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

By MARC GALIMAND,† BERTRAND PERROUD,‡ FRÉDÉRIC DELORME, ANNICK PAQUELIN, CLAIRE VIEILLE, HÉRÈ VE BOZOUKLIAN§ AND CLAUDINE ELMERICH*

Unité de Physiologie Cellulaire and URA 209 CNRS, Département des Biotechnologies, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France

(Received 29 November 1988; revised 27 January 1989; accepted 14 February 1989)

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).

† Present address: Mikrobiologisches Institut, ETH Zentrum, CH 8092 Zurich, Switzerland.
‡ Present address: Department of Biological Chemistry, University of California, Davis, CA 95616, USA.
§ Present address: CNES, 2 Place M. Quentin, 75039 Paris Cedex 01, France.

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., 1983).

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] (*A. brasilense* 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 (Franche & 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 (Franche & 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSUP202</td>
<td>pBR325</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; Cm&lt;sup&gt;a&lt;/sup&gt; T&lt;sup&gt;c&lt;/sup&gt; Mob&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pSUP202&lt;sup&gt;1&lt;/sup&gt;</td>
<td>pBR325</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; Cm&lt;sup&gt;a&lt;/sup&gt; Km&lt;sup&gt;6&lt;/sup&gt; Mob&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>ColE1</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt; Tra&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pVK100</td>
<td>pRK290</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt; T&lt;sup&gt;c&lt;/sup&gt; Mob&lt;sup&gt;a&lt;/sup&gt; cos</td>
<td>Knauf &amp; Nester (1982)</td>
</tr>
<tr>
<td>pUC18</td>
<td></td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; lacZ</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pBR322-Tn&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; T&lt;sup&gt;c&lt;/sup&gt; Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Denèfle et al. (1987)</td>
</tr>
<tr>
<td>pAB3</td>
<td>pSUP202</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; T&lt;sup&gt;c&lt;/sup&gt;; contains a 6.7 kb EcoRI fragment from <em>A. brasilense</em> Sp&lt;sup&gt;7&lt;/sup&gt;, carrying <em>nifHDK</em></td>
<td>Perroud et al. (1985); see Fig. 1</td>
</tr>
<tr>
<td>pAB10</td>
<td>pUC8</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; Km&lt;sup&gt;a&lt;/sup&gt;; contains <em>nif</em> DNA from <em>A. brasilense</em> Sp&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Elmerich et al. (1987); see Fig. 1</td>
</tr>
<tr>
<td>pAB16</td>
<td>pUC8</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; Km&lt;sup&gt;a&lt;/sup&gt;; contains <em>nif</em> DNA from <em>A. brasilense</em> Sp&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Elmerich et al. (1987); see Fig. 1</td>
</tr>
<tr>
<td>pCRA37</td>
<td>pMB9</td>
<td>T&lt;sup&gt;c&lt;/sup&gt;; contains four EcoRI fragments from <em>K. pneumoniae</em> M5al covering <em>hisDG</em> and <em>nifXUSVZMFLABQ&lt;sup&gt;(a)&lt;/sup&gt;</em></td>
<td>Cannon et al. (1977); see Fig. 4</td>
</tr>
<tr>
<td>pPC937</td>
<td>pACYC177</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt;; contains a 4.8 kb EcoRI-<em>XhoI</em> fragment from <em>K. pneumoniae</em> M5al carrying <em>nifXUSV</em> and part of <em>nifZ&lt;sup&gt;(a)&lt;/sup&gt;</em></td>
<td>Noret et al. (1985)</td>
</tr>
<tr>
<td>pGR113</td>
<td>pACYC184</td>
<td>T&lt;sup&gt;c&lt;/sup&gt;; contains a 2.2 kb EcoRI fragment from <em>K. pneumoniae</em> M5al carrying part of <em>nifE</em> and most of <em>nifN&lt;sup&gt;(a)&lt;/sup&gt;</em></td>
<td>Riedel et al. (1983)</td>
</tr>
<tr>
<td>pRS46</td>
<td>pSUP202</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt; Cm&lt;sup&gt;a&lt;/sup&gt;; contains a 13 kb <em>HindIII</em> fragment from <em>A. caulinodans</em> ORS571 carrying <em>nifHDK</em> and <em>nifE</em></td>
<td>Denèfle et al. (1987); see Fig. 4</td>
</tr>
<tr>
<td>pRS54</td>
<td>pHE3</td>
<td>T&lt;sup&gt;c&lt;/sup&gt;; contains a 9-5 kb <em>PstI</em> fragment from <em>A. caulinodans</em> ORS571 carrying <em>nifH2</em> and <em>fixABC</em></td>
<td>Noret &amp; Elmerich (1987); see Fig. 4</td>
</tr>
<tr>
<td>pRS55</td>
<td>pLA2917</td>
<td>Same insert as pRS54</td>
<td>Kaminski et al. (1988)</td>
</tr>
<tr>
<td>pRJ7128</td>
<td>pACYC184</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt;; contains a 3-4 kb <em>SmaI</em> fragment from <em>B. japonicum</em> II10 carrying <em>nifA</em> and <em>fixA</em></td>
<td>Fisher et al. (1986)</td>
</tr>
<tr>
<td>pRJ7011</td>
<td>pACYC177</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt;; contains a 3-6 kb <em>PstI</em>-<em>HindIII</em> fragment from <em>B. japonicum</em> II10 carrying <em>nifB</em></td>
<td>Ebeling et al. (1987)</td>
</tr>
</tbody>
</table>

* 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).
**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 BglII, EcoRI, HindIII, PstI and Sall. 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 KmR mutants obtained by marker exchange ranged between $10^{-5}$ and $10^{-6}$. The frequency of KmR 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 KmR 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 PstI 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 PstI 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 PstI fragment and to the appearance of 9 kb and 2.8 kb PstI fragments hybridizing to both probes (Fig. 2a, lanes 2 and 14). The Tn5 probe revealed three additional PstI 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, BamHI; Bg, BgII; H, HindIII; P, PstI; R, EcoRI; S, SalI (only two sites shown); Sm, SmaI (only one site shown); X, XhoI. 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 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 HindIII–BglII fragment part of the Km cartridge. Nitrogenase activity of the mutants in regions I, 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 HindIII 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 EcoRI–HindIII 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 PstI 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 PstI (P) restriction fragments. The two distal PstI sites are located at 0.55 kb of the extremities of the transposon. IS50, inverted repeats; NPTII/Km, localization of the neomycin phosphotransferase type I (NPTII) gene conferring resistance to kanamycin and neomycin (Rothstein et al., 1980).

Table 2. Nitrogenase activity of the KmR 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 ± SD of triplicate experiments.

<table>
<thead>
<tr>
<th></th>
<th>N-free medium</th>
<th>Plus glutamate</th>
<th>Nitrogenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp7</td>
<td>100 ± 21</td>
<td>100 ± 27</td>
<td></td>
</tr>
<tr>
<td>Region I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7309 nifH</td>
<td>12 ± 4</td>
<td>22 ± 15</td>
<td>1934 0.5 ± 0.3</td>
</tr>
<tr>
<td>7313</td>
<td>7 ± 2</td>
<td>20 ± 7</td>
<td>1935 0.8 ± 0.5</td>
</tr>
<tr>
<td>7339</td>
<td>4 ± 1.5</td>
<td>8 ± 1.5</td>
<td>1993 2 ± 1.6</td>
</tr>
<tr>
<td>7317 nifK</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>1936 0.7 ± 0.5</td>
</tr>
<tr>
<td>Region II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1413</td>
<td>60 ± 18</td>
<td>100 ± 13</td>
<td>1927 1.5 ± 1</td>
</tr>
<tr>
<td>1438</td>
<td>38 ± 16</td>
<td>14 ± 5</td>
<td>1928 0.9 ± 0.6</td>
</tr>
<tr>
<td>1451</td>
<td>95 ± 13</td>
<td>NT</td>
<td>1948 1.4 ± 0.8</td>
</tr>
<tr>
<td>1450</td>
<td>90 ± 17</td>
<td>NT</td>
<td>1986 70 ± 17</td>
</tr>
<tr>
<td>1453</td>
<td>95 ± 14</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>1429</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td></td>
</tr>
<tr>
<td>1403, 1469</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td></td>
</tr>
<tr>
<td>1462, 1447</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td></td>
</tr>
<tr>
<td>1439, 1420</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td></td>
</tr>
</tbody>
</table>
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 BglII (1) and BglII plus PvuII (2) and corresponding autoradiogram after hybridization with the 2.3 kb HindIII-SmaI 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 BamHI (1) and EcoRI plus SmaI (2) and corresponding autoradiogram after hybridization with the 5-6 kb PstI 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 SphI (1) and XhoI (2) and corresponding autoradiogram after hybridization with the 3 kb PstI 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 PstI plus BamHI (1), SalI plus XhoI (2) and SalI plus BglII (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 PstI (1), HindIII (2), SalI (3), EcoRI (4) after hybridization with a 1-9 kb SalI fragment containing nifB from B. japonicum.
nif and fix genes of Azospirillum

3 kb PstI fragment of pAB23 (Fig. 1) was used as a probe against restriction digests of pRS54, it revealed a 3.8 kb SphI fragment which covers fixABC (lane 1), a 1.4 kb XhoI fragment containing fixA and a 3 kb XhoI fragment containing fixBC (Kaminski et al., 1988; Fig. 4c). The two XhoI 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 PstI fragment (lane 1), a 3.7 kb SalI-XhoI fragment and a 4 kb SalI-BglII fragment (Fig. 4d), suggesting that a fixA homologous region is entirely contained within the 2.6 kb PstI-XhoI fragment. The same fragments plus the adjacent XhoI-BamHI fragment were revealed by the 3 kb XhoI fragment of pRS54 containing fixBC (data not shown), suggesting that fixBC overlapped the XhoI 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 SalI 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 PstI, a 14 kb HindIII, a 3 kb SalI and a 5 kb EcoRI 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 (Nair 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 (Nair 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.

The authors wish to thank Professor J.-P. Aubert for discussions, Professor H. Hennecke for the gift of plasmids, Dr D. Dean for information prior to publication and Dr A. Edelman for reading the manuscript. The authors would like to acknowledge the help of Mr A. Perrin and of Ms K. Giebeler in performing some of the experiments. B. P., F. D. and C. V. were recipients of predoctoral fellowships from the Ministère de la Recherche et de la Technologie and H. B. was the recipient of a fellowship from the École Polytechnique. This work was supported by research funds from the University Paris 7 and by a research contract from Elf Bio-Recherche, Entreprise Minière et Chimique, Rhône-Poulenc and CDF Chimie.

REFERENCES


