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# Partial characterization of *nif* genes from the bacterium *Azospirillum amazonense*

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## 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*.

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- *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. halopraeferens*, *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(2), and *A. doebereinerae* (Eckert B, Baller-Weber O, Kirchhof G, Halbritter A, Stoffels M and Hartmann A, unpublished results, and GenBank accession number AJ238576).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 *nifENXORF3,5*, *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 $^{33}$P-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 $MgCl_2$), 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.*

*brasilense*, 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 *Eco*RI 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 *Eco*RI 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 *Pst*I and *Sal*I restriction enzymes (Figure 1A, lanes 1P and 1S). A hybridization signal specific to *Sal*I 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 *Sal*I DNA fragment (15). Heterologous hybridization revealed that the *nifA* homologue in *A. amazonense* is also found in a S*al*I DNA fragment of approximately 3 kb (Figure 1D, lane 1).

The *glnB* homologous gene was local-

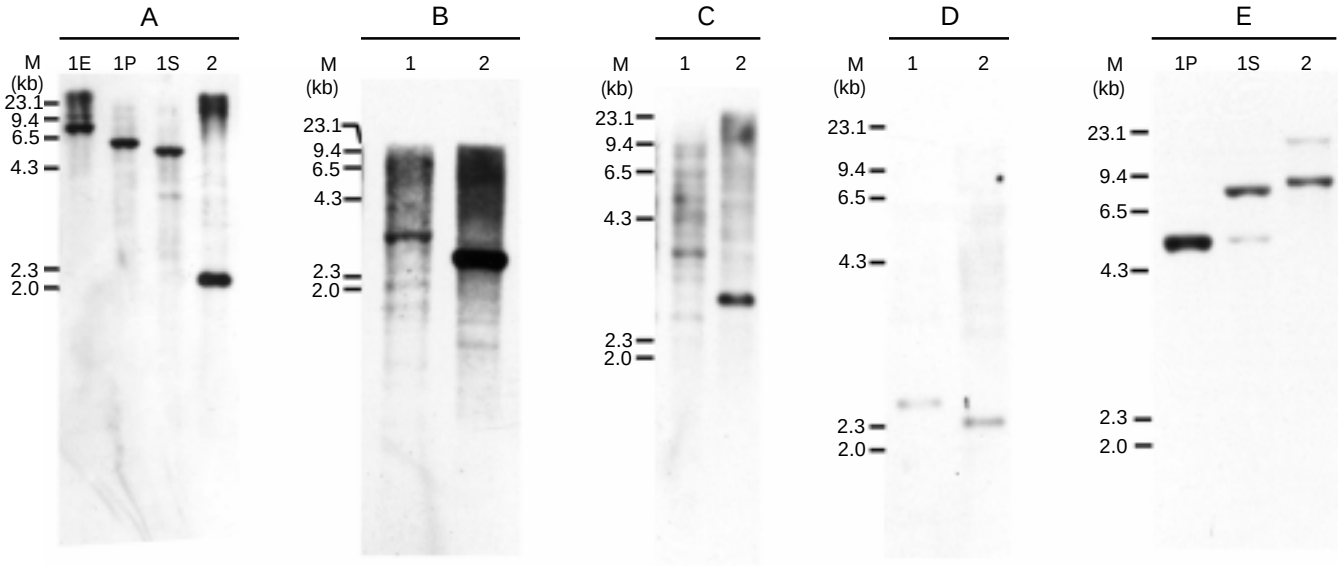


Figure 1. Hybridization pattern of *Azospirillum amazonense* total DNA with *Eco*RI (1E), *Pst*I (1P), and *Sal*I (1S) in *A*, *Sal*I in *B*, *C* and *D*, and *Pst*I (1P) and *Sal*I (1S) in *E* with the following probes: *nifD* in panel A; *nifEN* in panel B; *fixABC* in panel C; *nifA* in panel D, and *glnB* in panel E. Lane 2 represents *A. brasilense* total DNA digested with *Pst*I in *A*, *C* and *E*, or *Sal*I in *B* and *D*. Lane M contains λ *Hin*dIII as molecular marker.

ized within a 4.5-kb *Pst*I 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. amazonens*e 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 *nif*/*fix* genes. Several primers were selected from the sequences of the *nif*/*fix* 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

Table 1. Plasmids and probes.

| Probe | Characteristics | Reference |
|---|---|---|
| *nifHDK* | 6.5-kb *Eco*RI *A. brasilense* DNA fragment (*nifHDKORF1Y* operon) | 9 |
| *nifH* | 2.8-kb *Eco*RI/*Pst*I *A. brasilense* DNA fragment (*nifHDKORF1Y* operon) | 9 |
| *nifD* | *A. amazonense* PCR fragment | Present study |
| *glnB*/$P_{II}$ | *A. amazonense* PCR fragment | Present study |
| *nifA* | 2.5-kb *Sal*I *A. brasilense* DNA fragment (*nifA* gene) | Pedrosa F (with permission) |
| *nifEN* | 2.2-kb *Eco*RI/*Hin*dIII *A. brasilense* DNA fragment (*nifENX* operon) | Potrich D, Bressel T, Schrank IS and Passaglia L (unpublished results) |
| *fixABC* | 3.0-kb *Pst*I *A. brasilense* DNA fragment (*fixABCX* operon) | Gross J, Vedoy C and Schrank IS (unpublished results) |

Table 2. Oligonucleotide primers.

| Primer | Oligonucleotide sequence | Length (bp) | Amplification size (bp) | Annealing temperature |
|---|---|---|---|---|
| UAS1 | 5'CCTGACGCTGGCTCTGACGCTGG | 23 | 200 | 52°C |
| UAS2 | 5'CGCGAACTGGCACGGGGGATGCA | 23 | | |
| IGR-up | 5'ATCCCGACCCCGATCACGATG | 21 | 310 | 52°C |
| IGR-do | 5'CTTCTCGGGATACGCTTC | 18 | | |
| nifD-up | 5'ATCATCGGTGACTACAAC | 18 | 710 | 52°C |
| nifD-do | 5'ATCCATGTCGCGGCGAA | 17 | | |
| nifA-up | 5'CGCGGCGAGAGCGGCACC | 18 | na | 42°C |
| nifA-do | 5'GCGCCGTTCGCGCAGCGG | 18 | | |
| glnB-up | 5'GCCATCATTAAGCCGTTCAA | 20 | 250 | 56°C |
| glnB-do | 5'AAGATCTTGCCGTCGCCGAT | 20 | | |
| 16S-up | 5'TGATGGTTGTGGAGACTGTCAG | 22 | 400 | 52°C |
| 16S-do | 5'ATTTCACGCCTGACTTAAACA | 21 | | |

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

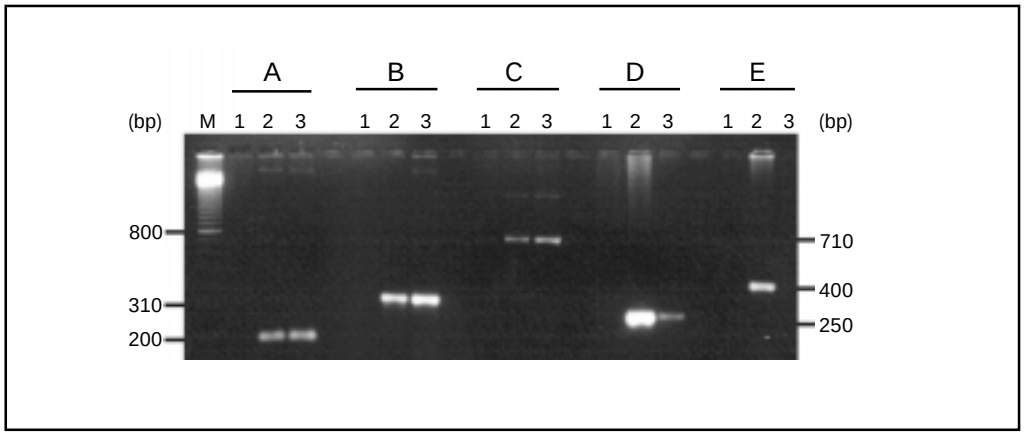


Figure 2. Specific PCR for amplification of *nif* genes from *Azospirillum amazonense*. Amplified products on 1.5% agarose gel were visualized with a UV transilluminator after ethidium bromide staining using the following primers: *panel A*, UAS; *panel B*, IGR; *panel C*, nifD; *panel D*, glnB, and *panel E*, rRNA from *A. amazonense* (lane 2) and *A. brasilense* (lane 3) total DNA. The negative control (lane 1) contains no template DNA in the reaction mixture. Lane M contains the 100-bp DNA ladder (Gibco/BRL).

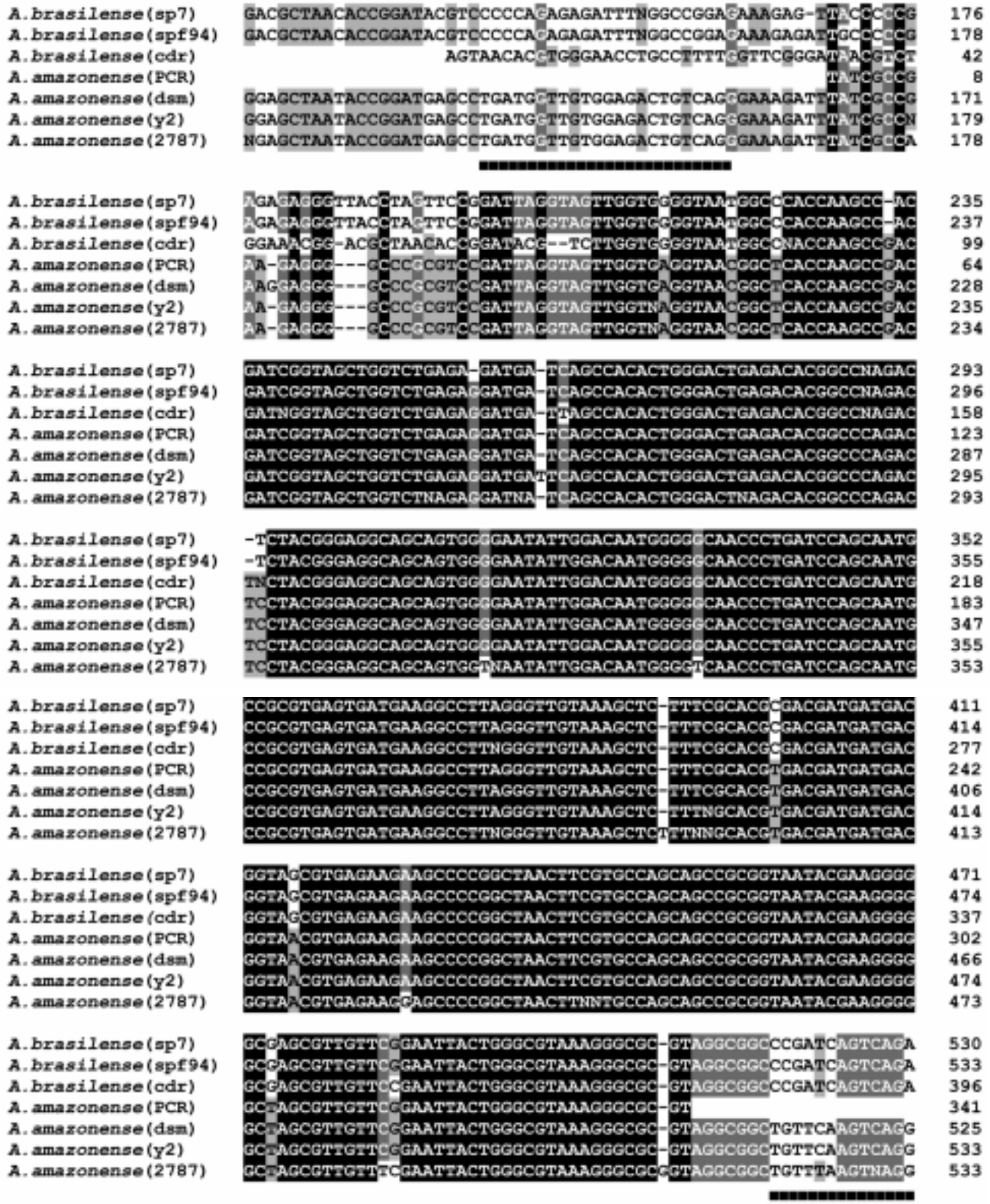


Figure 3 continued on the next page

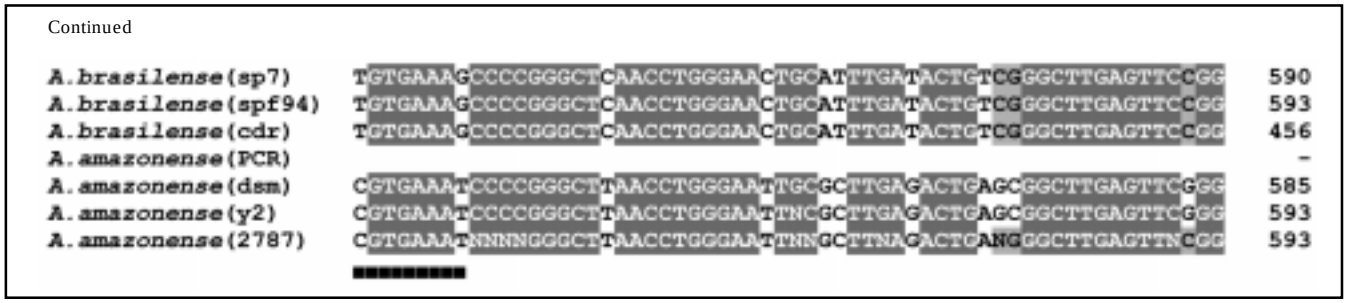


Figure 3. Multiple sequence alignment of the 16S rDNA genes from *Azospirillum amazonense* (GenBank accession Nos. X79735, Z29616, X79742) and the distantly related *A. brasilense* Sp7 (GenBank accession Nos. X79732, 79739, 79740). *A. amazonense* (PCR) represents the PCR DNA sequence obtained in the present study. The primers used here for the PCR amplification experiments are underlined. A black background indicates conserved residues in all aligned sequences, a dark grey background indicates conserved residues in at least 80% of the aligned sequences, and a light grey background indicates conserved residues in at least 60% of the aligned sequences. Multiple alignments were done with the PILEUP program, University of Wisconsin Genetics Computer Group, and the alignment editing was done using the GENDOC program considering the score table Dayhoff PAM 250 (26,27).


Figure 4. *Azospirillum amazonense nifD* protein partial sequence alignment with *A. brasilense* (GenBank accession No. M64344), *Herbaspirillum seropedicae* (GenBank accession No. Z54207) and *A. vinelandii* (GenBank accession No. M20568) NifD proteins. A grey background indicates similarity including the conserved amino acid substitutions while using the *A. amazonense* sequence as reference sequence. Amino acid-derived sequence from DNA sequencing obtained with primer nifD-up (A) and with primer nifD-do (B). The cysteine residue 275 is marked with an asterisk. Multiple alignments were done with the PILEUP program, University of Wisconsin Genetics Computer Group, and the alignment editing was done with the GENDOC program considering the Dayhoff PAM 250 score table (26,27).

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 $P_{II}$ 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 $P_{II}$ 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-

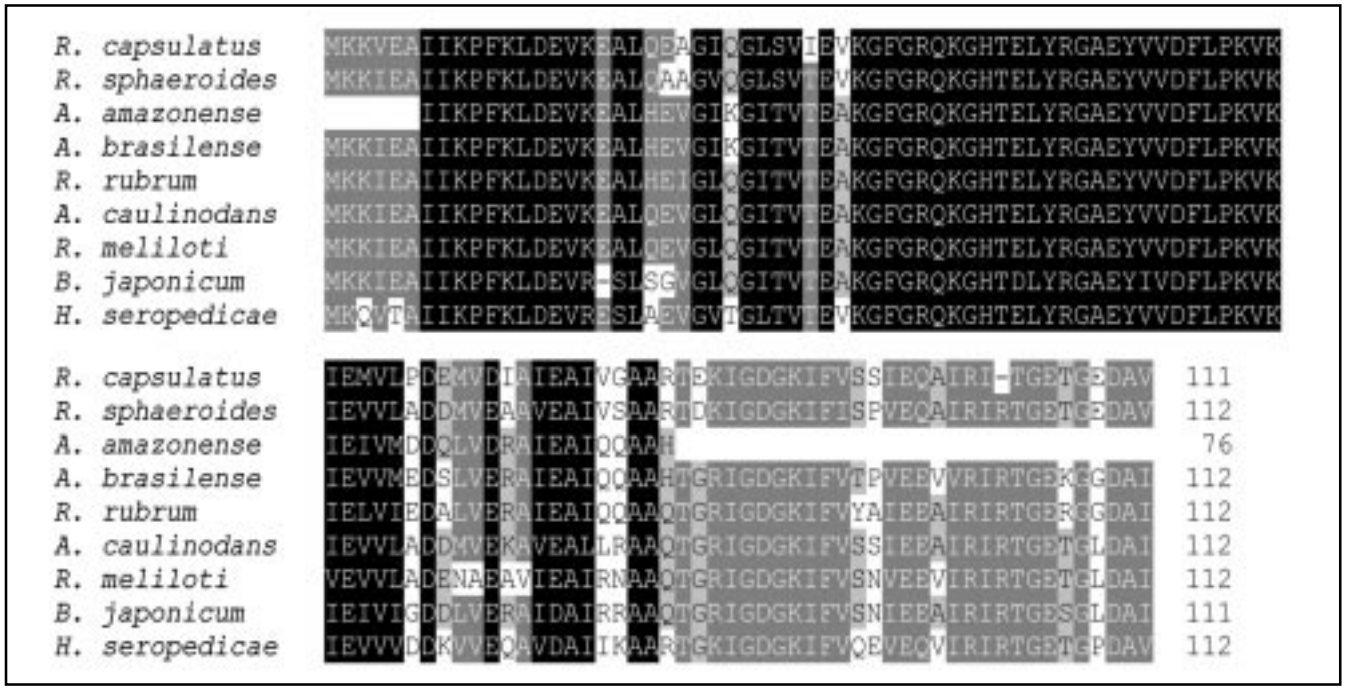


Figure 5. *Azospirillum amazonense glnB* partial deduced amino acid sequence alignment with *A. brasilense* (GenBank accession No. X51499), *Rhodobacter sphaeroides* (GenBank accession No. AF032116), *R. capsulatus* (GenBank accession No. U25953), *Rhodospirillum rubrum* (GenBank accession No. X84158), *Azorhizobium caulinodans* (GenBank accession No. Y10213), *Rhizobium meliloti* (GenBank accession No. U50385), *Bradyrhizobium japonicum* (GenBank accession No. M26753), and *Herbaspirillum seropedicae* (GenBank accession No. U86073) $P_{II}$ proteins. A black background indicates conserved residues in all aligned sequences, a dark grey background indicates conserved residues in at least 80% of the aligned sequences, and a light grey background indicates conserved residues in at least 6% of the aligned sequences. Multiple alignments were done with the PILEUP program, University of Wisconsin Genetics Computer Group, and the alignment editing was done with the GENDOC program considering the Dayhoff PAM 250 score table (26,27).

way of this microorganism may be similar to that of other *Azospirillum* species. The $P_{II}$ protein from *A. amazonense* revealed a high level of similarity when compared to $P_{II}$ from other diazotrophic bacteria (Figure 5).

The occurrence of duplicate copies of genes encoding $P_{II}$-like proteins now appears to be common among members of the $\alpha$ (*Azospirillum*) and $\gamma$ (*Klebsiella*) subdivisions of the Proteobacteria class. In *A. brasilense*, $P_{II}$ and $P_Z$ proteins are involved differently in nitrogen-dependent regulation of various physiological functions (25). The $P_{II}$ amino acid sequence is about 60% identical to that of $P_Z$ 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.

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