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# Functional *Bradyrhizobium japonicum* NifA Expression under a Hybrid *nptII-nifH* Promoter in *E. coli* and *Acetobacter diazotrophicus* SRT4

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**ABSTRACT. A hybrid promoter consisting of the in tandem fusion of the Tn5 *nptII* and the *Klebsiella pneumoniae nifH* promoters was constructed to study the functionality of the *nif* genes transcriptional activator NifA from *Bradyrhizobium japonicum* in two different host bacteria. β-galactosidase experiments in *E. coli* revealed that the hybrid *nptII-nifH* promoter can behave as a constitutive or a NifA-inducible promoter depending on the aeration conditions. Expression of the *B. japonicum* NifA from the hybrid *nptII-nifH* promoter (plasmid pBPF204) induced "in trans" *lacZ* transcription from the *Azotobacter chroococcum nifH* promoter in *E. coli* and *A. diazotrophicus* cells grown at low $pO_2$. Similarly, the plasmid pBPF204 increased nitrogenase activity in *A. diazotrophicus* cells grown under microaerobic conditions. Based on these results, we suggest that the *B. japonicum* NifA could function as an efficient $O_2$-sensitive transcriptional activator of *nif* genes in genetically distant diazotrophic bacteria.**

**RESUMEN. Un promotor híbrido derivado de la fusión de los promotores *nptII* del transposón Tn5 y *nifH* de *Klebsiella pneumoniae* fue construido para estudiar la funcionalidad del activador transcripcional NifA de *Bradyrhizobium japonicum* en los hospederos *E. coli* y *Acetobacter diazotrophicus*. Diferentes experimentos de expresión de ß-galactosidasa realizados en *E. coli*, demostraron que el promotor híbrido *nptII-nifH* puede funcionar constitutivamente o activarse por NifA en dependencia de las condiciones de aireación existentes. A baja presión de $O_2$ se comprobó, tanto en *E. coli* como en *A. diazotrophicus*, que la producción de NifA es capaz de inducir "in trans" la expresión de ß-galactosidasa a partir del promotor *nifH* de *Azotobacter chroococcum*. La expresión del NifA de *B. japonicum* incrementó la actividad nitrogenasa en *A. diazotrophicus* crecido en condiciones microaeróbicas. Del resultado obtenido se concluye que el NifA de *B. japonicum* puede funcionar como un activador de la transcripción de los genes *nif* de bacterias diazotróficas que están distantes genéticamente.**

## INTRODUCTION

Biological nitrogen fixation (BNF) is an important element of the nitrogen cycle in nature and it is of great importance to agriculture. BNF occurs at low oxygen pressure and constitutes an energy costly process which is repressed in the presence of assimilable nitrogen sources.[8]

In rhizobia, low-oxygen sensing and transmission of this signal to allow expression of nitrogen fixation genes (*nif* and *fix*) involve at least five regulatory proteins FixL, FixJ, FixK, NifA, and RpoN.[11] The NifA activates transcription of several genes and operons involved in nitrogen fixation and thus is needed in high amounts to avoid titration by the *nif* and *fix* promoters.[14] Transcriptional enhancers termed upstream activator sequence (UAS) that have the consensus sequence 5'-TGT-$N_{10}$-ACA function as NifA-binding sites in most NifA-dependent promoters, which become extremely strong upon activation, producing up to 20% of total cell proteins.[19,20] NifA-dependent gene activation occurs at so-called –24/-12 consensus promoters (5'-TGGYRYRYR-$N_4$-TTGCT) which are recognised by RNA polymerase containing the alternative sigma factor σ.[54,15,23] A DNA loop model has been proposed for the activation of NifA-dependent genes in *Bradyrhizobium japonicum*. In this model, direct contact between the activator protein NifA bound to the consensus UAS and the RNA polymerase holoenzyme bound to the promoter is facilitated,[19] thereby mediating an open complex formation and efficient transcription.

Nitrogen fixation genes are often functionally conserved among diazotrophic bacteria. In an attempt to increase nitrogen fixation efficiency in the sugarcane endophyte *Acetobacter diazotrophicus*,[9] we constructed a chimeric expression system in which the *B. japonicum nifA* gene[12] was placed under the control of a hybrid promoter formed by the fusion in tandem of the constitutive Tn5

*nptII* promoter and the *Klebsiella pneumoniae nifH* promoter. Considering that the *B. japonicum* NifA is sensitive to oxygen,[10] the hybrid system may induce itself a high production of active NifA only at the low $pO_2$ necessary for nitrogenase activity.

## MATERIALS AND METHODS

**Bacterial strains, plasmids and growth media.** The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown at 37°C in LB[21] supplemented with ampicillin (50 μg/ml), kanamycin (30 μg/ml) and tetracycline (12.5 μg/ml) as appropriate; or M9 medium[21] enriched with 1% (w/v) glucose, 0.02% (w/v) casein hidrolizate and 0.1 mM $FeSO_4$ *A. diazotrophicus* SRT4 was grown at 28°C in liquid LGI medium[6] enriched with 3% (w/v) glucose, 0.02% (w/v) yeast extract and 20 mM $NH_4Cl$, and supplemented with ampicillin (50 μg/ml), kanamycin (100 μg/ml) and tetracycline (20 μg/ml) For aerobic and microaerobic growth the cultures were shaken at 105 and 2 rpm respectively. The semisolid LGI medium[6] was used for nitrogenase assays.

**DNA manipulations.** Standard procedures were used for DNA manipulations.[21] Conjugative mating from *E. coli* to *A. diazotrophicus* was performed as previously described.[1]

**β-Galactosidase assays.** β-Galactosidase activity was determined using the Miller procedure.[17] *E. coli* strains and *A. diazotrophicus* SRT4 were grown at appropriate temperatures in enriched M9 and LGI media, respectively.

**Acetylene reduction assay.** The $C_2H_2$ reduction test was performed using 10% (v/v) $C_2H_2$ in the headspace of sealed vials containing *A. diazotrophicus* grown in semisolid medium. After one hour incubation at 28°C, $C_2H_4$ production was measured using a Pye Unicam Gas Chromatograph 4550 having an AL-FI glass column (1.5 m long and 4 mm diameter) and a flame ionization detector. The column temperature was 120 °C. The gas carrier was $N_2$ at a flux of 35 ml/min.

**Construction of plasmids.** Plasmid pBPF201 was constructed by inserting the 0.34-kb *Eco*RI/*Bgl*II (blunt-ended with Klenow) fragment containing the Tn5 *nptII* promoter from pUC4-KIXX into the *Eco*RI (filled in with Klenow) site of plasmid pMB1, which carries the *lacZ* gene under the *K. pneumoniae nifH* promoter. Plasmid pBPF202 was constructed by inserting the 0.67-kb *Sac*I/*Bam*HI fragment from pBPF201 containing the fused *nptII-nifH* promoter, into the same sites of pUC19. Plasmid pBPF203 was constructed to place the *Bradyrhyzobium japonicum nifA* gene under the hybrid *nptII-nifH* promoter from pBPF202. For that purpose, plasmid pRJ7523 was *Hin*dIII-linearized, filled in with Klenow and digested with *Pst*I to isolate the *B. japonicum nifA* gene in a 1.7-kb fragment, which was inserted into pBPF202 previously *Bam*HI-linearized, made blunt with *S1* nuclease and digested with *Pst*I. Plasmid pBPF204 was constructed by inserting the 2.3-kb *Hin*dIII blunt-ended fragment containing the *B. japonicum nifA* gene under the hybrid *nptII-nifH* promoter from pBPF203, into the *Bam*HI (filled in) site of the broad-host-range vector pKT230.

## RESULTS AND DISCUSSION

**Functionality of the hybrid *nptII-nifH* promoter in *E. coli*.** Plasmids pRJ7523 containing the *B. japonicum nifA* gene under the constitutive cat promoter and pBPF201 carrying the *lacZ* gene under the hybrid *nptII-nifH* promoter were both introduced into the *E. coli* strain 71-18. As control, cells were co-transformed with plasmids pRJ7523 and pMB1. β-galactosidase activity was deter-

**TABLE 1.** Bacterial Strains and Plasmids.

| Strains or plasmids | Relevant genotype or phenotype | Source or Reference |
|---|---|---|
| *E. coli* DH5α | F, 80d *lacZ*M15, hsdR R17, RecA1, (*lacZ*YA-argF) | 13 |
| *E. coli* 71-18 | (*lac*-proAB), thi, supE, [F', proAB, *lacI*ZM15] | 25 |
| *E. coli* S17-1 | pro, hsdR, recA, [RP4-2(Tc::Mu)(nptII::Tn7)] | 22 |
| *A. diazotrophicus* SRT4 | Wild type | 7 |
| Plasmid pUC4-KIXX | ColE1 replicon; $Ap^r$, $Kan^r$, $Ble^r$; 4.0 kb | 3 |
| Plasmid pUC19 | ColE1 replicon; $Ap^r$; 2.7 kb | 25 |
| Plasmid pKT230 | RSF1010 replicon; $Str^r$, $Km^r$; 11.9 kb | 2 |
| Plasmid pMB1 | pMC1403 derivative; Kpr*nifH::lacZ*; $Tc^r$, $Ap^r$; 10.2 kb | 5 |
| Plasmid pRJ7523 | p15A relicon; pr*cat::*Bj*nif*A; $Km^r$; 5.1 kb | 12 |
| Plasmid pBPF201 | pMC1403 derivative; pr*nptII*-Kpr*nifH::lacZ*; $Ap^r$; 10.5 kb | This work |
| Plasmid pBPF202 | ColE1 replicon; pr*nptII*-Kpr*nifH*; $Ap^r$; 3.9 kb | This work |
| Plasmid pBPF203 | ColE1 replicon; pr*nptII*-Kpr*nifH*::Bj*nif*A; $Ap^r$; 5.6 kb | This work |
| Plasmid pFAC11 | RK2 replicon, Acpr*nifH::lacZ*; $Tc^r$; 28.0 kb | * |
| Plasmid pBPF204 | RSF1010 replicon; pr*nptII*-Kpr*nifH::*Bj*nif*A; $Str^r$, $Km^r$; 16.9 kb | This work |

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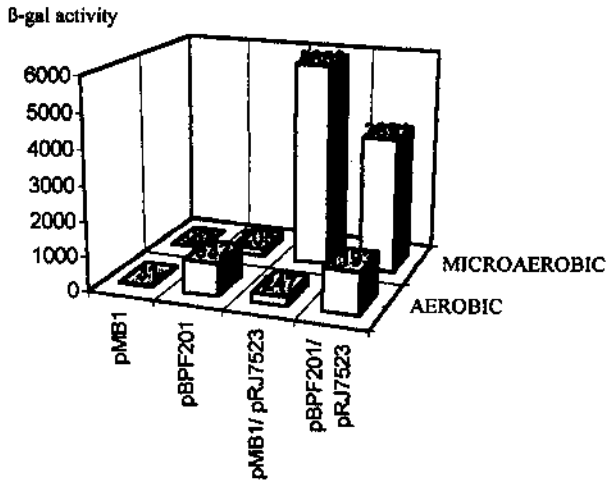


Fig. 1. Functionality of the hybrid *nptII-nifH* promoter in *E. coli* 71-18. Values are expressed in Miller units,[17] and represent the average of three replicas in two independent experiments.

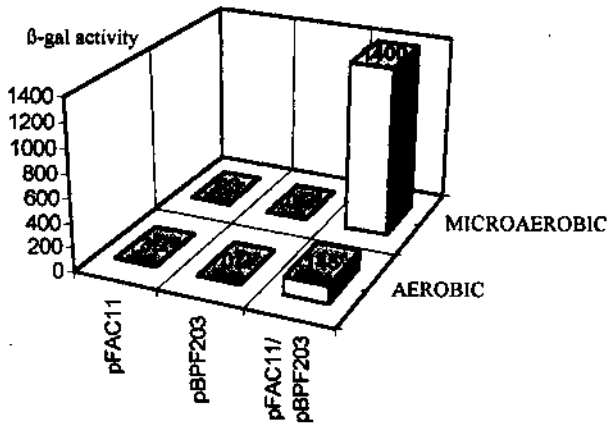


Fig. 2. Induction of the *A. chroococcum nifH* promoter by the *B. japonicum* NifA expressed from the ColE1-plasmid PBPF203 in *E. coli* strain DH5α. Values are given in Miller units,[17] and represent the average of three replicas in two independent experiments.

mined in bacteria grown under microaerobic and aerobic conditions.

As shown in Fig 1, β-galactosidase activity was about 30 fold higher in *E. coli* (pBPF201) than in *E. coli* (pMB1) irrespective of the aeration conditions, indicating that the hybrid promoter allowed constitutive expression of the *lacZ* gene. On the other hand, the presence of plasmid pRJ7523 highly increased LacZ production by plasmids pMB1 (pr*nifH::lacZ*) and pBPF201 (pr*nptII*-pr*nifH::lacZ*) only in bacteria grown under microaerobic conditions. This result is in agreement with the finding that activation of *nif* promoters by the *B. japonicum* NifA occurs at low $O_2$ pressure.[10] These results indicate that the hybrid *nptII-nifH* promoter can behave either as a constitutive or a NifA-inducible promoter in *E. coli.*

**NifA expression under the hybrid *nptII-nifH* promoter in *E.coli.*** To test the ability of the hybrid *nptII-nifH* promoter to direct efficient expression of the NifA activator protein in *E. coli,* the plasmid pBPF203 (carrying the *B. japonicum nifA* gene under the hybrid promoter) and the plasmid pFAC11 (carrying the *lacZ* gene under the *nifH* promoter from *Azotobacter chroococcum),* were simultaneously introduced into *E. coli* strain DH5α. ß-galactosidase activity was determined in cells grown under microaerobic and aerobic conditions.

*LacZ* expression from plasmid pFAC11 was only observed in presence of plasmid pBPF203 (Fig 2), indicating that the *A. chroococcum nifH* promoter can be activated by the *B. japonicum* NifA.

Under microaerobic conditions, NifA expression from the hybrid promoter (plasmid pBPF203) increased 400 fold the β-galactosidase production from plasmid pFAC11, as compared to *E. coli* carrying the plasmid pFAC11 alone. Weidenhaupt et al.[24] reported that *lacZ* expression in *E. coli* from a NifA-dependent promotor at low $pO_2$ was activated 11-fold by the *B. japonicum* NifA. Under aerobic conditions, β-gal activity decreased 10-fold in *E. coli* carrying the plasmids pFAC11 and pBPF203. The remaining *lacZ* expression from the *A. chroococcum nifH* promoter can be explained by an incomplete inactivation of NifA at the chosen aeration conditions.

The fact that pBPF203 could activate "in trans" heterologous *nif* promoters at low $pO_2$, suggests that the hybrid promoter-*B. japonicum nifA* gene cassette could also be self-inducible allowing a high production of the NifA activator protein under microaerobic conditions. However, the ability of the hybrid *nptII-nifH* promoter to induce "in cis" the *B. japonicum nifA* transcription needs to be demonstrated by using a *nifA::lacZ* transcriptional fusion.

**NifA expression under the hybrid *nptII-nifH* promoter in *A. diazotrophicus* SRT4.** To test the functionality of the *B. japonicum* NifA expression from the hybrid promoter in *A. diazotrophicus*, the cassette pr*nptII*-pr*nifH::* Bj*nifA* was subcloned into the broad-host-range vector pKT230. The resulting plasmid (pBPF204) was conjugally transferred into *A. diazotrophicus* strain SRT4, which already carried the plasmid pFAC11. β-galactosidase activity was determined at high and low $O_2$ pressures and compared to the values obtained for the control plasmids pFAC11 and pBPF204 each alone.

Table 2 shows ß-gal activities determined in *E. coli* DH5α and *A. diazotrophicus* SRT4. In *E. coli,* 2-galactosidase activity was high at low $pO_2$ in which the *B. japonicum* NifA is active. In *A. diazotrophicus* grown un-

**TABLE 2** Induction of the a *Chroococcum nifh* promoter by the *B. Japonicum nifa* expressed from the RSF1010-

| | *E. coli* strain DH5a | *E. coli* strain DH5a | *A. diazotrophicus* strain SRT4 | *A. diazotrophicus* strain SRT4 |
|---|---|---|---|---|
| Plasmids | $O_2$- | $O_2$+ | $O_2$- | $O_2$+ |
| pFAC11 | 3.3 ± 1.8* | 3.2 ± 1.6 | 28 ± 5 | 4.5 ± 3.3 |
| pBPF204 | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 |
| pFAC11 / pBPF204 | 1450 ±187 | 145 ± 19 | 72 ± 6 | 6.5 ± 3.5 |

* Values are given in Miller units,[17] and represent the average of three replicas in two independent experiments

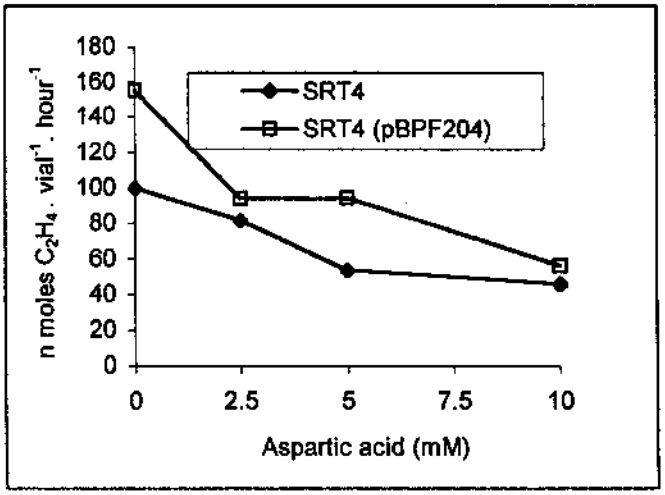


Fig. 3. Effect of aspartic acid concentration in the semisolid LGI medium on the nitrogenase activity of *A. diazotrophicus* strain SRT4 expressing the *B. japonicum nifA* gene from the hybrid *nptII-nifH* promoter. Values represent the average of three replicas in two independent experiments.

der microaerobic conditions the *B. japonicum* NifA produced from the hybrid *nptII-nifH* promoter (plasmid pBPF204) also induced "in trans" *lacZ* expression from the *A. chroococcum nifH* promoter (plasmid pFAC11) although at a much lower level. This reduction in *β*-galactosidase expression could be caused by titration of the *B. japonicum* NifA by the own *A. diazotrophicus nif* promoters. In *B. japonicum* a high level of the activator protein is required to avoid titration by NifA-dependent promoters.[14] On this basis, *A. diazotrophicus nif* promoters should contain the consensus NifA-binding sequence 5'-TGT-$N_{10}$-ACA[18] as it is also suggested by the increased nitrogenase activity observed in the presence of plasmid pBPF204 (Fig 3). A second explanation for the decreased induction of the *A. chroococcum nifH* promoter-*lacZ* gene fusion could be a poor transcription of the *B. japonicum nifA* gene from the hybrid *nptII-nifH* promoter in *A. diazotrophicus*. However, this hypothesis is not supported by the fact that expression of the *nptII* gene from its own promoter conferred high kanamycin resistance in strain SRT4.[1]

A low *β*-galactosidase activity was detected in the absence of plasmid pBPF204, suggesting the existence of a functional *A. diazotrophicus* NifA capable to induce "in trans" expression of the *A. chroococcum nifH* promoter-*lacZ* gene fusion. Recently, the *A. diazotrophicus nifA* gene was identified to be the first transcript of a *nif* operon.[16]

**Effect of aspartic acid on the nitrogenase activity of *A. diazotrophicus* SRT4 carrying the hybrid construct pBPF204.** The *β*-galactosidase experiments demonstrated that construct pBPF204, expressing the *B. japonicum nifA* gene from the hybrid *nptII-nifH* promoter, is able to activate "in trans" foreign *nifH* promoters in *E. coli* and *A. diazotrophicus* cells cultured at low $pO_2$. Thus, it was expected that the *B. japonicum* NifA could also induce transcription of native *nif* and *fix* genes in *A. diazotrophicus*. To support this hypothesis, *A. diazotrophicus* strain SRT4 carrying the plasmid pBPF204 was grown for 3 days under the microaerobic environment of the semisolid LGI medium supplemented with different concentrations of aspartic acid. Then, bacterial cultures were tested for nitrogenase activity by their ability to reduce acetylene.

Increasing concentrations of the amino acid in the growth media partially inhibited nitrogen fixation in both the *A. diazotrophicus* wild-type and the genetically modified strain (Fig. 3). Similarly, ammonia has been found to cause only partial inhibition of the *A. diazotrophicus* nitrogenase.[4] It is otherwise significant that the plasmid pBPF204 enhanced 1.6 fold the nitrogenase activity in the absence of aspartic acid. This result suggests that the *B. japonicum* NifA expressed from the hybrid *nptII-nifH* promoter could activate the *A. diazotrophicus nif* system. In the presence of aspartic acid, nitrogenase activity although decreased in the two strains, was always higher in the bacterium carrying the plasmid pBPF204. This finding could have practical implications on the use of *A. diazotrophicus* as a biofertilizer.

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