Genetics and Molecular Biology, 23, 4, 901-905 (2000)

Directed mutagenesis affects recombination in *Azospirillum brasilense nif* genes

C.P. Nunes[2], L.M.P. Passaglia[2], A. Schrank[1] and I.S. Schrank[1]

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 Tn*5* 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 N_2-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 NH_4^+ and O_2, 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 *nifENX*O RF3ORF5ORF6*Q*, ORF2*nifUSV*ORF4 and *fixABC* operons located, 3, 11, and 15 kb, respectively, downstream from the *nifHDK*ORF1*Y* operon (Passaglia, L., Frazzon, J. and Vedoy, C., unpublished results).

After the report of Elmerich and Franche (1982) on Tn*5*-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 Tn*5* 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 C_2H_2 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 10^8 cells/ml. The *E. coli*

[1]Departamento de Biologia Molecular e Biotecnologia, and [2]Departamento de Genética, Centro de Biotecnologia, UFRGS, Av. Bento Gonçalves, 9500 P.43421, Caixa Postal 15005, 91501-970 Porto Alegre, RS, Brasil.
Send correspondence to I.S.S. Fax: +55-51-336-2779. E-mail: irene@dna.cbiot.ufrgs.br

Table I - Bacterial strains used in the present study.

Bacteria	Strain	Relevant characteristics	Source/reference
Escherichia coli	JA221	*rec*A, *leu*B6, *trp*ES, *hsd*R$^-$, *hsd*M$^+$, *lac*Y	Clark and Carbon, 1978
	TG2	*sup*E, *hsd*D5, *thi*, D(*lac-pro*AB), (*srl-rec*A) 306::Tn*10*(*tet*^{r})	Sambrook *et al.*, 1989
	S17.1	*rec*A, *thi*, *pro*, *hsd*R$^-$, *hsd*M$^+$, Sm^r	Simon *et al.*, 1983
Azospirillum brasilense	Sp7	Amp^r, wild-type	ATCC29145
	Sp7Nif$^-$10	Amp^r, Km^r, *nif*D-mutant	This work

Table II - Plasmids used in the present study.

Plasmids	Relevant characteristics	Source/reference
pRK290X	Tc^r, incP, Tra$^-$	Alvarez-Morales *et al.*, 1986
pRK2013	Km^r, colicine E1, Tra$^+$	Ditta *et al*., 1980
pPH1JI	Gm^r, incP, Tra$^-$	Ditta *et al.*, 1980
pSUP202	Tc^r, Amp^r, Cm^r, Mob$^+$	Simon *et al*., 1983
pAbc6	pACYC184 + 6.5-kb *Eco*RI *nif*HDK region	This lab.
pRKm6X	Tc^r, Km^r, incP, 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 NH_4^+ 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

^{32}P-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 *nifHDK*ORF1*Y* operon. To construct pKm6, DNA from the pAbc6 plasmid was digested with *Pst*I and the 2.4-kb *nifD* DNA fragment was replaced with a 1.9-kb *Pst*I DNA fragment originating from partially digested Tn*5* DNA (Beck *et al*., 1982). The 6.0-kb *Eco*RI DNA fragment, carrying the Km^r-cartridge from the pKm6 (*nifHKm^{r}K*) plasmid, was subcloned into either *Eco*RI-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 *Eco*RI DNA fragment (Figure 1). This DNA fragment was originally cloned into an *Eco*RI-digested pACYC184 vector yielding pAbc6 (Araújo *et al*., 1988). Since pACYC184 has no *Pst*I sites, it was possible to replace the *nifD* gene with a Km^r-cartridge from Tn*5* (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

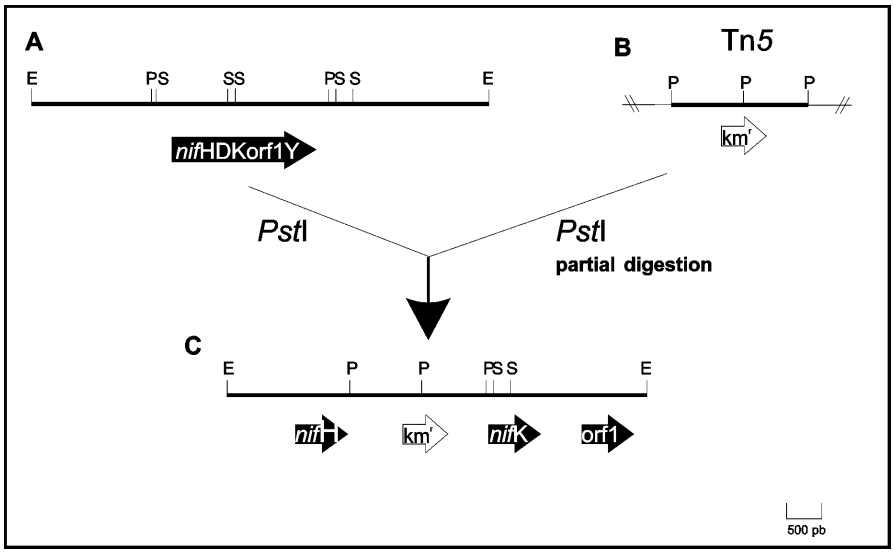

Figure 1 - Strategy for constructing the gene replacement cartridge. A) Physical map of the *Azospirillum brasilense nif* structural gene cluster. B) Partial physical map of the Tn*5* transposon. C) Physical map of the pKm6 plasmid containing the *nif*D gene replaced with the km^r-cartridge.

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^{r}K* 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 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 Tn*5* 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 Tn*5*, 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 *Eco*RI *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 *Eco*RI and hybridized against the ^{32}P-labelled 6.5-kb *Eco*RI fragment isolated from the wild-type *A. brasilense nif*HDK 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 *Eco*RI and hybridized against the 6.0-kb *Eco*RI 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 *nif*HKm^{r}K genes were no longer intact in this mutant. With wild-type DNA a 6.5-kb band representing the *nif*HDK genes was visualized (Figure 2A, lane 1). In the other three recombinants only faint bands were visualized.

To determine the presence of Tn*5* in all 12 mutants, total DNA was digested with *Pst*I and hybridized against the 0.9- and 1.0-kb *Pst*I fragments of Tn*5*. No hybridization was detected (data not shown), suggesting that the mutants had lost the Tn*5* DNA fragment, a fact probably explaining the weak hybridization signal obtained when the 6.0-kb *Eco*RI fragment isolated from the pKm6 plasmid was used as probe.

To further analyze the recombination events that occurred in the Sp7Nif$^-$10 mutant, total DNA was digested with *Pst*I and hybridized against four alternative probes (spanning *nifHDK*, *nifH*, *nifD*, or *nifK*). All four probes hybridized against the same 3.0-kb *Pst*I DNA fragment (Figure 2B), suggesting that several recombination events had occurred. Thus, the Sp7Nif$^-$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.

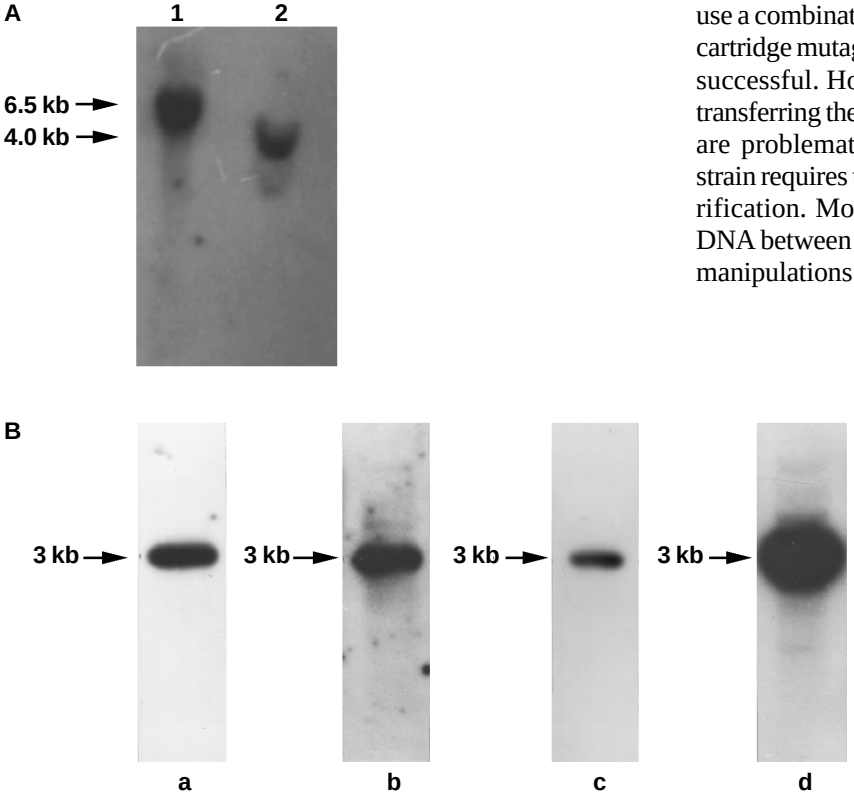

Figure 2 - Physical analysis of Km^r-transformants of *Azospirillum brasilense*. Southern hybridization between Sp7Nif$^-$10 and ^{32}P-labelled DNA probes. λ DNA digested with *Hin*dIII was used as molecular weight standard. A) Total Sp7 DNA (lane 1) and Sp7Nif$^-$10 DNA (lane 2) digested with *Eco*RI and hybridized with *nif*HKm^{r}K probe. B) Total Sp7Nif$^-$ 10 DNA digested with *Pst*I 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.

ACKNOWLEDGMENTS

The authors wish to thank Dr. H. Hennecke for providing the pRK290X and Dr. Fábio Pedrosa for providing pSUP202. C.P.N. received a scholarship from CNPq (Conselho Nacional de Pesquisa-Brasil) which, in addition to FAPERGS, supported this work through grants.

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 *nif*D por um segmento de DNA do transposon Tn*5* 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.

REFERENCES

Abdel-Salam, M.S. and **Klingmüller, W.** (1987). Transposon Tn5 mutagenesis in *Azospirillum lipoferum*: isolation of indole acetic acid mutants. *Mol. Gen. Genet. 210*: 165-170.

Alvarez-Morales, A., Betancourt-Alvarez, M., Kaluza, K. and **Hennecke, H.** (1986). Activation of the *Bradyrhizobium japonicum nif*H and *nif*DK is dependent on promoter-upstream DNA sequences. *Nucleic Acids Res. 14*: 4207-4227.

Araújo, E.F., Zaha, A., Schrank, I.S. and **Santos, D.S.** (1988). Characterization of DNA segments adjacents to the *nif*HDK genes of *Azospirillum brasilense* Sp7 by Tn5 site-directed mutagenesis. In: *Azospirillum IV: Genetics, Physiology and Ecology* (Klingmüller, W., ed.). 4th edn. Proceedings of the Fourth Bayrenth *Azospirillum* Workshop, Bayrenth, Germany, p. 16.

Arnold, W., Rump, A., Klipp, W., Priefer, O. and **Pühler, A.** (1988). Nucleotide sequence of a 24,206 base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. *J. Mol. Biol. 203*: 715-738.

Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. and **Schaller, H.** (1982). Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene 19*: 327-336.

Clark, L. and **Carbon, J.** (1978). Functional expression of cloned yeast DNA in *Escherichia coli:* Specific complementation of Argininosuccinate lyase (*arg*H) mutation. *J. Mol. Biol. 120*: 517-532.

Ditta, G.S., Stanfield, S., Corbin, D. and **Helinski., D.R.** (1980). Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA 77*: 7347-7351.

Dixon, R.A. (1984). The genetic complexity of nitrogen fixation. *J. Gen. Microbiol. 130*: 2745-2755.

Elmerich, C. and **Franche, C.** (1982). *Azospirillum* Genetics: plasmids, bacteriophages and chromosome mobilization. *Physiol. Ecol. 3*: 9-17.

Faure, D., Bouillant, M.L. and **Bally, R.** (1994). Isolation of *Azospirillum lipoferum* 4T Tn*5* mutants affected in melanization and laccase activity. *Appl. Environ. Microbiol. 60*: 3413-3415.

Haaker, H. and **Veeger, C.** (1984). Enzymology of nitrogen fixation. *Trends Biochem. Sci. 9*: 188-192.

Jacobson, M.R., Brigle, K.E., Bennett, L.T., Setterquist, R.A., Wilson, M.S., Cash, V.L., Beynon, J.L., Newton, W.E. and **Dean, D.R.** (1989). Physical and genetic map of the major *nif* gene cluster from *Azotobacter vinelandii*. *J. Bacteriol. 171*: 1017-1027.

Jara, P., Quiviger, B., Laurent, P. and **Elmerich, C.** (1983). Isolation and genetic analysis of *Azospirillum brasilense* Nif- mutants. *Can. J. Microbiol. 29*: 968-972.

Nelson, L.M. and **Knowles, R.** (1978). Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. *Can. J. Microbiol. 24*: 1395-1403.

Passaglia, L.M.P., Nunes, C.P., Zaha, A. and **Schrank, I.S.** (1991). The *nif*HDK operon in the free-living nitrogen-fixing bacteria *Azospirillum brasilense* sequentially comprises genes H,D,K, a 353-bp ORF and gene Y. *Braz. J. Med. Biol. Res. 24*: 649-675.

Postgate, J.R. and **Cannon, F.C.** (1981). The molecular and genetic manipulation of nitrogen fixation. *Philos. Trans. R. Soc. Lon. 292*: 589-599.

Sambrook, J., Fritsch, E.F. and **Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Simon, R., Priefer, U. and **Pühler, A.** (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology 1*: 784-790.

Singh, M. and **Klingmüller, W.** (1985). Problems and prospects of site-directed transposon mutagenesis in *Azospirillum*. In: *Azospirillum III: Genetics, Physiology and Ecology* (Klingmüller, W., ed.). Spring Verlag, Heidelberg, Germany, p. 20.

Singh, M. and **Klingmüller, W.** (1986). Transposon mutagensis in *Azospirillum brasilense*: isolation of auxotrophic and Nif-mutants and molecular cloning of the mutagenized *nif*DNA. *Mol. Gen. Genet. 202*: 136-142.

Vande Broek, A., Gool, A.P.V. and **Vanderleyden, J.** (1989). Electroporation of *Azospirillum brasilense* with plasmid DNA. *FEMS Microbiol. Lett. 61*: 177-182.

Vanstockem, M., Michiels, K. and **Vanderleyden, J.** (1987). Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: physical analysis of Tn*5* and Tn*5*-mob insertion mutants. *Appl. Environ. Microbiol. 53*: 410-415.

Willison, J.C., Pierrard, J. and **Hübner, P.** (1993). Sequence and transcript analysis of the nitrogenase structural gene operon (*nif*HDK) of *Rhodobacter capsulatus*: evidence for intramolecular processing of *nif*HDK mRNA. *Gene 133*: 39-46.

(Received March 14, 2000)