Directed mutagenesis affects recombination in *Azospirillum brasilense* nif genes

C.P. Nunes¹, L.M.P. Passaglia², A. Schrank¹ and I.S. Schrank¹

**Abstract**

In order to improve the gene transfer/mutagenesis system for *Azospirillum brasilense*, gene-cartridge mutagenesis was used to replace the nif/D gene with the Tn5 kanamycin resistance gene. The construct was transferred to *A. brasilense* by electrotansformation. Of the 12 colonies isolated using the suicide plasmid pSUP202 as vector, only four did not show vector integration into the chromosome. Nevertheless, all 12 colonies were deficient in acetylene reduction, indicating an Nif⁻ phenotype. Four Nif⁻ mutants were analyzed by Southern blot, using six different probes spanning the nif and Km' genes and the plasmid vector. Apparently, several recombination events occurred in the mutant genomes, probably caused mainly by gene disruption owing to the mutagenesis technique used: resistance gene-cartridge mutagenesis combined with electrotansformation.

**INTRODUCTION**

*Azospirillum brasilense* can grow diazotrophically using a molybdenum-dependent nitrogenase. Nitrogenase, the enzyme that catalyses biological nitrogen fixation, consists of two protein components: iron and molybdenum iron. Native Fe protein is a homodimer of approximately 68-kDa subunits, while the MoFe protein is a tetrameric complex with four [4Fe-4S] centers and two iron-molybdenum cofactors (Dixon, 1984; Haaker and Veeger, 1984). In most N₂-fixing organisms, the nitrogenase structural genes are organized in a single operon and transcribed in the order nifH-D-K (Arnold et al., 1988; Jacobson et al., 1989; Willson et al., 1993). The nifH gene codes for subunits of the Fe protein and for the MoFe protein nifD, and nifK genes code for α and β subunits, respectively. Transcription of these genes, in general, is repressed by both NH₄⁺ and O₂, and occurs only under nitrogen-limiting conditions (Nelson and Knowles, 1978; Postgate and Cannon, 1981).

*Azospirillum* spp. fix nitrogen under free-living conditions and in association with grasses. The nif structural genes from *A. brasilense* have been sequenced and present the same sequential organization found in other nitrogen-fixing bacteria (Passaglia et al., 1991). Analysis of the molecular genetics of nitrogen fixation in *A. brasilense* revealed a 45-kb DNA region, comprised of the nif/ENXO RF3ORF5ORF6OQ, ORF2nifUSVORF4 and fixABC operons located, 3, 11, and 15 kb, respectively, downstream from the nifHDKORF1Y operon (Passaglia, L., Frazzon, J. and Vedoy, C., unpublished results).

After the report of Elmerich and Franche (1982) on Tn5-induced auxotroph mutants in *A. brasilense*, different protocols have been suggested for increasing the efficiency of transposon mutagenesis in this organism. The suicide plasmid pSUP202 was used successfully to deliver Tn5 into the *Azospirillum* genome (Singh and Klingmüller, 1986; Abdel-Salam and Klingmüller, 1987; Faure et al., 1994). Plasmids of the P incompatibility group can also be transferred to *A. brasilense*, and plasmid pRK290 has been used as a vector to isolate Nif⁺ mutants of *A. brasilense* (Jara et al., 1983).

We established an efficient gene disruption system for inducing site-specific mutations in *A. brasilense*, and then used it to isolate NifD mutants.

**MATERIAL AND METHODS**

Bacterial strains, growth conditions and nitrogenase derepression

Several *E. coli* strains and one *A. brasilense* strain were used (Tables I and II). Growth of *E. coli* strains carrying either hybrid nif-containing plasmids or vectors, and preparation, restriction enzyme digestion, and ligation of hybrid plasmid DNAs were performed as described previously (Sambrook et al., 1989). The wild-type and mutant strains of *A. brasilense* were cultured in Nfb medium (Ditta et al., 1980) supplemented with ammonium chloride to a final concentration of 40 mM, when a fixed source of nitrogen was introduced into the medium. For nitrogenase synthesis induction, all cultures were grown in nitrogen-free Nfb medium for 24 h. *In vivo* nitrogenase activity was measured by C₂H₂ reduction in nitrogen-free, semi-solid Nfb medium (0.175% agar) (Nelson and Knowles, 1978).

Bacterial mating and electroporation

The *A. brasilense* cultures were grown overnight at 30°C in LB broth to a density of 10⁶ cells/ml. The *E. coli*
donor cells were grown at 37°C overnight in LB broth, diluted 10-fold, and grown for another 2 h to exponential phase. Samples of donor and acceptor cells (1:3 ratio) were passed through nitrocellulose filters, placed on LB agar plates, and incubated for 16 h at 30°C. Filters containing either donor or recipient cells were incubated as controls. Conjugal and control growth patches were resuspended in 0.85% NaCl solution, washed several times, diluted, and spread on selective plates. The pRK290X derivative was mobilized into A. brasilense by triparental mating, as described by Ditta et al. (1980). Mutants, in which the wild-type region is to be replaced with the mutated DNA fragment, should be obtained by introducing the IncP plasmid pPH1JI into the pKm6X-carrying A. brasilense strain, followed by selection on both kanamycin and gentamycin. Km’ exconjugants were selected on minimal Nfb medium supplemented with NH₄⁺ and 30 µg/ml of kanamycin.

Electroporation of A. brasilense was carried out using the Gene Pulser Apparatus (Bio-Rad). The procedure used was based on the method described by Vande Broek et al. (1989). The pSUP202 suicide derivative pSUP6 was electrotransferred to A. brasilense and transformants were selected for kanamycin resistance. Plasmid pRK290X was used as control.

Hybridization procedures

32P-labelled probes were prepared by nick translation (Sambrook et al., 1989). Southern hybridization was carried out at 68°C under conditions described previously.

### Table I - Bacterial strains used in the present study.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TG2</td>
<td>supE, hsdD5, thi, D(lac-proAB), (srl-recA) 306::Tn10(tet')</td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td></td>
<td>S17.1</td>
<td>recA, thi, pro, hsdR, hsdM, Sm'</td>
<td>Simon et al., 1983</td>
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<td>Azospirillum brasilense</td>
<td>Sp7</td>
<td>Amp', wild-type</td>
<td>ATCC29145</td>
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<tr>
<td></td>
<td>Sp7Nif'10</td>
<td>Amp', Km', nifD-mutant</td>
<td>This work</td>
</tr>
</tbody>
</table>

### Table II - Plasmids used in the present study.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRK290X</td>
<td>Tc', incP, Tra'</td>
<td>Alvarez-Morales et al., 1986</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km', colicine E1, Tra'</td>
<td>Ditta et al., 1980</td>
</tr>
<tr>
<td>pPH1JI</td>
<td>Gm', incP, Tra'</td>
<td>Ditta et al., 1980</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Tc', Amp', Cm', Mob'</td>
<td>Simon et al., 1983</td>
</tr>
<tr>
<td>pAbc6</td>
<td>pACYC184 + 6.5-kb EcoRI nifHDK region</td>
<td>This lab.</td>
</tr>
<tr>
<td>pKm6X</td>
<td>Tc', Km', incP, Tra'</td>
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<td>pSUP6</td>
<td>Tc', Km', Amp', Mob'</td>
<td>This paper</td>
</tr>
<tr>
<td>pKm6</td>
<td>Tc', Km'</td>
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</tr>
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</table>

A. brasilense nitrogenase structural genes have already been isolated, and their complete nucleotide sequence has been determined. These genes are clustered and arranged in the nifHDKorf1Y operon. To construct pKm6, DNA from the pAbc6 plasmid was digested with PsI and the 2.4-kb nifD DNA fragment was replaced with a 1.9-kb PsI DNA fragment originating from partially digested Tn5 DNA (Beck et al., 1982). The 6.0-kb EcoRI DNA fragment, carrying the Km’-cartridge from the pKm6 (nifHkmK) plasmid, was subcloned into either EcoRI-digested pRK290X or pSUP202 plasmid vectors, generating plasmids pRKm6X and pSUP6, respectively.

**RESULTS AND DISCUSSION**

Isolation of A. brasilense nif mutants

The A. brasilense nitrogenase structural genes are clustered and arranged as follows: promoter-nifH-nifD-nifK in a 6.5-kb EcoRI DNA fragment (Figure 1). This DNA fragment was originally cloned into an EcoRI-digested pACYC184 vector yielding pAbc6 (Araújo et al., 1988). Since pACYC184 has no PsI sites, it was possible to replace the nifD gene with a Km’-cartridge from Tn5 (Figure 1). In the resulting pKm6 plasmid, the Km’-cartridge is flanked by 1.7-kb and 2.3-kb DNA fragments from the A. brasilense nifHDK cluster. The size of these fragments should be sufficient to direct homologous recombination in A. brasilense genome, yielding Nif’ mutants with a deleted nifD gene. To date, mutagenized plasmids have been transferred to A. brasilense only by conjugation (Elmerich and Franche, 1982; Singh and Klingmüller, 1985; Vanstockem et al., 1987). However, Vande Broek et al. (1989) developed an electroporation protocol for DNA delivery into A. brasilense. We used both conjugation and electroporation techniques to isolate Nif’ mutants from A. brasilense.

Typical mating conditions are outlined in Material and Methods. The A. brasilense strain used as DNA acceptor...
Azospirillum brasilense nifD gene disruption

Is resistant to ampicillin up to 200 µg/ml and very sensitive to kanamycin (less than 5 µg/ml). Spontaneous mutants resistant to kanamycin (25 µg/ml) were undetected in control experiments. In order to generate A. brasilense NifD mutants we used two different plasmids: pRKm6X and pSUP6. In the first experiment, nifHKm mutagenized genes were transferred to the wild-type strain and the stable replicating vector (pRK290X) was removed using another plasmid belonging to the same incompatibility group (pPH1), thus making it easier to detect the mutagenized phenotype. Azospirillum exconjugants bearing pRKm6 were isolated on Nfb medium containing 30 µg/ml kanamycin at maximum frequencies of 10⁸ per recipient cell. Several experiments of triparental mating were carried out to remove the replicating vector and to generate Nif- mutants. A. brasilense Sp7 carrying pRKm6X was used as acceptor and E. coli strains JA221, carrying pPH1 (Gm r ), and JA221, carrying pRK2013, were used as donors. Approximately 60,000 colonies were screened for Km r and Gm r transconjugants, all of which were also resistant to tetracycline (Tc r ), indicating that pRKm6X had integrated into the A. brasilense chromosome (cointegrate formation). Singh and Klingmüller (1986) have also reported a failed attempt to isolate Nif- mutants using the stable replicative plasmid vector pRK290. In addition, we used vectors which were unable to replicate in the recipient bacterium and which were lost after transfer into the recipient cell.

Successful mutagenesis of A. brasilense using conjugation methods has been reported previously (Elmerich and Franche, 1982; Abdel-Salam and Klingmüller, 1987; Singh and Klingmüller, 1986; Vanstockem et al., 1987), and has been achieved using either stable replicative or suicide vectors and transposon Tn5 as the mutagenic element. We failed to isolate mutants from A. brasilense using this method. However, we then used a resistance gene-cartridge mutagenesis technique instead of transposon Tn5, which involved the cloning of a DNA fragment containing an antibiotic resistance gene into the genes to be mutagenized. Since the Km'-cartridge is unable to transpose, no interference occurs due to further transposition in the recipient cell genome.

Transformation of Azospirillum by electroporation has proven to be an efficient method for DNA transfer in this bacterium (Vande Broek et al., 1989), and the feasibility of electrotransformation of A. brasilense Sp7 with pSUP6 was analyzed as outlined in Material and Methods, using the broad host range plasmid pRK290X as control throughout the study. Under these conditions 12 Ap r plus Km r colonies were obtained. To determine the integration of the Km'-cartridge into the A. brasilense chromosome, we assayed the colonies for tetracycline resistance. Four out of 12 colonies tested did not acquire pSUP202-encoded tetracycline resistance, confirming the absence of the vector DNA which was further confirmed by the absence of hybridization between total DNA from the transformants and 32P-labelled pSUP202 plasmid DNA. The remaining eight colonies were Tc r, indicating that, since this marker is coded by a non-transposable gene present in the pSUP202

Figure 1 - Strategy for constructing the gene replacement cartridge. A) Physical map of the Azospirillum brasilense nif structural gene cluster. B) Partial physical map of the Tn5 transposon. C) Physical map of the pKm6 plasmid containing the nifD gene replaced with the km r-cartridge.
vector, pSUP202 had been integrated into the genome. This
was confirmed by the absence of free plasmid DNA corre-
sponding in size to pSUP202 and by positive hybridization
on a Southern blot of total DNA from transformants probed
with the vector (data not shown).

When the 12 potential Nif− mutants isolated were
assayed for acetylene-reducing activity they proved to be
completely deficient (0.02-0.4% of wild-type total activ-
ity), confirming a Nif− phenotype for all isolated trans-
formants.

Physical analysis of A. brasilense mutants

To further characterize the mutagenized 6.5-kb EcoRI
A. brasilense genomic region, total DNA from the 12 Ap\(^r\)
and Km\(^r\) mutants isolated after electroporation was analyzed
by Southern blot. Total DNA was digested with EcoRI and
hybridized against the \(^{32}P\)-labelled 6.5-kb EcoRI fragment
isolated from the wild-type A. brasilense nifHDK DNA
region. Hybridization was detected only in genomes of the
four Ap\(^r\), Km\(^r\) and Tc\(^r\) mutants (data not shown). Due to lack
of hybridization of the remaining eight Nif− mutants, we
further analyzed only the four mutants that did not show
vector integration. Therefore, total DNA from Sp7Nif− 9,
Sp7Nif− 10, Sp7Nif− 11, and Sp7Nif− 13 was digested
with EcoRI and hybridized against the 6.0-kb EcoRI frag-
ment isolated from the pKm6 plasmid (Figure 1). Only one
band of 4.0 kb was visualized in strain Sp7Nif− 10 (Figure
2A, lane 2), showing that after the recombination event
nifHKm\(^r\)K genes were no longer intact in this mutant. With
wild-type DNA a 6.5-kb band representing the nifHDK
genes was visualized (Figure 2A, lane 1). In the other three
recombinants only faint bands were visualized.

To determine the presence of Tn5 in all 12 mutants,
total DNA was digested with PstI and hybridized against the
0.9- and 1.0-kb PstI fragments of Tn5. No hybridization
was detected (data not shown), suggesting that the
mutants had lost the Tn5 DNA fragment, a fact probably
explaining the weak hybridization signal obtained when the
6.0-kb EcoRI fragment isolated from the pKm6 plasmid
was used as probe.

To further analyze the recombination events that oc-
curred in the Sp7Nif− 10 mutant, total DNA was digested
with PstI and hybridized against four alternative probes
(spanning nifHDK, nifH, nifD, or nifK). All four probes
hybridized against the same 3.0-kb PstI DNA fragment (Fig-
ure 2B), suggesting that several recombination events had
occurred. Thus, the Sp7Nif− 10 mutant partially lost both
the nifH, nifD, and nifK genes and the entire kanamycin
resistance gene.

Narrow host range plasmids, based on pACYC184 and
pBR325 replicons, such as a pSUP202 plasmid, can be trans-
ferred to A. brasilense but are unable to replicate in this
bacterium. Such plasmids have been used as suicide vehicles
to deliver transposons in Azospirillum and the transposon
mutagenesis technique has been efficient in generating dif-
ferent Nif− mutants in this bacterium (Elmerich and Franche,
1982; Abdel-Salam and Klingmüller, 1987; Singh and
Klingmüller, 1986; Vanstockem et al., 1987). Attempts to
use a combination of nif-gene deletion and resistance gene-
cartridge mutagenesis of A. brasilense genes have also been
successful. However, all mutants have been obtained by
transferring the plasmids using conjugation methods, which
are problematic because counterselection of the donor
strain requires time-consuming rounds of single-colony pu-
rification. Moreover, since attempts to transfer plasmid
DNA between Azospirillum strains have so far failed, many
manipulations involve passage through E. coli cells.

Figure 2 - Physical analysis of Km\(^r\)-transformants
of Azospirillum brasilense. Southern hybridization
between Sp7Nif− 10 and \(^{32}P\)-labelled DNA probes. λ
DNA digested with HindIII was used as molecular
weight standard. A) Total Sp7 DNA (lane 1) and
Sp7Nif− 10 DNA (lane 2) digested with EcoRI and
hybridized with nifHKm\(^r\)K probe. B) Total Sp7Nif−
10 DNA digested with PstI and hybridized with
nifHDK (a), nifH (b), nifD (c), and nifK (d) probes.
We have used a novel combination of nifD-gene deletion with kanamycin gene-cartridge mutagenesis and electrottransformation. The method proved to be useful in isolating a number of A. brasilense Nif- mutants, all of which were completely defective in nitrogen fixation. However, the technique should be studied in more detail since other genome regions were also lost. Recombination events in A. brasilense are still poorly understood. We suggest that gene disruption combined with electrottransformation is the major cause of genome rearrangements by illegitimate recombination, producing deletions not only of the target gene but also of adjacent regions.

ACKNOWLEDGMENTS

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RESUMO

Com o objetivo de melhorar os sistemas de transferência gênica e mutagênese para Azospirillum brasilense, a técnica de mutagênese através do uso de um gene marcador (“gene-cartridge mutagenesis”) foi utilizada para subordinar a região genômica de A. brasilense correspondente ao gene nifD por um segmento de DNA do transposon Tn5 contendo o gene que confere resistência ao antibiótico canamicina. A construção foi transferida para a linhagem de A. brasilense por eleetrotransformação. Doze colônias transformadas foram isoladas com o plasmídeo suícid pSUP202 servindo como vetor. Dessas, somente quatro não possuíam o vetor integrado no cromossomo da bactéria. Independência da integração ou não do vetor, as 12 colônias foram deficientes na redução do gás acetileno, evidenciando o fenótipo Nif-: Quatro mutantes Nif- foram transformados através da técnica de Southern blot, utilizando-se seis diferentes fragmentos contendo genes nif, de resistência à canamicina e do vetor como sondas. Os resultados sugerem a ocorrência de eventos recombinacionais variados no genoma dos mutantes. A combinação entre a disrupção gênica através da técnica de mutagênese utilizada e eleetrotransformação foi, provavelmente, a causa principal do rearranjo genômico ocorrido nessas bactérias.

REFERENCES


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