

Directed mutagenesis affects recombination in *Azospirillum brasilense* *nif* genes

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Abstract

In order to improve the gene transfer/mutagenesis system for *Azospirillum brasilense*, gene-cartridge mutagenesis was used to replace the *nifD* gene with the Tn5 kanamycin resistance gene. The construct was transferred to *A. brasilense* by electrotransformation. 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^r 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 electrotransformation.

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; Willison *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 *nifENXO* RF3ORF5ORF6Q, ORF2*nifUSVORF4* 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 plas-

mid 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 10⁸ cells/ml. The *E. coli*

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Table I - Bacterial strains used in the present study.

Bacteria	Strain	Relevant characteristics	Source/reference
<i>Escherichia coli</i>	JA221	<i>recA</i> , <i>leuB6</i> , <i>trpES</i> , <i>hsdR</i> ⁻ , <i>hsdM</i> ⁺ , <i>lacY</i>	Clark and Carbon, 1978
	TG2	<i>supE</i> , <i>hsdD5</i> , <i>thi</i> , <i>D(lac-proAB)</i> , (<i>srl-recA</i>) 306::Tn10(<i>ter</i>)	Sambrook <i>et al.</i> , 1989
	S17.1	<i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> ⁻ , <i>hsdM</i> ⁺ , <i>Sm</i> ^r	Simon <i>et al.</i> , 1983
<i>Azospirillum brasilense</i>	Sp7	Amp ^r , wild-type	ATCC29145
	Sp7Nif ⁻ 10	Amp ^r , Km ^r , <i>nifD</i> -mutant	This work

Table II - Plasmids used in the present study.

Plasmids	Relevant characteristics	Source/reference
pRK290X	Tc ^r , <i>incP</i> , Tra ⁻	Alvarez-Morales <i>et al.</i> , 1986
pRK2013	Km ^r , colicine E1, Tra ⁺	Ditta <i>et al.</i> , 1980
pPH1J1	Gm ^r , <i>incP</i> , Tra ⁻	Ditta <i>et al.</i> , 1980
pSUP202	Tc ^r , Amp ^r , Cm ^r , Mob ⁺	Simon <i>et al.</i> , 1983
pAbc6	pACYC184 + 6.5-kb <i>EcoRI nifHDK</i> region	This lab.
pRKm6X	Tc ^r , Km ^r , <i>incP</i> , Tra ⁻	This paper
pSUP6	Tc ^r , Km ^r , Amp ^r , Mob ⁺	This paper
pKm6	Tc ^r , Km ^r	This paper

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. Conjugation and control growth patches were resuspended in 0.85% NaCl solution, washed several times, diluted, and spread on selective plates. The pRK290X derivative pRKm6X 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 *IncP1* plasmid pPH1J1 into the pKRm6X-carrying *A. brasilense* strain, followed by selection on both kanamycin and gentamycin. Km^r 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

(Singh and Klingmüller, 1986). To verify results of marker exchange, total DNA was isolated from putative mutants, digested with the appropriate restriction enzyme and hybridized against labelled DNA fragments.

Plasmid constructions

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 *PstI* and the 2.4-kb *nifD* DNA fragment was replaced with a 1.9-kb *PstI* DNA fragment originating from partially digested Tn5 DNA (Beck *et al.*, 1982). The 6.0-kb *EcoRI* DNA fragment, carrying the Km^r-cartridge from the pKm6 (*nifHKm^rK*) 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 *PstI* sites, it was possible to replace the *nifD* gene with a Km^r-cartridge from Tn5 (Figure 1). In the resulting pKm6 plasmid, the Km^r-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 into the *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

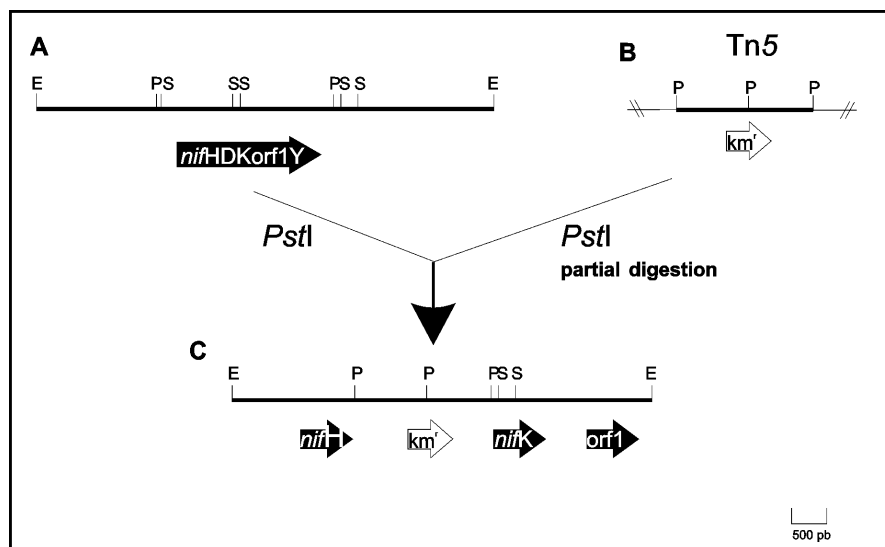


Figure 1 - Strategy for constructing the gene replacement cartridge. A) Physical map of the *Azospirillum brasilense nif* structural gene cluster. B) Partial physical map of the Tn5 transposon. C) Physical map of the pK6 plasmid containing the *nifD* gene replaced with the *km^r*-cartridge.

is resistant to ampicillin up to 200 $\mu\text{g/ml}$ and very sensitive to kanamycin (less than 5 $\mu\text{g/ml}$). Spontaneous mutants resistant to kanamycin (25 $\mu\text{g/ml}$) 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^rK* 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 $\mu\text{g/ml}$ kanamycin at maximum frequencies of 10^{-8} 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.

The narrow host range plasmid pSUP202, a mobilizable derivative of pBR325, was transferred to *A. brasilense* using the special mobilizing *E. coli* strain S17.1 (Simon *et al.*, 1983). This strain has the RP4 plasmid integrated into its chromosome and hence can mobilize plasmids having the same *mob* site of IncP plasmids as pSUP202 (Simon *et al.*, 1983). After mating *E. coli* S17.1 (bearing pSUP6) with *A. brasilense*, *Km^r* plus *Ap^r* exconjugants were selected

on Nfb minimum medium. Although a transfer frequency of 10^{-7} per recipient cell was achieved, we were unable to isolate any transconjugant sensitive to tetracycline (*Tc^s*). The results obtained with pSUP6 also indicated its integration into the *A. brasilense* chromosome.

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^r*-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^r*-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 ^{32}P -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

vector, pSUP202 had been integrated into the genome. This was confirmed by the absence of free plasmid DNA corresponding 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 activity), confirming a Nif⁻ phenotype for all isolated transformants.

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 ³²P-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^s 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* fragment 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^rK* 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 occurred 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 (Figure 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 a pSUP202 plasmid, can be transferred 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 different 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 purification. Moreover, since attempts to transfer plasmid DNA between *Azospirillum* strains have so far failed, many manipulations involve passage through *E. coli* cells.

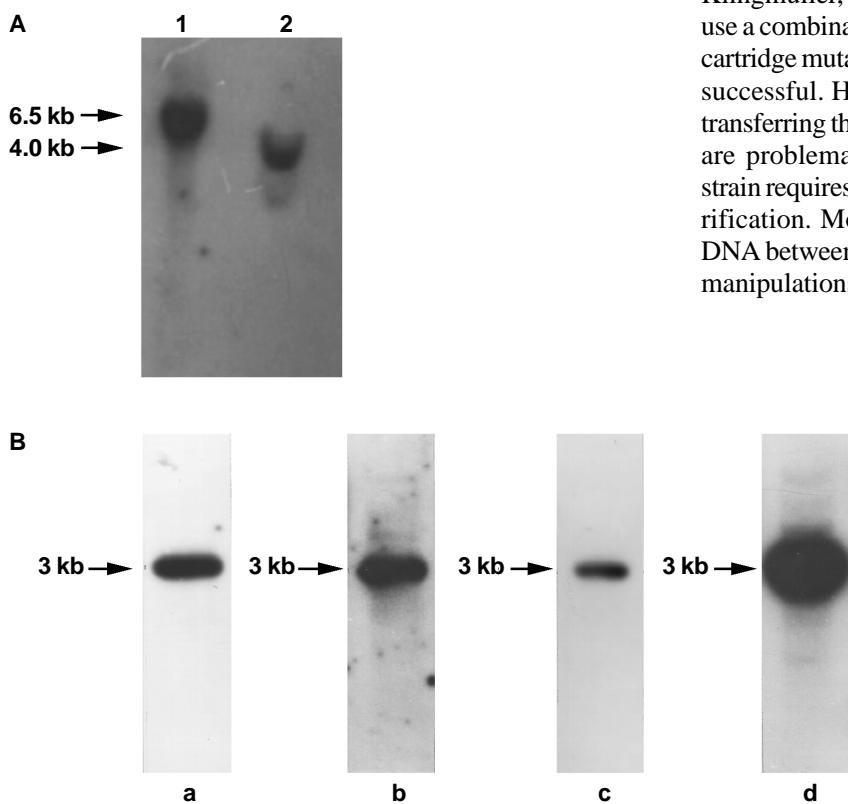


Figure 2 - Physical analysis of Km^r-transformants of *Azospirillum brasilense*. Southern hybridization between Sp7Nif⁻ 10 and ³²P-labelled DNA probes. λ DNA digested with *HindIII* was used as molecular weight standard. A) Total Sp7 DNA (lane 1) and Sp7Nif⁻ 10 DNA (lane 2) digested with *EcoRI* and hybridized with *nifHKm^rK* 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 electrotransformation. 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 electrotransformation is the major cause of genome rearrangements by illegitimate recombination, producing deletions not only of the target gene but also of adjacent regions.

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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 substituir 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 eletrotransformação. Doze colônias transformantes foram isoladas com o plasmídeo suicida pSUP202 servindo como vetor. Dessas, somente quatro não possuíam o vetor integrado no cromossomo da bactéria. Independentemente da integração ou não do vetor, as 12 colônias foram deficientes na redução do gás acetileno, evidenciando o fenótipo Nif⁻. Quatro mutantes Nif⁻ foram analisados 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 eletrotransformação foi, provavelmente, a causa principal do rearranjo genômico ocorrido nessas bactérias.

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