Partial characterization of nif genes from the bacterium Azospirillum amazonense

Abstract

Azospirillum amazonense revealed genomic organization patterns of the nitrogen fixation genes similar to those of the distantly related species A. brasilense. Our work suggests that A. brasilense nifHDK, nifENX, fixABC operons and nifA and glnB genes may be structurally homologous to the counterpart genes of A. amazonense. This is the first analysis revealing homology between A. brasilense nif genes and the A. amazonense genome. Sequence analysis of PCR amplification products revealed similarities between the amino acid sequences of the highly conserved nifD and glnB genes of A. amazonense and related genes of A. brasilense and other bacteria. However, the A. amazonense non-coding regions (the upstream activator sequence region and the region between the nifH and nifD genes) differed from related regions of A. brasilense even in nitrogenase structural genes which are highly conserved among diazotrophic bacteria. The feasibility of the 16S ribosomal RNA gene-based PCR system for specific detection of A. amazonense was shown. Our results indicate that the PCR primers for 16S rDNA defined in this article are highly specific to A. amazonense and can distinguish this species from A. brasilense.

Key words
- Azospirillum amazonense
- nif genes
- glnB
- 16S rDNA

Introduction

Bacteria belonging to the genus Azospirillum are found associated with forage grasses, cereals and agriculturally important crops from various geographical origins. This genus consists of seven species, A. lipoferum, A. brasilense, A. amazonense, A. halopraeorferens, A. irakense (1), A. largomobile (2), and A. doebereinerae (Eckert B, Baller-Weber O, Kirchhof G, Halbritter A, Stoffels M and Hartmann A, unpublished results, and GenBank accession number AJ238576). Worldwide inoculation experiments using a variety of crops have shown that Azospirillum species can contribute to the nitrogen economy (3,4). In addition, experiments with sugarcane and other crops suggest either a synergistic or an additive effect when A. amazonense is used in combination with other diazotrophics as inoculant (5).

A. amazonense differs in several important characteristics when compared to other Azospirillum species. Its nitrogenase activity has lower oxygen tolerance, its ability to use sucrose as carbon source is different, and the most remarkable difference is its sensitivity to alkaline pH (6). The genetic relationship existing between A. amazonense and other Azospirillum species was analyzed by 16S...
rDNA restriction fragment length polymorphism (7). The phylogenetic analysis, based on 16S rDNA sequences, confirmed that *A. amazonense* and *A. irakense* form one cluster and that the closely related species *A. brasilense* and *A. lipoferum* form a second cluster together with *A. halopraeferens* (8).

During the last few years, nitrogen fixation (*nif*) genes have been isolated from a variety of diazotrophic organisms. In *A. brasilense* a DNA fragment of 25,100 bp encompassing the *nif* region was shown to contain the *nifHDKORF1Y* (9), the *ORF2nifUSVORF4* (10), an *mcp*-like gene, the *nifENXORF3* and *fdxAnifQ*, and the *fixABCX* (Gross J, Vedoy C and Schrank IS, unpublished results) transcriptional units. However, to date, few genes were isolated from *A. amazonense* and none of them could be related to the biological nitrogen fixation process.

We report here the application of PCR and Southern hybridization to characterize some of the *nif* genes from *A. amazonense*. We have also analyzed the amplification products from two conserved regions within the *nifD* and *glnB* genes. Moreover, we have developed primers that specifically amplify *A. amazonense* DNA and allow us to distinguish between this species and *A. brasilense*.

**Material and Methods**

**Bacterial strains and growth conditions**

*A. brasilense* Sp7 (ATCC29145) and *A. amazonense* (ATCC35119) were grown on NFB and LGI minimal media, respectively, as previously described (6,11).

**DNA manipulation and sequence analysis**

All DNA manipulations were performed using standard techniques (12) and instructions provided by suppliers of material, enzymes or reagents. Total DNA extraction of *A. amazonense* was performed as previously described (13) and Southern blot analysis was performed with the ECL Direct Nucleic Acid Labeling and Detection Systems (Amersham Pharmacia Biotech, Uppsala, Sweden). The nucleotide sequence determination was performed by the chain-termination method of Sanger et al. (14) using 32P-dNTPs and the ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham). The PCR products were purified using the GFX PCR kit from Amersham. Analysis of DNA sequences and comparison with nucleotide and deduced protein sequences from other organisms were performed using the GCG (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI, USA) computer programs (licensed to CENARGEN-EMBRAPA, Brasília, DF, Brazil).

**Amplification conditions**

PCR amplification of the target sequences was performed using a DNA thermal cycler (MJ Research, Waltham, MA, USA). The PCR mixture contained the reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl2), 200 µM of each dNTP, 30 pmol of each primer, 1 U of *Taq* polymerase (CenBiot enzymes, Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil), template DNA, and distilled water to a final concentration of 25 µl. The reaction mixture was subjected to PCR under the following conditions: heat denaturation at 94°C for 5 min and then an additional 35 cycles with heat denaturation at 94°C for 30 s, annealing (at a temperature defined for each set of primers, see Table 2) for 30 s, and DNA extension at 72°C for 30 s. After the last cycle, samples were maintained at annealing temperature for 5 min followed by 72°C for 10 min. PCR products were analyzed by gel electrophoresis (12). Primers listed in Table 2 were purchased from Oligo ETC. & Oligo Therapeutics Inc. (Wilsonville, OR, USA).

**Results and Discussion**

**A. amazonense nif genes: hybridization and PCR**

In order to understand the molecular organization of some of the *nif* genes from *A. amazonense* and to establish their relationship with the well-known *nif* genes from *A.
We used two different approaches. Initially, DNA fragments from different nif operons already characterized in *A. brasilense* were used in Southern blot hybridization to analyze the relatedness between the two *Azospirillum* species. The hybridization patterns of *A. amazonense* DNA are shown in Figure 1. The nif structural genes from *A. amazonense* are localized in a 6.5-kb EcoRI DNA fragment (Figure 1A) as previously found for *A. brasilense* genome (13). In *A. brasilense* and other diazotrophic bacteria the nifHDK genes are organized in a single transcriptional unit (9). To determine whether the *A. amazonense* nif genes are clustered in a similar manner as found in *A. brasilense* and other nitrogen-fixing organisms, we used sequences from nif genes (Table 1) from *A. brasilense* as DNA hybridization probes. Three different probes, which consist of the entire nifHDK operon (data not shown), the nifH gene alone (data not shown) from *A. brasilense*, and a region of the nifD gene (Table 1) from *A. amazonense*, showed homology with the same DNA fragment from *A. amazonense* (Figure 1A, lane 1E). This result suggests that the region comprising a 6.5-kb EcoRI DNA fragment probably contains the nifHD homologous in *A. amazonense* and that the genes are organized in a single operon. Nevertheless, the hybridization pattern of the nifD gene amplified from *A. amazonense* revealed a different restriction pattern when *A. amazonense* and *A. brasilense* total DNA was digested with PstI and SalI restriction enzymes (Figure 1A, lanes 1P and 1S). A hybridization signal specific to SalI DNA fragments was obtained when *A. amazonense* total DNA was hybridized with *A. brasilense* nifENX and fixABC probes, respectively (Figure 1B, lane 1 and Figure 1C, lane 1).

In order to understand the nature of the *A. amazonense* genes that regulate nitrogen fixation we have isolated and characterized the nifA and glnB genes, which are responsible for the regulation of other nif genes and operons in *A. brasilense* (15,16). The presence of genes homologous to glnB and nifA was detected by hybridization of *A. amazonense* DNA with two different probes (Table 1). The entire nifA gene from *A. brasilense* is present within a 2.5-kb SalI DNA fragment (15). Heterologous hybridization revealed that the nifA homologue in *A. amazonense* is also found in a SalI DNA fragment of approximately 3 kb (Figure 1D, lane 1).

The glnB homologous gene was local-
ized within a 4.5-kb PstI DNA fragment from *A. amazonense* (Figure 1E, lane 1P). Faint hybridization signals were detected in *A. amazonense* total DNA and also with *Pst*I-digested *A. brasilense* total DNA (Figure 1E, lane 2). Genes homologous to *glnB* have been found in *A. brasilense*, *Herbaspirillum seropedicae* and other bacteria, suggesting the presence of two copies of *glnB*-like genes in these organisms (17, 18). Since no *Pst*I restriction site was found within *glnB* or *glnZ* genes from *A. brasilense* or in the *glnB* gene from *A. amazonense*, the presence of two hybridization fragments in both strains may represent similarity between the probe and the two *glnB*-like genes.

The results presented here are the first to reveal homology between *A. brasilense* *nif* genes and *A. amazonense* total DNA. Taken together, these results suggest that the *nifHDK*, *nifENX* and *fixABC* operons and the *nifA* and *glnB* genes may be structurally homologous to *A. amazonense* counterpart genes.

The second approach to the understanding of the nature of nitrogen fixation in *A. amazonense* was based on PCR amplification of regions within the *niffix* genes. Several primers were selected from the sequences of the *niffix* genes from *A. brasilense*. The primers listed in Table 2 represent, except for *nifA*, those that revealed positive and conclusive results after PCR amplification and DNA sequencing. Three sets of primers were designed from regions within the *A. brasilense* *nifHDK* operon. The promoter region of this operon shows two overlapping upstream activator sequences (UAS) as the only potential NifA-binding sites (19). To date this organization seems to be unique to *A. brasilense* and may be an atypical NifA-binding site interacting with two dimers of NifA, as proposed to occur in other *nif* promoters (20). UAS primers define a region of approximately 200 bp in the *nifH* promoter sequence encompassing the two overlapping UAS. The second pair of IGR primers is derived from the intergenic region between *nifH* and *nifD* and amplifies a 310-bp region. In *A. brasilense* three inverted repeat structures have been found downstream from the *nifH* stop codon (9). Transcription analysis revealed the presence of one 1.1-kb transcript corresponding to the *nifH* gene (21). The *nifD* primer pair is specific for a 710-bp region of the *nifD* gene containing a highly conserved amino acid sequence and a serine residue which is present only in the *nifD* gene of *A. brasilense* (9).

Amplification of *A. amazonense* DNA with the above selected primers (Table 2) resulted in fragments of the predicted size (Figure 2A,B,C). All three amplified DNA

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### Table 1. Plasmids and probes.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifHDK</td>
<td>6.5-kb EcoRI <em>A. brasilense</em> DNA fragment (nifHDKORFLY operon)</td>
<td>9</td>
</tr>
<tr>
<td>nifH</td>
<td>2.8-kb EcoRI/PstI <em>A. brasilense</em> DNA fragment (nifHDKORFLY operon)</td>
<td>9</td>
</tr>
<tr>
<td>nifD</td>
<td><em>A. amazonense</em> PCR fragment</td>
<td>Present study</td>
</tr>
<tr>
<td>glnB/Py</td>
<td><em>A. amazonense</em> PCR fragment</td>
<td>Present study</td>
</tr>
<tr>
<td>nifA</td>
<td>2.5-kb Sall <em>A. brasilense</em> DNA fragment (nifA gene)</td>
<td>Pedrosa F (with permission)</td>
</tr>
<tr>
<td>nifEN</td>
<td>2.2-kb EcoRI/HindIII <em>A. brasilense</em> DNA fragment (nifENX operon)</td>
<td>Potrich D, Bressel T, Schrank IS and Passaglia L (unpublished results)</td>
</tr>
<tr>
<td>fixABC</td>
<td>3.0-kb PstI <em>A. brasilense</em> DNA fragment (fixABCX operon)</td>
<td>Gross J, Vedoy C and Schrank IS (unpublished results)</td>
</tr>
</tbody>
</table>

### Table 2. Oligonucleotide primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
<th>Length (bp)</th>
<th>Amplification (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS1</td>
<td>5’CCTGACGCTGCTGACGCTGG</td>
<td>23</td>
<td>200</td>
<td>52⁰C</td>
</tr>
<tr>
<td>UAS2</td>
<td>5’CGCGAAGCTGCACGACGCA</td>
<td>23</td>
<td></td>
<td>52⁰C</td>
</tr>
<tr>
<td>IGR-up</td>
<td>5’ATCCCGACCCCGATACGAT</td>
<td>21</td>
<td>310</td>
<td>52⁰C</td>
</tr>
<tr>
<td>IGR-do</td>
<td>5’CTTCTGGGATACGCTTC</td>
<td>18</td>
<td></td>
<td>52⁰C</td>
</tr>
<tr>
<td>nifD-up</td>
<td>5’ATCATGGTGACTACAAC</td>
<td>18</td>
<td>710</td>
<td>52⁰C</td>
</tr>
<tr>
<td>nifD-do</td>
<td>5’ATCATCTGGGCGGGGAA</td>
<td>17</td>
<td></td>
<td>52⁰C</td>
</tr>
<tr>
<td>nifZ-up</td>
<td>5’CGCGCGAGAGCGCGAC</td>
<td>18</td>
<td></td>
<td>42⁰C</td>
</tr>
<tr>
<td>nifZ-do</td>
<td>5’GCACCTGCTCCGACGCG</td>
<td>18</td>
<td></td>
<td>56⁰C</td>
</tr>
<tr>
<td>glnB-up</td>
<td>5’GCCATATTTAGCCGGCTTCA</td>
<td>20</td>
<td>250</td>
<td>56⁰C</td>
</tr>
<tr>
<td>glnB-do</td>
<td>5’AGATCTTGGCCGTGCAGC</td>
<td>20</td>
<td></td>
<td>56⁰C</td>
</tr>
<tr>
<td>16S-up</td>
<td>5’TGATGGTTTGGAGACTGTCAG</td>
<td>22</td>
<td>400</td>
<td>52⁰C</td>
</tr>
<tr>
<td>16S-do</td>
<td>5’ATTTCAGCGCTGACCTAAAACA</td>
<td>21</td>
<td></td>
<td>52⁰C</td>
</tr>
</tbody>
</table>

UAS = upstream activator sequences; IGR = intergenic region; na = no amplification; do = downstream; up = upstream.
fragments were used to probe total *A. amazonense* DNA, revealing homology with the same *Eco*RI fragment encompassing the *nifHD* genes (Figure 1A). To further characterize the PCR products, all DNA fragments were purified and sequenced. The sequences for the UAS and IGR products showed very little similarity to the *A. brasilense* counterparts (data not shown). Only the region amplified within the *nifD* gene is similar to other *nifD* gene sequences (discussed below). These results indicate that the *A. amazonense* non-coding regions described above differ from related *A. brasilense* regions even among nitrogenase structural genes which are highly conserved among diazotrophic bacteria.

A set of primers was designed (Table 2) from previously published sequences of *nifA* and *glnB* genes from *A. brasilense* (15,22). A PCR product of approximately 250 bp was detected in *A. amazonense* DNA (Figure 2D, lane 1). This product hybridized with total *A. amazonense* DNA (Figure 1E, lanes 1P and 1S) and, after DNA sequencing, revealed homology with P II proteins from different organisms (discussed below). Surprisingly, no amplification product was visualized when *nifA* target primers were applied to *A. amazonense* DNA although these primers were designed on the basis of a highly conserved region found among NifA proteins from *A. brasilense*, *A. lipoferum* and related bacteria. This unexpected result suggests that the *A. amazonense* *nifA* gene may have greater differences in DNA sequence than found among other *nifA* genes previously characterized.

### Amplification of *A. amazonense* 16S rDNA

To overcome problems with misinterpretation of the PCR results, a PCR amplification system was developed to specifically detect *A. amazonense*. The 16S rRNA sequence from *Azospirillum* species was obtained from the GenBank database. Multiple sequence alignments to other 16S rRNA gene sequences revealed one region of considerable sequence divergence from the closest relatives, as shown in Figure 3. Two primers (16S-up and 16S-do) were constructed, one based on the variable region and the other based on a lower sequence divergence region (Table 2). The PCR product generated with *A. amazonense* was 400 bp long (Figure 2E, lane 2) and was confirmed by direct sequencing (Figure 3). No PCR product was detected when *A. brasilense* DNA was the template (Figure 2E, lane 3). Using this methodology we could specifically detect and differentiate DNA templates from these two *Azospirillum* species.

### Interspecies conservation of *nif/gln* genes

A sequence comparison of the *A. amazonense* and *A. brasilense* *nifD* genes revealed a high degree of similarity at both the DNA and amino acid levels (Figure 4). One relevant difference between *A. amazonense* and *A. brasilense* NifD proteins is located at position 275 (relative to *A. brasilense* NifD sequence; 9). The *A. brasilense* *nifD* gene product contains a total of five cysteine residues, four of which are highly conserved...
Figure 3 continued on the next page
among the other nifD genes with respect to both positions and adjacent sequences (9). Of the five conserved cysteine residues proposed to act as ligands to the iron-clusters of dinitrogenase, the cysteine at position 275 is substituted by a serine on the A. brasilense predicted sequence (Figure 4) (9). The highly conserved nifD region amplified from A. amazonense shows a cysteine residue at position 275 (Figure 4), suggesting that the substitution observed in the A. brasilense nifD gene is not conserved among other Azospirillum species and appears to be unique among diazotrophic bacteria.

The regulation of nitrogen fixation in A. lipoferum and A. amazonense is less well understood than in A. brasilense. The regulation is controlled both transcriptionally and post-translationally in A. brasilense and A. lipoferum (23). In A. brasilense, the expression of the nifHDK operon is positively regulated by NifA. In this bacterium nifA is expressed under conditions both compatible and incompatible with nitrogen fixation. NifA activity is modulated by the PII protein (encoded by glnB), the intracellular signal transmitter, in response to the nitrogen status of the cell. The glnB gene of A. lipoferum was isolated, but the PII protein has not yet been characterized (24). The isolation of part of the glnB gene from A. amazonense is the first evidence that the nitrogen regulatory path-
The occurrence of duplicate copies of genes encoding PII-like proteins now appears to be common among members of the \( \alpha \) (Azospirillum) and \( \gamma \) (Klebsiella) subdivisions of the Proteobacteria class. In \( A. \) brasilense, PII and PZ proteins are involved differently in nitrogen-dependent regulation of various physiological functions (25). The PII amino acid sequence is about 60% identical to that of PZ and could explain the detection of two hybridization signals in \( A. \) brasilense and \( A. \) amazonense Southern blots (Figure 1E, lane 1S and lane 2).

In this report, we present distinct lines of evidence showing that the nif gene organization and regulation in \( A. \) amazonense differ, in some aspects, from those of the best characterized \( A. \) brasilense. Although our results indicate the presence of counterparts of genes nifHDK, nifENX, fixABC, nifA, and glnB in \( A. \) amazonense, we found differences in restriction sites and non-coding sequences suggesting that the organization of these genes may differ from that of the related \( A. \) brasilense. These results support the suggestion by Fani et al. (8) that \( A. \) amazonense isolates may be members of a taxonomic cluster that is clearly distinct from the closely related \( A. \) brasilense and \( A. \) lipoferum species.

Acknowledgments

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