Activation of the *Bradyrhizobium japonicum* *nifH* and *nifDK* operons is dependent on promoter-upstream DNA sequences

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ABSTRACT

Previous analysis of *B.japonicum* *nifH*'- and *nifD*'-'*lacZ* translational fusions showed that these promoters could be activated by the *K.pneumoniae* *nifA* plus the *E.coli* *ntrA* gene products. To study the functions of the DNA 5' to these promoters, plasmids carrying deletions in this region were constructed and analyzed in vivo in a heterologous system consisting of an *E. coli* (*NtrA*⁺) background with a plasmid that constitutively expresses the *K. pneumoniae* *nifA* gene. Activation of the *B.japonicum* promoters was completely dependent on sequences located between positions -165 and -100, relative to the start of transcription. Some of the *nifD* deletion-fusions were mobilized to the wild-type *B.japonicum* and the exconjugants tested in an *ex planta* micro-aerobic system, and also used to infect soybean seedlings. The time course of derepression was followed by assaying β -galactosidase activity from samples withdrawn from the microaerobic cultures or from root-nodule extracts. The results conclusively show that in the homologous system the sequences upstream of the promoter are required to achieve wild-type activity.

INTRODUCTION

Nitrogen fixation, the enzymic conversion of N₂ to ammonia, is a complex process which can be carried out by several groups of prokaryotes, among them the free-living diazotroph *Klebsiella pneumoniae*. In this bacterium, nitrogen fixation requires the concerted function of 17 contiguous *nif* genes distributed in 7 or 8 operons (1). This complex regulon is controlled at two levels: one external level which links nitrogen fixation to a regulatory system controlling the utilization of alternative nitrogen sources when ammonia becomes limiting, and a second internal level which acts specifically on the *nif* genes.

Control at the first level is under the nitrogen regulation (*ntr*) system, known in several enterobacteria, which regulates operons such as those required for the catabolism of arginine (*aut*), proline (*put*) and histidine (*hut*) (2). This system also regulates the complex *p*₁-*p*₂-*glnA*-*p*₃-*ntrBC* operon which comprises *glnA*, the gene for glutamine synthetase, and two *ntr* regulatory

genes, ntrB and ntrC (3,4,5).

The operons under ntr control and the nif genes have a characteristic promoter structure distinct from the consensus -10 and -35 regions of the E. coli operons (1,6). A third ntr gene unlinked to glnA, namely ntrA, has been proposed to specify a distinct sigma factor for the recognition of ntr-activatable promoters in K.pneumoniae (7,8,9); such is the case for the ntrA products of Salmonella typhimurium (10) and E.coli (11). In view of these results it has been proposed that the ntrA gene should be renamed rpoN (11).

The ntrC gene product is a bifunctional DNA-binding protein which, under conditions of nitrogen excess behaves as a repressor of the glnA operon (3,4, 12), but under nitrogen limitation behaves as an activator of operons under ntr control (13,14). The ability of the ntrC product to act as a repressor or an activator is under the control of the ntrB gene product (3,4). Data obtained from E.coli indicate that information regarding the nitrogen/carbon status of the cell flows from the products of glnB and glnD, which are components of the glutamine synthetase adenylylation system, to the ntrB regulatory protein and from this to the ntrC gene product which is the final effector acting directly on gene expression (2,15).

Under conditions of low nitrogen, the ntrC gene product activates one of the nif operons, nifLA (13,14). This initiates the second, nif-specific level of control. The nifA gene product is a nif-specific activator (16,17,18) whilst the product of nifL counteracts the activation mediated by nifA (19,20, 21,22).

Activation of the ntr and nif operons by the ntrC and nifA proteins respectively requires the product of the rpoN(ntrA) gene (23,24). This dependence on the same sigma factor as well as some physical similarities between both effector molecules suggested an evolutionary relationship for both products and perhaps for both nifLA and ntrBC operons and, in fact, it was shown that the nifA gene product can substitute for the ntrC protein as activator of operons such as hutC and nifLA (14,23,25).

In N_2 fixing organisms other than K.pneumoniae, the analysis of the regulatory circuitry has not yet been so fully developed. Nevertheless it has been shown that the promoters of nif genes from Rhizobium and Bradyrhizobium species, and from rhizobia of the cowpea miscellany and Parasponia groups have a sequence which fits into the consensus proposed for the K.pneumoniae nif genes (for

promoter sequences see Ref. 26). The same nif consensus sequence has also been found in Azotobacter vinelandii (27,28).

In addition, it has been shown that the K.pneumoniae nifA product can activate expression of nif genes in A.vinelandii and A.chroococcum (29) and in Azospirillum brasilense (30). In R.meliloti both the K.pneumoniae nifA and the E.coli ntrC gene products can activate the nifHDK and fixABC operons (24,31,32). Also, the K.pneumoniae nifA product can activate the nifH and nifDK operons of B.japonicum; however, in this case the K.pneumoniae ntrC gene product does not have an effect on these nif promoters (26).

A gene corresponding to the K.pneumoniae nifA cistron has been found in R.meliloti (32,33,34,35), R.leguminosarum (36,37,38) and B.japonicum (39). In R.meliloti a gene analogous to the K.pneumoniae ntrC gene has also been identified (40).

Wild-type levels of activity from the K.pneumoniae nifLA consensus promoter require not only the products of the nifA and rpoN(ntrA) genes, but also sequences at least 150 base pairs (bp) upstream of the point of transcription initiation (13). The same is true for the nifHDKY operon where sequences up to the -136 position are required (41,42,43). Recently, a similar effect was identified when 5' deletions of the R.meliloti nifHDK promoter were assayed in an E.coli background using the K.pneumoniae nifA gene product as activator. However, the requirement for the DNA upstream of the R.meliloti nifHDK promoter disappeared when expression from this promoter was assayed in 5-week old alfalfa root-nodules (44).

We have now tested the effect of sequences upstream of the B.japonicum nifH and nifDK promoters making use of a series of Bal31- and restriction endonuclease-generated 5' deletions of nifH'-'lacZ and nifD'-'lacZ translational fusions. The nifH- and nifD-lacZ deletion fusions were both tested in a heterologous system composed of an E.coli background and the K.pneumoniae nifA activator. It was found that sequences extending up to the -165 position were required to achieve full activity from these promoters. Furthermore, and in contrast to the results obtained in R.meliloti, a stringent requirement for the upstream sequences was also observed when deletion fusions of the B.japonicum nifD promoter were assayed in a microaerobic (ex planta) system and in soybean root-nodules.

Table 1. Strains and plasmids used

Strains	Relevant genotype or phenotype ^a	Reference
<i>E.coli</i> ET8000	rbs lacZ::IS1 gyrA	(45)
<i>E.coli</i> MC1061	Δ (lacIPOZYA)X74 hsdR	(46)
<i>B.japonicum</i> 110spc4	Spc ^R	(47)
Plasmids		
pMC1403	lac'ZYA bla	(46)
pMC71A	Kp-nifA ⁺ cat	(18)
pRJ1008	Bj- ϕ (nifD'-lacZ)hyb bla	(26)
pRJ1009	Bj- ϕ (nifH'-lacZ)hyb bla	(26)
pRK2013	Km ^R tra	(48)
pRK290	Tc ^R	(49)
pRK290X	as pRK290 plus XhoI	this work

^a Bj = *B.japonicum*, Kp = *K.pneumoniae*

MATERIALS AND METHODS

Strains and plasmids

These are listed in Table 1.

DNA manipulation and cloning

Restriction and DNA-modifying enzymes were obtained from commercial sources and used according to the manufacturer's instructions. Standard procedures for DNA cloning were used (50).

Media and growth conditions for *E.coli* and *B.japonicum*

E.coli was normally grown in LB. Nitrogen-free medium (NFDm) (51) was used supplemented with 200 μ g/ml of filter-sterilized glutamine plus appropriate antibiotics. Minimal medium (MM) was as described (52) but NaCl and CaCl₂ were omitted. Indicator medium (MM-Xgal) for strains carrying lacZ fusions was MM plus casaminoacids (250 μ g/ml), yeast extract (250 μ g/ml), 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal) (30 μ g/ml) and appropriate antibiotics. Chloramphenicol was used at 40 μ g/ml and ampicillin at 200 μ g/ml.

B.japonicum 110spc4 was routinely grown on PSY medium (47). When required, tetracycline was used at 100 μ g/ml, spectinomycin at 200 μ g/ml and Xgal at 30 μ g/ml. Microaerobic (*ex planta*) cultures have been described (53). Plant infection tests were done by inoculating soybean seedlings (*Glycine max* (L.) Merr. cv. Clark-L1) and growing the plants in a climatized chamber (54).

Construction of 5' deletion derivatives of the *nifD'*-'*lacZ* fusion plasmid pRJ1008

The translational *lacZ* fusion vector pMC1403 contains a 17 bp polylinker with sites for *EcoRI*-*SmaI*-*BamHI* just upstream from the 9th codon of the *E.coli lacZ* gene. The *B.japonicum nifD'*-'*lacZ* fusion plasmid, pRJ1008 (Fig. 1A), comprises a 560 bp *EcoRI*-*BamHI* fragment cloned in pMC1403 (26). The 560 bp insert includes 23 bp of pBR322 DNA and 534 bp of *B.japonicum nifD* DNA, including 428 bp upstream the site of transcription initiation.

Deletions of DNA upstream the promoter region were constructed by cutting pRJ1008 at the unique *EcoRI* site and treating the linear molecules with the exonuclease Bal31. After inactivation of the exonuclease, the DNA was digested with *BamHI*. This step generated a series of fragments of *B.japonicum* DNA with a common 3' *BamHI* end and different 5' blunt ends. These fragments were then recloned into *SmaI*/*BamHI*-digested pMC1403 vector, thus generating plasmids in which the insert was flanked by a 5' *EcoRI* site and a 3' *BamHI* site at the *B.japonicum* DNA/*lacZ* junction. This procedure ensured that all the deletion derivatives of pRJ1008 retained similar sequences upstream the *EcoRI* site as the parent plasmid.

The plasmids obtained by the procedure described above were pRJ1010/11/12/14/15/16/17 and 18 (Fig. 1A). Other deletion derivatives were constructed using specific restriction sites. Plasmid pRJ1019 was constructed by cutting pRJ1008 at the unique *EcoRI* and *BglII* sites, the ends were "filled in" and the plasmid religated. To construct pRJ1020 the plasmid pRJ1008 was digested at the unique *EcoRI* and *SphI* sites, both ends were "filled in" and the plasmid religated. In the new *EcoRI*/*SphI* junction, the *EcoRI* site was regenerated. Plasmid pRJ1021 was obtained by first digesting pRJ1008 with *XmaIII* which generated more than one fragment, the ends were "filled in" and the DNA digested with *BamHI*. The blunt-*BamHI* fragment carrying *B.japonicum* DNA was purified and recloned into *SmaI*/*BamHI*-cut pMC1403 vector.

To determine the 5' end-points for those deletion derivatives generated with the exonuclease Bal31, the plasmids were cut at their unique *EcoRI* site and labelled with (α -³²P)dATP and Klenow polymerase. After inactivation of the polymerase the DNA was digested with *BamHI* and the DNA loaded onto a 6% polyacrylamide sequencing gel. The samples were run alongside a dideoxy sequencing ladder generated from an M13mp8 clone carrying the *EcoRI*-*BamHI* fragment of pRJ1008 which had been digested with *BamHI* after the elongation reaction in

order to generate a common 5' end for the sequencing reaction. The inserts in plasmids pRJ1020 and 1021, which had a defined 5' end-point, were also sized by the above-mentioned procedure, together with the BglII-BamHI fragment of pRJ1008, in order to have internal controls. The sizes could be estimated to an accuracy of +/- 1 to 2 bp (data not shown).

Construction of 5' deletion derivatives of the *nifH*'-'*lacZ* fusion plasmid pRJ1009

Plasmid pRJ1009 contains approximately 880 bp of B.japonicum *nifH* DNA between the EcoRI and BamHI sites of pMC1403 (Fig. 1B) (26). From pRJ1009 four deletion derivatives were constructed. Plasmid pRJ1070 was generated by cutting at the unique BglII and HindIII sites, "filling in" and religation. This generated a 67 bp internal deletion. During the same experiment a clone with a 96 bp deletion was found, pRJ1071. We suspect that it has been generated by an unknown, contaminating endonuclease activity. DNA sequencing of this clone has established that the remaining DNA has not suffered additional alterations. Plasmids pRJ1072 and 1073 are EcoRI-HindIII and EcoRI-BglII deletions, respectively, generated after cutting, "filling in" and religation.

Subcloning 5' deletions into a wide host-range plasmid

To mobilize the B.japonicum promoter region plus the *lacZ* DNA contained in all the pMC1403 fusion derivatives, a modification was introduced into the wide host-range vector pRK290. A specific 10 bp linker (5'CCCTCGAGCC3') was synthesized using a DNA synthesizer from Applied Biosystems (model 380A, Foster City, CA). Plasmid pRK290 was cut at the unique EcoRI site, "filled in", and the linker was introduced. The linker regenerated the EcoRI site and introduced a unique XhoI restriction site; the new vector was called pRK290X. The pMC1403-derived plasmids were digested with EcoRI, which cuts at the 5' end of the relevant B.japonicum DNA, and with SalI which cuts downstream of the *lacA* gene in pMC1403. The fragments thus generated contained the B.japonicum DNA upstream of *nifD* and *nifH* plus the *lacZYA* genes of the fusion vector.

The EcoRI-SalI fragments were subcloned into EcoRI/XhoI-cut pRK290X, and the new constructs were transferred to B.japonicum 110spc4 by a three-parental mating using pRK2013 as helper plasmid (49). It is important to mention that when the B.japonicum exconjugants carrying the pRK290X-based *lacZ* fusions were grown on PSY-Xgal-tetracycline they gave rise to light-blue colonies after ca. 10 days at 30°C. The wild-type B.japonicum strain without a fusion plasmid gave rise only to white colonies if grown on PSY-Xgal medium.

Estimation of stability of pRK290X-based fusion plasmids

Precultures used to inoculate the microaerobic cultures were grown without selection for the fusion plasmids (tetracycline) because this antibiotic seems to interfere in *nif* derepression (B. Regensburger, pers. commun.). To investigate the stability of all the pRK290X-fusion plasmids, samples from these cultures were streaked onto PSY-Xgal without tetracycline. For a strain harboring pRJ1025 the following results were obtained: after ca. 15 days at 30°C a mixture of colonies was observed in which 70% were blue-segmented colonies and the remaining colonies were 8% blue and 22% white. Samples taken at the end of the microaerobic growth (9 days) and plated on PSY-Xgal revealed ca. 40% blue-segmented colonies and ca. 54% and 6% of white and blue colonies, respectively. In the case of the plant tests the culture used to infect the soybean seedlings was grown in the presence of tetracycline and, in this case, samples streaked onto PSY-Xgal revealed about 79% blue-segmented colonies and about 14% and 7% blue and white colonies, respectively. In all these tests there was no significant variation in stability among the different fusion plasmids.

To confirm the presence of bacteroids harboring the *lacZ* fusion plasmid, some nodules from 21 day-old plants were taken and halved, some others were used to cut 200 µm thick section. In both cases the nodule samples were incubated in saline-phosphate buffer (52) containing 5 µg/ml of Xgal. The colour reaction was followed using a stereo microscope and when the samples developed blue colour they were transferred to glutaraldehyde.

Assays of β -galactosidase activity

The β -galactosidase (β -gal) assay for the *E.coli* strains carrying the different fusion plasmids has been described (26). In every case the assays were done in duplicate from at least three independent cultures. Measurement of β -gal activity in the microaerobic system was done by taking 110 µl samples of the cultures and using two 25 µl aliquots for duplicate assays as described (55). From the remaining sample, 50 µl were used to measure A_{600} after dilution of the samples. Activity of β -gal was calculated as Miller units (55). For each strain three independent cultures were assayed in duplicate.

To measure β -gal activity from root-nodule bacteroids all the nodules from one plant were collected in an Eppendorf tube containing a small amount of powdered glass and 100 µl of ice-cold Z-buffer (55) plus 0.5M mannitol. The nodules were crushed with a blunted glass pipette which fitted well into the

Fig. 1. Map of *B.japonicum* *nifD*- and *nifH*-*lacZ* fusion plasmids. A. Deletion derivatives of the wild-type *nifD*-*lacZ* fusion plasmid (pRJ1008). The deletion end points were mapped with an accuracy of ± 1 to 2 bp (for details see Materials and Methods). The plasmid numbers in parenthesis correspond to the pRK290X-based derivatives (see text). The hatched area between E and C is a portion of pBR322 sequence. B. Deletion derivatives of the wild-type *nifH*-*lacZ* fusion plasmid (pRJ1009). The sizes of the deletions were proven by DNA sequencing. The asterisk (*) indicates the spontaneous deletion (Δ) endpoint of pRJ1071. The following symbols apply to both sections (A) and (B): open bars denote vector (pMC1403) DNA; the filled boxes labelled "P" indicate the *nif* consensus promoters; ($\blacktriangle$ - $\triangle$) marks the positions of the TGT-N₁₀-ACA sequences; restriction sites are B (*Bam*HI), Bg (*Bgl*II), C (*Cla*I), E (*Eco*RI), H (*Hind*III), Sp (*Sph*I), Xm (*Xma*III).

Eppendorf tube. More Z-buffer-mannitol was added to a final volume of 0.5, 1.0 or 1.5 ml depending on the amount and size of the nodules. The Eppendorf tubes were centrifuged (10 min/4°C/2500 rpm) to pellet the cell debris. From the supernatant, appropriate aliquots were taken to measure β -gal activity (55). In this case, and because of the small size and number of nodules during the first days after infection of the seedlings, protein was measured using the Bio-Rad assay since this was more reliable than measurement of optical density. β -Gal activity was calculated as specific activity (nmol o-nitrophenol/min/mg protein) (55).

Sequence homology analysis

Comparison of upstream sequences from different organisms was done using a program based on the algorithm of DIAGON (56). It was developed for an IBM-AT

Fig. 2. Activation of *B.japonicum* *nifD-lacZ* fusions in a heterologous system (*E.coli* ET8000). The abscissa depicts the *nifD* promoter region and the positions of the deletion end-points of the various plasmids as shown in Fig. 1A. The symbol ($\blacktriangle-\triangle$) marks the positions of the TGT-T₁₀-ACA sequences, and the filled box labelled "P" indicates the *nif* consensus promoter. The plotted values represent *K.pneumoniae* *nifA*-mediated activation, since background β -gal activity obtained in the absence of *Kp nifA* (ca. 0.5%) was subtracted in all instances.

computer and an EPSON FX100 printer. The program was written using TURBO Pascal (I. Lorencez-Gonzalez, unpublished).

RESULTS

Effect of 5' deletions on *nifA*-dependent activation of the *B.japonicum nifD* and *nifH* promoters

Plasmids pRJ1008, pRJ1009 and their deletion derivatives (Fig. 1) were used to transform the *E.coli* strain ET8000. The *K.pneumoniae nifA* product was supplied in trans from pMC71A which is a plasmid that constitutively produces the *nifA* gene product and which is compatible with the *lacZ* fusion plasmids (3,18,26).

Effect of deletions on the *nifD* promoter. The results obtained for pRJ1008 and its deletion derivatives are shown graphically in Fig. 2. Wild-type levels of activity, those obtained for pRJ1008, were maintained by plasmids pRJ1019, 1010 and 1021. Between pRJ1021 and pRJ1011 there is a drop in activity equiva-

Table 2. Activation of *B.japonicum* *nifH-lacZ* fusions in a heterologous system

Plasmid	β -Gal activity (%)	
	---	+Kp NifA protein
pRJ1009	0.4	100.0
pRJ1070	0.3	1.2
pRJ1071	0.4	1.0
pRJ1072	0.4	3.1
pRJ1073	0.4	4.2

The background was *E.coli* ET8000. The *K.pneumoniae* *nifA* product was provided in trans from pMC71A. See Fig. 1B for description of plasmids.

lent to about 50% of the wild-type. Activity drops further for pRJ1012 (to about 40%) and reaches a minimum for pRJ1014. The low level (ca. 7%) obtained with pRJ1014 is maintained throughout plasmids pRJ1015, 1016 and 1017. The activity then drops to zero when the deletions reach into the *nif* promoter (pRJ1020 and 1018).

From the graph in Fig. 2 it is possible to distinguish two regions that affect *nif* gene expression: one between the *nif* consensus promoter up to position -100 and a second one from position -101 to -166. The results show that wild-type levels of activity from the *B.japonicum* *nifD* promoter, in the heterologous system, can only be achieved if the second region is intact. The fact that all the deletion plasmids have similar sequences upstream of the 5' *EcoRI* site shows that vector sequences cannot substitute for the deleted regions, suggesting that within the second region there is a specific DNA sequence responsible for the effect observed. The low level of activation obtained from plasmids with deletions between -100 and the promoter region may represent the activity due solely to the *nif* promoter.

Effect of deletions on the *nifH* promoter. Activation analysis of pRJ1009 and its deletion derivatives showed that wild-type levels of activation from the *B.japonicum* *nifH* promoter also depend on upstream DNA (Table 2). In the cases of pRJ1070 and 1071, both of which contain internal deletions, activities were only about 1.2% of the wild-type which demonstrates that other *B.japonicum* DNA cannot substitute for the DNA lost within the deleted regions. Both the internal deletions even gave a lower level of expression than deletions which entirely remove the upstream *B.japonicum* DNA (Table 2). One may interpret these results on the basis that the internal deletions result in positioning of upstream sequences closer to the transcription start site and hence would negatively interfere with promoter activity.

Effect of 5' deletions on activation of the *nifD* promoter in the *B. japonicum* background

The requirement for upstream sequences for the activation of the *B. japonicum* *nifD* and *nifH* promoters, as measured in *E. coli*, could potentially be an artifact due to the heterogeneity of the system. To test this possibility the *B. japonicum* *nifD/lacZ* regions of plasmids pRJ1008, 1021, 1011, 1014 and 1020 were cloned into pRK290X (Fig. 1A) and mobilized into *B. japonicum* 110spc4. Activities from these *nifD*'-'*lacZ* fusion plasmids, pRJ1025, 1040, 1037, 1038, 1039, were assayed in cells grown in microaerobic (*ex planta*) conditions as well as from root-nodule bacteroids.

Microaerobic (*ex planta*) assays. It was possible that the upstream DNA regions could be required only during the first stages of derepression; therefore, if activity was measured late, differences may not have been detected. Thus, derepression was followed from the day the cultures were inoculated. The results (Fig. 3A) show that wild-type levels of β -gal activity cannot be obtained from strains carrying deletion derivatives. Surprisingly, in this experiment pRJ1040 does not reach wild-type levels as compared to the corresponding plasmid (pRJ1021) in the heterologous system. All the other plasmids behave similarly as compared to the heterologous system.

During this experiment acetylene reduction, from parallel cultures of *B. japonicum* without plasmids as well as from strains carrying *lacZ*-fusion plasmids, was first detected at day 7. The internal control, *B. japonicum*/pRJ1039 showed almost undetectable levels of β -gal, and a culture of *B. japonicum*/pRJ1025 grown under aerobic conditions did not show derepression (Fig. 3A). Assays in root-nodule bacteroids. *B. japonicum* strains carrying pRJ1025, 1037, 1038, 1039 and 1040 as well as a plasmid-free strain were used to infect soybean seedlings. β -Gal assays from root-nodule bacteroids were started from day 8 for *B. japonicum*/pRJ1025 and from day 10 for the other strains. Up to day 13 all the nodules from one plant were assayed for each strain, and from day 15 all the nodules from two plants were assayed independently and the results averaged.

The results from this experiment (Fig. 3B) were very similar to those obtained in the microaerobic cultures. Again none of the strains carrying deletion derivatives reached wild-type levels of β -gal activity. In this case the β -gal activity from plasmid pRJ1040 deviates even further from the wild-type

Fig. 3. Derepression kinetics of *B. japonicum* *nifD-lacZ* fusions in a homologous system (*B. japonicum* 110spc4). A. β -Gal activities from fusion plasmids in cells grown under free-living, microaerobic conditions. B. β -Gal activities from fusion plasmids in soybean root-nodule bacteroids. Strains were used that contained the following plasmids (cf. Fig. 1A): pRJ1025 (●), pRJ1040 (△), pRJ1037 (■), pRJ1038 (○), pRJ1039 (□), pRJ1039 or *B. japonicum* 110spc4 without plasmids (◻), and pRJ1025 in cells grown under full aerobiosis (▲).

levels of activity and in fact the β -gal activity from this plasmid is now closer to that of pRJ1037 than to the β -gal activity of pRJ1025. Apart from plasmid pRJ1040 the pattern of activity obtained in this experiment resembles closely that obtained in the microaerobic and the heterologous systems.

DISCUSSION

Stability of pRK290X-derived fusion plasmids

It is evident from our results that without selective pressure the pRK290X-derived plasmids were highly unstable, however, it appeared as if during growth in the microaerobic cultures the rate of plasmid loss was the same for all the different constructions. Moreover, we estimate that under the microaerobic growth conditions only about 12 generations of *B. japonicum* would have occurred, thus minimising the effect of plasmid loss. Hence, the differences in β -gal derepression from the individual plasmids were not due to different plasmid instabilities. In the plant-infection tests the instability was apparently not as severe since the precultures were grown in the presence of tetracycline. Furthermore, 5 randomly chosen nodules (out of 18) from one 21-

day old plant gave a positive reaction to the Xgal test (data not shown) which indicated that most (if not all) nodules contained bacteria harbouring the fusion plasmids. Nevertheless, plasmid loss occurred within the nodules since distribution of the blue colour (Xgal reaction) in nodule cross-sections was not homogeneous (not shown).

Effect of upstream sequences on the activation of the *B.japonicum* *nifD* and *nifH* promoter in the heterologous system

When the *B.japonicum* *nifD* deletions and the *nifH* derivatives pRJ1072 and 1073 were made, vector (pMC1403) sequences were substituting the deleted regions. However, when *nifH*-internal deletions were constructed, as in plasmids pRJ1070 and 1071, *B.japonicum* DNA and not vector sequences replaced the deleted regions. Regardless of the origin of the replacing DNA in the deletion derivatives, the effect of removing the sequences upstream of the -115 position for *nifH* or -101 for *nifD* was always the same: a reduction of more than 90% of the wild-type promoter activity. These results suggest that the deleted sequences are highly specific since they cannot be simply substituted by other DNA sequences.

For the *nifD* promoter, plasmid pRJ1014 seems to delimit the region where the upstream sequences have an effect, since the activity observed with this plasmid is not significantly different from pRJ1015, 1016 and 1017. The level of activity from all these plasmids is about 6-7% of the wild-type (Fig. 2). This low level drops to zero when the deletions reach into the *nif* consensus promoter as in plasmids pRJ1018 and 1020. The low level (6-7%) of residual activity observed would depend only on the presence of a functional *nif* promoter, unless there is another influencing sequence between positions -65 and the *nif* promoter.

Effect of upstream deletions on activation of the *nifD* promoter in *B.japonicum*

From the results shown in Fig. 3A and B it is clear that removal of DNA upstream of the *nifD* promoter has a strong effect on the capacity of this promoter to be activated. The data also confirm that the results obtained when using the *E.coli* background are not an artifact of the system. In Fig. 3A it can be seen that during the first 4 days of microaerobic growth the activity from all the plasmids, except pRJ1039, runs parallel at a level between 3% and 8%. After day 4, when *nif* derepression begins, the effect of the upstream deletions becomes evident. That the activity from pRJ1039 is significantly lower than that from the other plasmids suggests that the low level of activity ob-

Fig. 4. Comparison of *nifD* and *nifH* upstream sequences from *B.japonicum* (Bj.) with the *nifD* upstream sequence from cowpea *Rhizobium* sp. IRC78 (IRC). The *nif* consensus promoters are underlined, the TGT-N₁₀-ACA sequences are boxed, and the 4 regions of homology, identified by computer analysis, between the *nifD* genes of *B.japonicum* and IRC78 (see text and Fig. 5) are numbered and enclosed within arrows. The asterisks mark identical nucleotides.

served before derepression is due solely to the intact *nif* consensus promoter. Also, since the upstream deletions have an effect on activation only after the onset of derepression we conclude that the low level of activation during the first 4 days is *nifA*-independent and that *nifA*-mediated activation of this promoter depends on the integrity of the upstream sequences.

In the heterologous system β -gal activity from plasmid pRJ1021 reaches wild-type levels of activity (Fig. 2); however, activity from the corresponding plasmid pRJ1040 in the *B.japonicum* background is not higher than 65% (Fig. 3A,B). At least 2 explanations may be possible. (i) Wild-type levels of activity in the *B.japonicum* background may require more DNA upstream of the -165 position. (ii) In pRJ1021 the sequences 5' to the *EcoRI* site are derived from pMC1403 and in pRJ1040 from pRK290X; hence, it may be possible that the sequences from pRK290X have an influence in this particular construction.

Possible identity of the upstream sequences

The effect of deletions upstream of a *nif* promoter was first noticed in the *nifLA* operon of *K.pneumoniae* (13). In this case the capacity for *nifA*- or

Fig. 5. Comparison of *nif*-upstream sequences from 10 different N_2 fixing bacteria. Each square represents the graphic output from the pairwise analysis of the sequences as produced by the DIAGON program. The four regions of homology identified between the *nifD*-upstream regions of *B.japonicum* and *IRc78* are also shown in Fig. 4. Double lines separate slow-growing rhizobia (genus *Bradyrhizobium*), fast-growing rhizobia, and *K.pneumoniae*. *Nif* genes from the following bacteria were compared (References in parentheses): *B.japonicum* (Bj) *nifD* and *nifH* (58,67); cowpea *Rhizobium* *IRc78* (IRc) *nifD* (68); *Parasponia Rhizobium* (PR) *nifH* and *nifD* (69,70); *R.meliloti* (Rm) *nifH* and *fixA* (71); *R.phaseoli* (Rp) *nifH* (72); *R.trifolii* (Rt) *nifH* (60); *K.pneumoniae* (Kp) *nifH* (73).

ntrC-mediated activation was gradually reduced in deletions extending from -150 to -28, and the deletion ending at -28 still retained its capacity to be activated by the *nifA* or *ntrC* products (13). A similar gradual decrease in activity from deletion mutants (from -98 to -40) of the *E.coli* *tyrT* promoter has also been observed (57). In this case it has been proposed that the role of the upstream DNA may be to bind additional RNA polymerase molecules which may then slide down to the promoter region (57). A similar mechanism may explain the observations on the *nifLA* promoter.

The involvement of more specific upstream sequences in *nif* regulation was first proposed for the *K.pneumoniae* *nifH* gene (41). In this case a G to T transversion at position -136 abolished the *Nif*-inhibition effect normally

associated with a multi-copy nifH promoter (20), and was believed to be due to titration of nifA product by the strong nifH promoter (20,41). The sequence around the G residue at -136 in the K.pneumoniae nifH promoter is TTGTTC. We found similar sequences in the B.japonicum nifD and nifH promoters at -157 and -140, respectively. At the same time work done to investigate the upstream sequences of the K.pneumoniae nif promoters which affect nifA-mediated activation revealed a wider consensus: TGT-N₁₀-ACA (43).

When this consensus is used, two similar regions are evident in each of the B.japonicum nif promoters (Fig. 4). In the nifD promoter the region with the TGT sequence centered at -157 was originally published as having 9 bp in-between TGT and ACA (58); however, reevaluation of the original data and analysis of new sequence data indicates the presence of an A residue at position -146 (data not shown). In the second sequence, the TGT is located at position -109. For the nifH promoter the sequences occur at -140 and -111.

Since the 5' TGT-N₁₀-ACA 3' sequence has rotational symmetry this could mean that the 3'-ACA-N₁₀-TGT 5' could also be an upstream sequence of a divergently transcribed, potential nif promoter. This has been proposed in the case of the K.pneumoniae nifH promoter where a second upstream element located at -180 appears to belong to the divergently transcribed ORF (43). In the case of the B.japonicum nif genes both TGT-N₁₀-ACA sequences upstream of each promoter seem to play an active role in regulation since deletions that remove only the copy located farthest upstream leaving the promoter proximal copy intact drastically affect activation of either the nifH (pRJ1073) or nifD (pRJ1011 and 1012) promoters (Table 2 and Fig. 2). Furthermore, Tn5 mutagenesis and sequence analysis have shown that no other nif or fix genes appear to be located immediately upstream of either nif operon (54,59).

Comparison of upstream sequences from different N₂-fixing microorganisms

The published promoter-upstream sequences of nif genes from 10 different bacteria were compared to each other. The 3' end was the point of transcription initiation or 12 bp downstream of the conserved GC residues of the nif promoter in those cases where the start of transcription was not known. A window of 19 bp was used (approximately the size of the nif consensus promoter) together with a significance level of 0.001% (i.e. the homologies found have a 0.001% probability of being random).

The result of the comparison for each pair of sequences is shown graphically in Fig. 5. As an example of the stringency level of the comparisons one

can see that 4 "diagonals" appear when the B.japonicum nifD and the IRc78 nifD sequences are compared. The actual sequences and the 4 homologous regions are shown in Fig. 4 together with the B.japonicum nifH sequence.

From Fig. 4 it can be seen that for B.japonicum and the IRc78 strain conservation of the upstream regions begins just before the promoter-distal TGT-N₁₀-ACA copy and that the homology extends immediately downstream of each of the two TGT-N₁₀-ACA sequences. It is also interesting to note that in the slow growers conservation is stronger between similar nif genes of two different species (B.japonicum and IRc78 nifD/nifD, B.japonicum and Parasponia Rhizobium nifH/nifH) than between nif genes of the same organism (B.japonicum nifD/nifH or Parasponia Rhizobium nifD/nifH).

From Fig. 5 it is also evident that despite the homologies among the slow growers (Bradyrhizobium) there is very little homology to fast growers (Rhizobium), at most only at the level of the nif promoter. Nevertheless the fast growers have a certain degree of homology among themselves and neither group shows significant homology to the K.pneumoniae nifH upstream region. These results disagree with the notion of nif genes having nif-specific and species-specific regions (60) since, at this stringency level of comparison, no species-specific sequences were apparent within the fast or slow growers and no nif-specific sequences were shared (besides in some cases at the nif promoter) between fast and slow growers.

The strong conservation of the sequences upstream the promoter region of slow growers leads to further speculations. (i) The additional sequences immediately downstream of the two TGT-N₁₀-ACA copies may also play a role in regulation, which in this work may not have been evident if the effect was obscured by the more drastic consequences of removing one or both of the TGT-N₁₀-ACA sequences. (ii) The fact that there is no significant homology between the overall upstream sequences of nifD and nifH of B.japonicum, whereas interspecies conservation of these genes is high, suggests that these promoters may be regulated somewhat differently, perhaps only on a quantitative basis.

In the light of this last point it is important to mention that the R.meliloti nifH upstream sequences do not seem to be required to achieve wild-type levels of expression when present in a R.meliloti background but do have an effect when activation is tested in E.coli (44). In contrast, the results presented here show that the B.japonicum nifD promoter region strongly depends on these sequences in both heterologous and homologous backgrounds. The explana-

tion for the difference observed may be that R.meliloti has only one TGT-N₁₀-ACA upstream sequence and activation in the nodule environment may not depend strongly on it. Alternatively, the relatively high residual activity of the intact nif consensus promoter in the R.meliloti upstream deletion mutants (44) could have obscured the contribution of the upstream sequences in mature bacteroids. Another possibility that cannot be completely excluded is that the B.japonicum and R.meliloti nifH promoters may have a similar regulation and different to that of B.japonicum nifD.

Possible role of the promoter-upstream sequences

When the K.pneumoniae nifH promoter is cloned in a multi-copy plasmid and introduced into a wild-type K.pneumoniae strain, the cells become phenotypically Nif⁻ (20,41). The explanation for this Nif-inhibition effect was that the multi-copy nifH promoter could titrate out the product of the nifA gene thus inhibiting or drastically reducing expression from weaker nif promoters (20). Titration could occur if the nifA product would bind directly to the nifH promoter. The observation that mutations in the promoter, and a point mutation at position -136, could abolish the multi-copy effect (41,42) indirectly supported the idea of nifA product-DNA interaction.

Analysis of the nifA DNA-sequence has shown that the nifA product has a potential DNA-binding site near its carboxy-terminal end (61). Furthermore, the nif upstream consensus sequence TGT-N₁₀-ACA turns out to be a subset of the TGTGT-N₆₋₁₀-ACACA sequence which has been proposed as the consensus DNA-binding site for the gene products of lacI, crp, araC, lexA, galR and others (62).

In the K.pneumoniae system it has recently been shown that the TGT-N₁₀-ACA sequence upstream of the nifH promoter is functionally independent of its orientation, is cis acting, and its position upstream can be translocated within 2 kb and still remains active (43).

All the evidence presented above strongly suggests that the nifA product binds to the upstream DNA sequence of the nif promoters, presumably at the TGT-N₁₀-ACA site. Once bound it could (i) modify the tertiary structure of the DNA such that it brings closer two otherwise distant regions, (ii) stabilize a certain DNA conformation required for efficient promoter activity or, (iii) bind another regulatory protein or RNA polymerase itself (43,63).

Activation of some genes in eukaryotes require upstream sequences called "enhancers", as in the activation of some SV40 early genes by Sp1 protein or

activation of heat-shock genes of *Drosophila* by HSTF protein (63). In yeast there are also examples of positive regulation involving upstream activating sequences (UAS) such as the induction of *CYC1* by the *HAP1* gene product (64), and activation of *GAL1* by the *GAL4* product (65,66). In the latter case the *GAL4* protein binds to the UAS sequences and then, apparently, it needs to make contact with another protein in order to exert its activator function (66).

In the case of the prokaryotic upstream sequences described for *B.japonicum* in this work, and those of *K.pneumoniae* (43), their mode of action remains to be elucidated. However, a new level of complexity in prokaryotic regulation is now evident.

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