fixABC* is localized in the major cluster.

A DNA region of 29 kb, containing the major *nif* cluster of *Azotobacter vinelandii*, was entirely sequenced by Jacobson *et al.* (1989) and 12 new ORFs were identified, interspersed among the identified *nif* genes. Several of these ORFs were preceded by a typical *ntr*-type promoter or were likely to be cotranscribed with known *nif* operons. However, these new ORFs may not be required for diazotrophic growth (Jacobson *et al.*, 1989). It is tempting to speculate that some of the ORFs identified in the major *Azotobacter vinelandii* *nif* cluster may be also present in *Azospirillum*. In particular, it would be interesting to determine if region III and the two insertions in region II (1413 and 1451) which impaired maximal *nif* expression corresponded to any of the ORFs found in *Azotobacter*.

In this work, identification of the *nif* loci was based on the Nif phenotype of Tn5 insertions recombined into the host genome. It can be seen in Fig. 1 that some DNA regions located between the identified loci have not been mutagenized and remain unexplored. It cannot be excluded, therefore, that *nif* loci other than those shown in Fig. 1 are present in the major *nif* cluster of *Azospirillum*.

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