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The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of *nif* gene expression and formation of determinate symbiosis

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In the slow-growing soybean symbiont, *Bradyrhizobium japonicum* (strain 110), a *nifA*-like regulatory gene was located immediately upstream of the previously mapped *fixA* gene. By interspecies hybridization and partial DNA sequencing the gene was found to be homologous to *nifA* from *Klebsiella pneumoniae* and *Rhizobium meliloti*, and to a lesser extent, also to *ntrC* from *K. pneumoniae*. The *B. japonicum nifA* gene product was shown to activate *B. japonicum* and *K. pneumoniae nif* promoters (using *nif*::*lacZ* translational fusions) both in *Escherichia coli* and *B. japonicum* backgrounds. In the heterologous *E. coli* system activation was shown to be dependent on the *ntrA* gene product. Site-directed insertion and deletion/replacement mutagenesis revealed that *nifA* is probably the promoter-distal cistron within an operon. NifA$^-$ mutants were Fix$^-$ and pleiotropic: (i) they were defective in the synthesis of several proteins including the *nifH* gene product (nitrogenase Fe protein); the same proteins had been known to be repressed under aerobic growth of *B. japonicum* but derepressed at low O_2 tension; (ii) the mutants had an altered nodulation phenotype inducing numerous, small, widely distributed soybean nodules in which the bacteroids were subject to severe degradation. These results show that *nifA* not only controls nitrogenase genes but also one or more genes involved in the establishment of a determinate, nitrogen-fixing root nodule symbiosis.

Key words: Bradyrhizobium/gene regulation/*nif* genes/nitrogen fixation/root nodule symbiosis

Introduction

The formation of a nitrogen-fixing root-nodule symbiosis between *Rhizobium* or *Bradyrhizobium* species and legumes involves a series of differential gene activation steps, both in the bacterial and plant symbionts, which are subject to complex genetic control. In the bacterial symbiont two regulatory genes have been identified recently, one of which (*nodD*) is involved in early steps of nodule initiation, whereas the other one (*nifA*) is thought to be involved at a later stage when derepression of symbiotic nitrogen fixation occurs. Mulligan and Long (1985) have shown that in *Rhizobium meliloti* the product of *nodD*, together with an unknown factor in the host plant root exudate, is required to activate the expression of *nodABC* genes, the products of which then elicit the earliest morphological change in the host plant known as root-hair curling. *NifA*-like regulatory genes have been identified in *R. meliloti* (Szeto *et al.*, 1984) and *R. leguminosarum* (Schetgens *et al.*, 1985), and have been shown to be required for the expression of the nitrogenase structural genes *nifH* (coding for the Fe protein) and *nifDK* (coding for the MoFe protein).

The *nifA* gene has originally been identified in a non-symbiotic diazotroph, *Klebsiella pneumoniae*, in which the *nifA* gene product, together with the product of *ntrA*, functions as a specific transcriptional activator for all *nif* operons, while the expression of *nifA* itself is regulated by general nitrogen control, i.e. it is strongly dependent on activation by the product of *ntrC* (for review, see Dixon, 1984). *NifA*- and *ntrC*-controlled promoters are characterized by common consensus promoter sequences (Beynon *et al.*, 1983; Dixon, 1984), and *nifA* and *ntrC* are functionally and structurally homologous (Ow and Ausubel, 1983; Merrick, 1983; Buikema *et al.*, 1985).

The *R. meliloti nif*-specific regulatory gene has been found to be homologous to both the *nifA* and *ntrC* genes of *K. pneumoniae* (Buikema *et al.*, 1985; Weber *et al.*, 1985). This gene, however, is more likely a *nifA*-like gene since Ausubel *et al.* (1985) have recently reported on the identification of a *R. meliloti ntrC*-like gene: interestingly, a *R. meliloti ntrC* mutant was not severely affected in symbiotic nitrogen fixation, whereas it was unable to grow on minimal media containing arginine, proline or aspartate as the sole nitrogen source. This suggests that in *R. meliloti* the *ntrC* product is not absolutely required for *nifA* expresion during symbiosis.

In slow-growing rhizobia (genus *Bradyrhizobium*) no symbiotic regulatory genes have been described thus far. In *B. japonicum*, the soybean symbiont, we had previously identified the nitrogenase structural genes and a number of further symbiotic genes (*nifB, fixA, fixBC, nodABC*; Kaluza and Hennecke, 1984; Fuhrmann and Hennecke, 1984; Fuhrmann *et al.*, 1985; Lamb and Hennecke, 1986). The *nifDK, nifH, nifB* and *fixA* promoters were shown to carry the typical *nif* consensus promoter sequence known from *K. pneumoniae* (Kaluza and Hennecke, 1984; Fuhrmann and Hennecke, 1984; Fuhrmann *et al.*, 1985; Chelm *et al.*, 1985). Using translational *nifD*::*lacZ* and *nifH*::*lacZ* fusions it was then demonstrated that the *B. japonicum* promoters can be activated by the *K. pneumoniae* NifA protein but not by the product of *ntrC* (Alvarez-Morales and Hennecke, 1985). These findings suggested that an analogous *nifA*-like regulatory gene may be present in the genome of *B. japonicum*. Here we report on the identification and mapping of a *nifA*-like gene in *B. japonicum* (strain 110) and demonstrate that its product functions as a transcriptional activator for *nif* promoters. Using NifA$^-$ mutants and taking advantage of the free-living, microaerobic culture system (which, in the wild-type, leads to derepression of nitrogenase activity *ex planta*) we provide evidence that *nifA* also regulates the synthesis of further proteins known to be under oxygen control. Furthermore *nifA* appears to control functions that determine the frequency of nodulation and the persistence of bacteroids in infected soybean nodule cells.

Results

NifA-specific interspecies homology, and mapping of a nifA-homologous region upstream of fixA

To identify potential *nifA* and/or *ntrC*-homologous genes, total genomic DNA of *B. japonicum* as well as DNA from a number

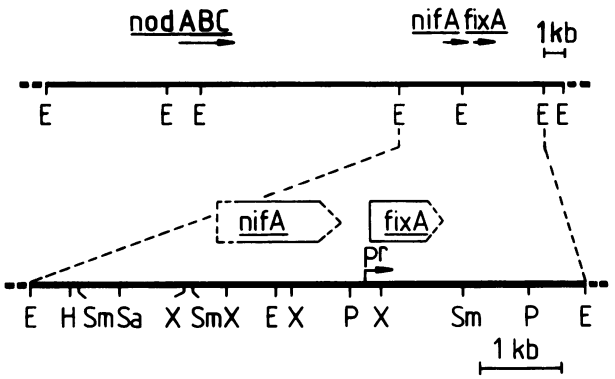

Fig. 1. Physical map of the *B. japonicum nodABC–nifA–fixA* region. The mapped region is contained within cosmid pL48-11G. The approximate location of the genes was determined by interspecies hybridization, partial DNA sequence analysis and mutational analysis. Pr = *fixA* promoter (Fuhrmann *et al.*, 1985). Restriction sites: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I.

Plasmid(s) in *E. coli* MC1061	Relevant structure of *B. japonicum nifA* expression plasmids	β-Galactosidase activities (Miller units)
— —		9 ± 6
pRJ1025 —		49 ± 17
pRJ1025+pRJ7515	(Pv/Sm) nifA P	4141 ± 622
pRJ1025+pRJ7521	Δ P	8368 ± 1283
pRJ1025+pRJ7519	Δ P	7264 ± 1230
	500 bp	
pRJ1025+pMC71A		9220 ± 2453

Fig. 2. Activation of the *B. japonicum nifD* promoter by the *nifA* gene product. β-Galactosidase values represent averages of at least three independent experiments. The sizes of the deletions (Δ) were measured by restriction enzyme analyses. Open bars denote *B. japonicum* DNA controlled by the promoter of the pBR329-derived chloramphenicol acetyltransferase gene. The junction between the previous *Pvu*I and *Sma*I sites is indicated (Pv/Sm); P = *Pst*I.

of cosmid or plasmid clones (carrying previously identified *nif, fix* and *nod* genes) were probed in Southern blot hybridizations with ^{32}P-labelled DNA fragments specific for *K. pneumoniae nifA* and *ntrC* (see Materials and methods for precise description of the probes used). The *ntrC* probe was completely internal to the gene whereas the *nifA* probe also contained 219 bp from the 5′ end of the adjacent *nifB* gene, which had previously led to the identification of a *nifB*-homologous gene (Fuhrmann *et al.*, 1985). Both probes hybridized to the same DNA region located immediately upstream of the previously identified *fixA* gene (Fuhrmann *et al.*, 1985). Hybridization to the *nifA* probe was always stronger than with the *ntrC* probe, even under conditions of low stringency (data not shown). A restriction map of the *nifA*-homologous region between *nodABC* and *fixA* is given in Figure 1. Hybridization to total *B. japonicum* DNA did not uncover any additional region with unambiguous *nifA* or *ntrC*-specific homology.

To confirm the hybridization data, we established the nucleotide sequence around the *Eco*RI site (Figure 1) within the *nifA/ntrC*-homologous region. The deduced amino acid sequence of one of the six possible reading frames showed 57% homology to the *K. pneumoniae* NtrC protein (between Gln_{225} and Thr_{280}), 71% to the *K. pneumoniae* NifA protein (between Lys_{297} and Thr_{352}), and 70% to the *R. meliloti* NifA protein (between Val_{285} and Thr_{340} (Buikema *et al.*, 1985). Because of the higher homology to *nifA* the corresponding *B. japonicum* region is tentatively called '*nifA*'. From the sequencing it was also possible to determine the orientation and the approximate location of the *nifA*-like gene as depicted in Figure 1.

Activation of nif promoters by the nifA gene product

An *in vivo* assay was established to obtain functional proof that the putative *B. japonicum nifA* gene codes for a transcriptional activator of other *nif* genes. The principle was to construct a $NtrA^+Lac^-$ *E. coli* strain with two compatible plasmids, one of which carried a *B. japonicum nifD::lacZ* fusion, and another one that expressed *nifA* constitutively, and activation of the *nifD* promoter was then monitored as β-galactosidase (β-gal) activity (Alvarez-Morales and Hennecke, 1985). The relevant components of the assay were as follows: (i) *E. coli* strain MC1061 served as recipient for plasmid transformations; (ii) plasmid pRJ1025 (Tc^R) carried the *B. japonicum nifD::lacZ* fusion in a pRK290 replicon; (iii) a 1.9-kb *Sma*I/*Pst*I fragment carrying *nifA* (Figure 1) was cloned into the *Pvu*II/*Pst*I site of pBR329 to give pRJ7515, thereby eliminating chloramphenicol and ampicillin resistance, and forcing *nifA* expression from the pBR329 promoter of the *cam* gene (Figure 2); to bring this promoter closer to *nifA*, derivatives of pRJ7515 were obtained as unidirectional, *Bal*31-generated deletions of the 5′ end of the *nifA*-like gene (plasmids pRJ7521 and pRJ7519). In addition, the remaining tetracycline resistance gene of all three plasmids was destroyed by cloning a 2347-bp *Xho*I fragment from Tn*5*, coding for kanamycin resistance (Km^R), into the *Sal*I site. This allows us to maintain selective pressure on *E. coli* MC1061 for the simultaneous presence of pRJ1025 (Tc^R) and the *nifA* expression plasmids (Km^R).

Figure 2 shows that the presence of pRJ1025 (*nifD::lacZ*) alone gave only low to background β-gal activity. In the presence of constitutive *nifA* expression the *nifD* promoter was activated 100- to 200-fold. Similar activation was obtained previously using pMC71A which constitutively expressed *K. pneumoniae nifA* from the *tet* promoter of pACYC184 (Alvarez-Morales and Hennecke, 1985; see also Figure 2, last line). The activation of *B. japonicum nifA* (Figure 2) was strongly dependent on *nifA* being expressed from an external promoter as no activation was obtained when *nifA* was cloned in the opposite orientation relative to the *cam* promoter (not shown). We have not, however, further investigated whether the *cam::nifA* hybrids shown in Figure 2 represent translational fusions in addition to being transcriptional fusions. Since pRJ7521 resulted in the most pronounced activation (Figure 2) a 2.1-kb *Bam*HI/*Pst*I fragment carrying the pBR329 promoter plus *nifA* was cloned into pACYC177 to give pRJ7523. This plasmid was compatible with a number of other available *nif::lac* fusion plasmids, and was then used for the experiment in Table I.

Table I (first and second columns) shows that, in the *E. coli* ET8000 background, the *B. japonicum nifA* gene product not only activates *B. japonicum* promoters (*nifD* and *nifH*) but also the *K. pneumoniae nifH* and *nifL* genes. (To avoid activation of *nifL* by the NtrC protein, the *nifL::lacZ* fusion was tested in the *glnAntrBC* deletion strain ET8894.) Furthermore, it was shown that activation of *B. japonicum* and *K. pneumoniae nif* promoters

Table I. Activation of *B. japonicum* and *K. pneumoniae nif* promoters by *K. pneumoniae* and *B. japonicum nifA* products in *E. coli*: dependence on *ntrA*

Plasmids	Relevant genotype	β-Gal activities from *nif–lacZ* translational fusions[a]		
		In ET8000 wild-type	In ET8894 Δ(*glnAntrBC*)	In ET8045 *ntrA*::Tn*10*
pRJ1008	Bj-*nifD'-'lacZ*	53 ± 12	ND	44 ± 4
pRJ1008/pMC71A	Bj-*nifD'-'lacZ*/Kp-*nifA*[c]	15 846 ± 5200	ND	28 ± 6
pRJ1008/pRJ7523[b]	Bj-*nifD'-'lacZ*/Bj-*nifA*[c]	1066 ± 607	ND	40 ± 14
pRJ1009	Bj-*nifH'-'lacZ*	24 ± 13	ND	26 ± 1
pRJ1009/pMC71A	Bj-*nifH'-'lacZ*/Kp-*nifA*[c]	5760 ± 1338	ND	22 ± 1
pRJ1009/pRJ7523[b]	Bj-*nifH'-'lacZ*/Bj-*nifA*[c]	146 ± 80	ND	25 ± 4
pMB1	Kp-*nifH'-'lacZ*	72 ± 16	ND	34 ± 1
pMB1/pMC71A	Kp-*nifH'-'lacZ*/Kp-*nifA*[c]	21 052 ± 3460	ND	37 ± 4
pMB1/pRJ7523[b]	Kp-*nifH'-'lacZ*/Bj-*nifA*[c]	3283 ± 2140	ND	42 ± 3
pRD532	Kp-*nifL'-'lacZ*	ND	88 ± 8	ND
pRD532/pMC71A	Kp-*nifL'-'lacZ*/Kp-*nifA*[c]	ND	4015 ± 376	ND
pRD532/pRJ7523[b]	Kp-*nifL'-'lacZ*/Bj-*nifA*[c]	ND	447 ± 300	ND

[a]Mean values of three independent experiments with two parallel cultures each: ND = not determined.
[b]Note that activation by pRJ7523 in this particular *E. coli* background (ET8000 or derivatives of it) is much lower than in *E. coli* MC1061 (cf. Figure 2). One possible reason for this may be the *gyrA* marker in ET8000 which potentially interferes with expression from plasmid pRJ7523.
[c]Constitutive *nifA* expression.

by the *B. japonicum* NifA protein was dependent on the presence of the *E. coli* NtrA protein (Table I, third column).

To rule out completely any potential artifact because of using the heterologous *E. coli* system, similar experiments were also performed in a homologous *B. japonicum* background. For this purpose DNAs carrying the entire *B. japonicum* and *K. pneumoniae nif::lac* fusions were cloned into a derivative of the wide host-range plasmid pRK290 (details of the constructions will be reported elsewhere), and then these plasmids (pRJ1025, pRJ1024, pKP1026, pKP1027) were mobilized into *B. japonicum* 110*spc4* by conjugation. A *nif::lac* fusion plasmid with a deletion of the *nifD* promoter (pRJ1039) served as negative control. Table II (first column) shows that free-living, microaerobic cultivation of these *B. japonicum* strains led to clear derepression of β-gal activity from the *B. japonicum nifD* and *nifH* promoters as well as from the *K. pneumoniae nifH* promoter. Only low levels of derepression were obtained from the *K. pneumoniae nifL* promoter. In a non-revertible *B. japonicum* NifA$^-$ mutant background (see next paragraph for the construction of mutant A9) the corresponding promoter activities (except *nifL*) were reduced by >90% which again proves their dependence on activation by the NifA protein (Table II, second column). There is some residual activity left which must stem from leaky, *nifA*-independent expression of *nif* promoters in this homologous background: as the control with pRJ1039 shows, this leaky expression requires an intact *nif* promoter. It is not known whether this low level expression is dependent on additional cellular factors.

Table II. Activation of *B. japonicum* and *K. pneumoniae nif* promoters in *B. japonicum*[a]

Plasmid	Relevant genotype	Relative β-gal activities (%)	
		In 110*spc4* wild-type	In A9 *nifA*::*aph*
None		0.5	ND
pRJ1039	Bj-Δ*nifD'-'lacZ*	0.6	ND
pRJ1025	Bj-*nifD'-'lacZ*	100	6.4
pRJ1024	Bj-*nifH'-'lacZ*	95	7.2
pKP1026	Kp-*nifH'-'lacZ*	56	4.5
pKP1027	Kp-*nifL'-'lacZ*	8.7	7.7

[a]*B. japonicum* strains were grown under free-living, microaerobic conditions. No selective pressure was exerted on plasmid-containing strains, as the presence of antibiotics such as tetracycline markedly inhibit derepression of Nif activity; ND = not determined.

Mutational analysis of the B. japonicum nifA region

B. japonicum nifA mutants were constructed by creating site-directed insertion or deletion/replacement mutations within and upstream of the *nifA*-like gene. Figure 3 lists a set of five mutant strain pairs. Each pair carries a particular kanamycin resistance gene cartridge from Tn*5* (see Materials and methods) in both orientations. This should help to recognize any possible out-reading promoter activity originating from the inserted fragment, e.g. from the promoter of the aminoglycoside phosphotransferase-3'-II (*aph*) gene. Mutations in strains A13/A14 and A11/A12, respectively, are insertions in the single *Hin*dIII and *Sal*I sites upstream of *nifA*. In strains A7/A8 a 1.4-kb *Sma*I fragment upstream of *nifA* was deleted and replaced by a Km^R cartridge. Similarly, in strain A1/A4 and A9/A10 two different *Xho*I fragments from the 5' end of *nifA* and internal to the gene, respectively, were deleted and replaced by Km^R cartridges. All mutants were tested for nodulation and symbiotic nitrogen fixation on soybean (*Glycine max*) roots (Fix phenotype). In addition, nitrogenase activity of all strains was determined under free-living, microaerobic culture conditions (Nif phenotype). The results are given in Figure 3.

All strains were able to nodulate but the nodulation properties differed substantially (these results will be discussed in a separate paragraph below). *NifA* deletion mutants A9 and A10 are clearly Fix$^-$ and Nif$^-$ (Figure 3) which proves the identity of *nifA* as a *nif* gene. The insertion in strain pair A13/A14 which is located 1.7 kb upstream of *nifA* does not significantly affect Fix and Nif activity; hence, this area does not appear to carry an essential symbiotic gene. All other strains show an interesting dependence on the orientation of the cloned *aph* fragment: (i) when the *aph* promoter is reading in the opposite direction to *nifA* (in strains A12, A8 and A4) none of the strains shows any detectable Fix or Nif activity; (ii) when the *aph* promoter reads in the same direction as *nifA* there is Fix activity, the amount of which depends on the type of insertion. Strain A11 has full

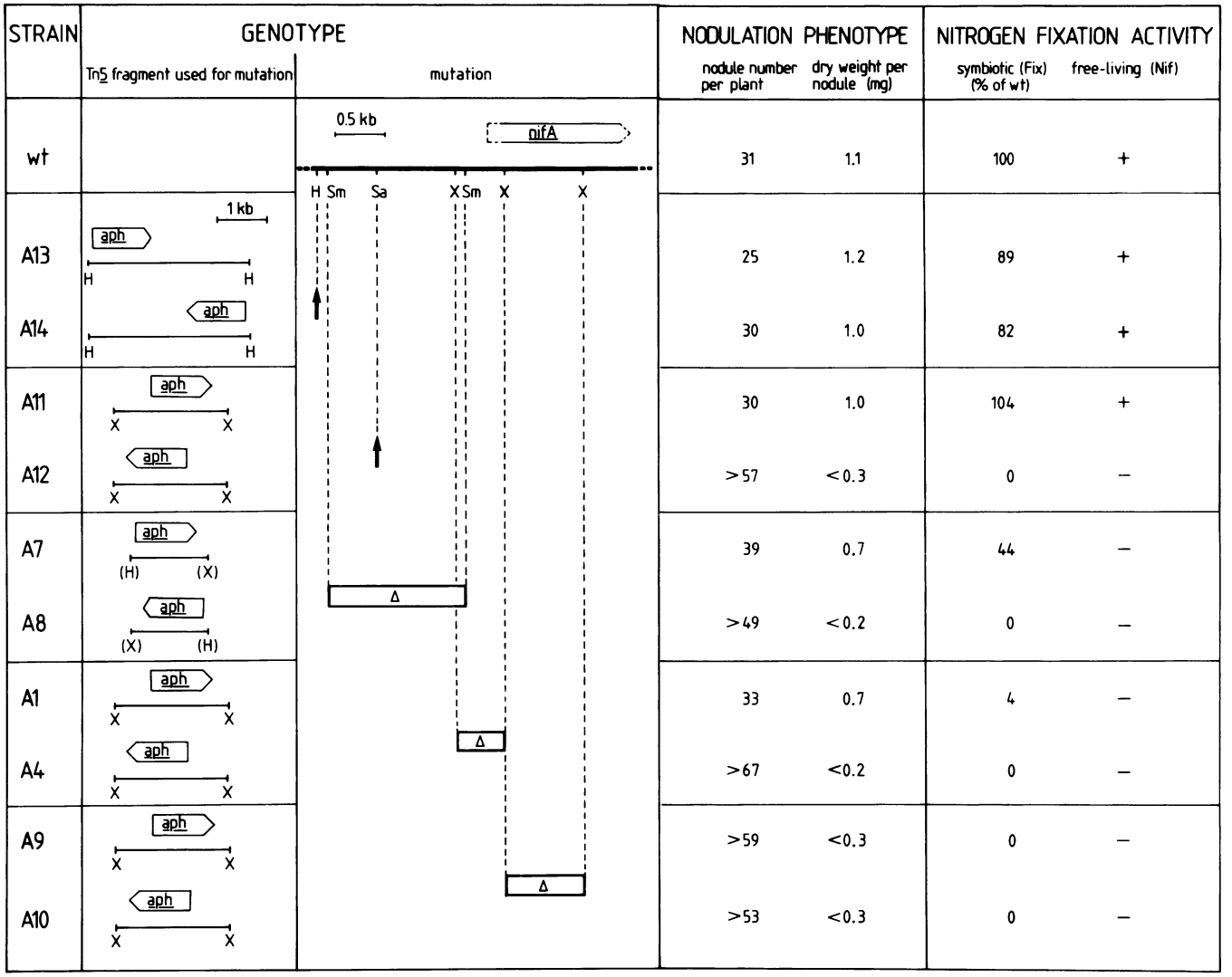

Fig. 3. Mutational analysis of the *B. japonicum nifA* region and phenotypic analysis of mutants. The left part shows the Tn*5*-derived fragments (in both orientations) used for insertion or replacement. Restriction sites in parentheses were destroyed after fill-in reactions and blunt-end ligations; *aph* = aminoglycoside phosphotransferase-3′-II gene coding for kanamycin resistance. The central drawing shows the positions of insertions (vertical arrows) and deletions (Δ); each of the five mutations corresponds to one mutant strain pair indicated on the left. Restriction sites are abbreviated as in Figure 1. The following explanations concern the four phenotypic traits given on the right. (i) Usually, seven plants (20 days after infection) were scored to determine the average nodule number; the symbol (>) indicates that many more infections have taken place which, however, did only lead to bump-like structures. (ii) Concerning nodule dry weight the symbol (<) indicates that the average values must be considered to be smaller because of the presence of those bumps that could not be weighed. (iii) 100% symbiotic Fix activity corresponds to 120 μmol ethylene produced/h/g nodule dry weight. (iv) Free-living Nif activity varied substantially so that only (+) or (−) symbols are given; (+) corresponds to values between 3.5 and 17.1 nmol ethylene accumulation per ml culture volume within 8 days after inoculation, whereas (−) means that no acetylene reduction was detected at all.

Fix and Nif activity like the wild-type. Strain A7 has >50% reduced Fix activity and zero Nif activity. Although the free-living assay system is not a very sensitive one the reproducible nature of this result suggests that the mutation in strain A7 indeed affects free-living N_2 fixation (viz. acetylene reduction) more than symbiotic fixation. Strain A1 has only 4% residual Fix activity and no Nif activity, which may be explained by the fact that a substantial amount of the *nifA* 5′ region is deleted. What little activity is left possibly stems from a truncated and/or fused NifA protein that is still partially active. This idea is consistent with a result of Buikema *et al.* (1985) who found that the NH_2-terminal end of the NifA protein is not well conserved, i.e. this protein domain may not be of absolute functional importance.

Taken together these results suggest that in strains A11, A7, and A1 the orientation of the *aph* fragment supports transcriptional read-through into *nifA* while in strains A12, A8 and A4 the inverse orientation of the inserted fragment exerts strong polarity on *nifA* expression. The likely conclusion is that *nifA* is part of a transcriptional unit which starts between the *Hin*dIII and *Sal*I sites >1.2 kb upstream of *nifA* (Figure 3).

B. japonicum nifA gene controls expression of multiple proteins

The role of the NifA protein as a positive regulator of the expression of other *nif* or *fix* genes leads to the expectation that a NifA$^-$ mutant would fail to synthesize a number of corresponding gene products. This was analysed by two-dimensional polyacrylamide gel electrophoresis of protein extracts prepared from free-living, microaerobic cultures of strains listed in Figure 3. Cells were labelled with L-[^{35}S]methionine to make protein spots visible by autoradiography. Previous work has thus identified the *nifH* gene product (nitrogenase Fe protein; Hahn *et al.*, 1984). Unfortunately the synthesis of the *nifD* and *K* products is not

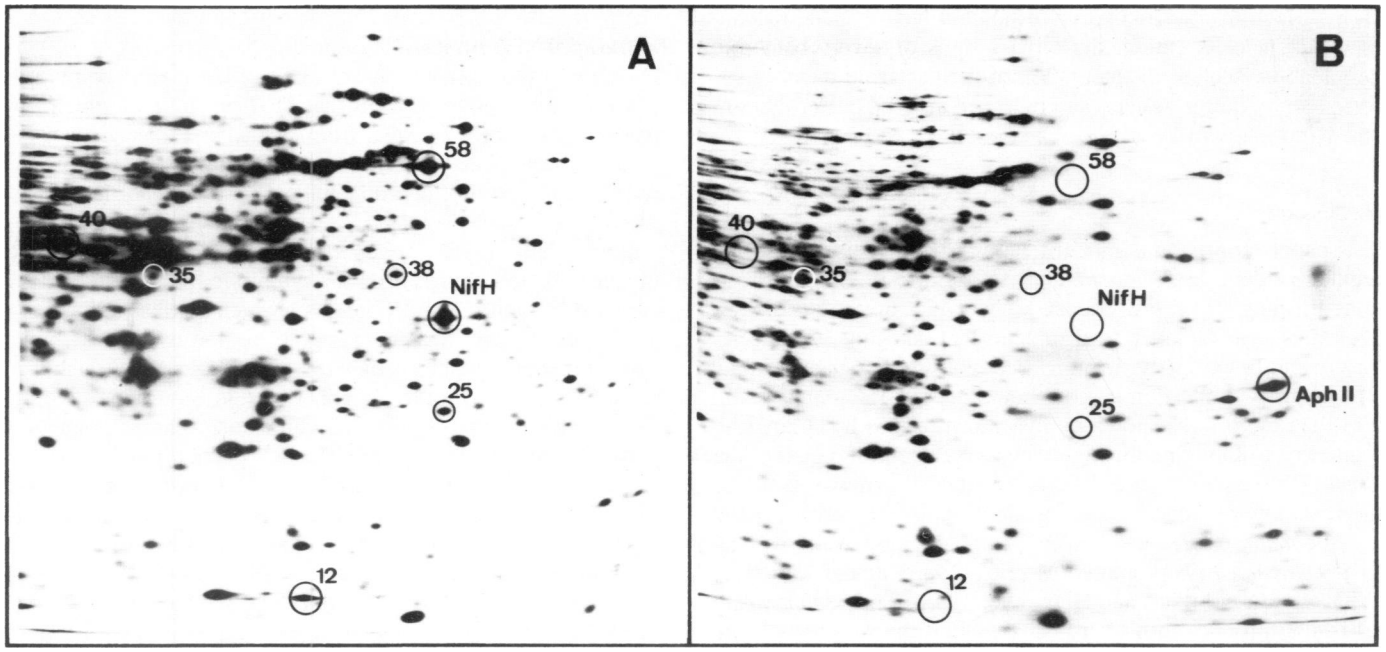

Fig. 4. Autoradiographs of two-dimensional gel electrophoretic analyses of ^{35}S-labelled proteins synthesized in free-living, microaerobic cultures of *B. japonicum* strains. **A** shows wild-type proteins, **B** shows mutant A8 proteins. The position of the *aph* gene product is marked in **B** (AphII). The other proteins encircled in black ink are 'microaerobic' proteins which are missing in the NifA$^-$ mutant; one of them is the *nif*H gene product (NifH). Numbers refer to mol. wts ($\times 10^3$). A 35-K protein encircled in white ink is specifically derepressed in the NifA$^-$ mutant.

well resolved by this gel system, but the experiments of Figure 2, and Tables I and II have already shown that the expression of the *nifDK* operon is activated by the *B. japonicum* NifA protein.

The result of this analysis was that all Fix$^-$ strains investigated (A12, A8, A4, A9) exhibited the same protein pattern which differed from Fix$^+$ strains (wild-type, A13, A14, A11, A7, A1) by the complete absence of at least six prominent, well-resolved protein spots. Figure 4 illustrates this observation, exemplified by comparing the wild-type protein pattern (Figure 4A) with that of mutant A8 (Figure 4B). One spot corresponds to the identified NifH protein (mol. wt 31 500) while the other five as yet unidentified proteins have mol. wts of ~12 000, 25 000, 38 000, 40 000 and 58 000. In mutants A7 and A1, which have diminished Fix activity, these proteins were still synthesized but in strongly reduced amounts. Thus, the synthesis of all six proteins is dependent on functionally intact *nifA*. Interestingly, those six proteins were known to be absent in the wild-type under aerobic growth conditions (Regensburger *et al.*, 1986). Another observation was that NifA$^-$ mutants (in contrast to the wild-type) markedly derepressed the synthesis of a 35-K protein which characteristically appears in the wild-type under conditions of aerobic growth.

Nodulation frequency, normal nodule development and bacteroid persistence are nifA-dependent

From previous studies we knew that mutations in *nif* and *fix* genes, such as *nifD, nifK, nifH* (Hahn and Hennecke, 1984; Hahn *et al.*, 1984), *nifB, nifE, fixA, fixB* and *fixC* (S.Ebeling and M.Gubler, unpublished data) did not significantly alter the nodulation phenotype of corresponding *B. japonicum* mutants as compared with the wild-type. It was surprising to learn that this was not the case with mutants A12, A8, A4, A9 and A10. Their defective nodulation phenotype could be seen at least at three levels.

(i) The frequency of nodulation was increased. Figure 3 shows that under our nodulation assay conditions the wild-type elicited the formation of ~30 nodules per soybean plant located mainly along the tap root, whereas NifA$^-$ mutants produced twice as many nodules plus countless bump-like structures both of which were widely distributed all over the root system.

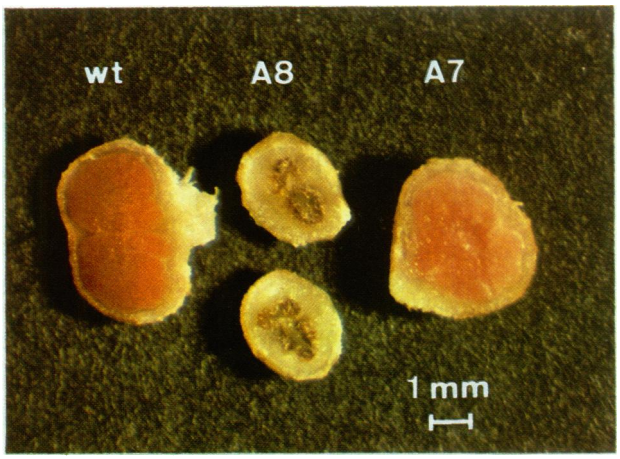

Fig. 5. Morphology of infected nodules 20 days after inoculation of soybean seedlings. Cross-sections of nodules were observed through a stereomicroscope. Nodules infected by the wild-type (wt) and by mutant A7 are red inside, whereas nodules with mutant A8 are pale with dark-brown zones.

(ii) The average dry weight per nodule infected by NifA$^-$ mutants was about one fourth of wild-type-infected nodules. (Dry weight was measured only of those larger nodules which one could pick from roots; Figure 3.) The interior of the Fix$^-$ nodules looked yellowish-white indicating lack of functional leghemoglobin production. Not later than 20 days after infection the Fix$^-$ nodules appeared to become degraded progressively losing their internal physical structure. This is shown in Figure 5 in which mutant A8-infected nodules show dark-brown zones of necrotic appearance.

(iii) Electron microscopic investigation has revealed that infected plant cells exhibit the typical signs of severe bacteroid degradation, such as disintegration of peribacteroid membranes, formation of larger vesicles and presence of lysed bacteroid remnants (not shown).

Discussion

This paper reports on the identification of a *nifA*-like symbiotic regulatory gene in *B. japonicum*, and demonstrates that mutations affecting *nifA* expression are highly pleiotropic. Several lines of evidence can be taken as arguments that this gene, both structurally and functionally, is more analogous to *nifA* than to *ntrC*. (i) The gene in question is more homologous to *K. pneumoniae nifA* than to *ntrC*, as shown by hybridization and by comparing sequences from within the highly conserved central region of the genes (Buikema *et al.*, 1985). (ii) It was known that the *K. pneumoniae ntrC* gene product fails to activate the *B. japonicum nifH* and *nifD* promoters as well as the *K. pneumoniae nifH* promoter (Merrick, 1983; Sundaresan *et al.*, 1983; Alvarez-Morales and Hennecke, 1985). The *B. japonicum nifA* gene product, however, behaves like the *K. pneumoniae* NifA protein in activating the *B. japonicum nifH* and *nifD* promoters and the *K. pneumoniae nifH* promoter (Figure 2, Tables I and II). (iii) The close proximity of the *nifA*-like gene to another *fix* gene (*fixA*; Figure 1) and to the common nodulation genes (*nodABC*; Lamb and Hennecke, 1986) suggests that it belongs to a symbiosis-specific regulation system rather than to a general nitrogen control circuitry. (iv) All Fix^- mutants shown in Figure 3 grow like the wild-type in minimal medium with glutamate or proline as the sole nitrogen sources. (v) The involvement of an *ntrC*-like gene in genetic regulation of nitrogen fixation in *B. japonicum* is difficult to conceive since free-living or symbiotic, N_2-fixing *B. japonicum* cells largely repress the necessary enzymes to assimilate the ammonia they produce, in contrast to N_2-fixing *K. pneumoniae* cells in which the ammonia assimilation pathway is derepressed (for review see Miflin and Cullimore, 1984). This does not exclude the possibility that *B. japonicum* harbours an *ntrC*-like gene that is needed for the regulation of nitrogen metabolism in aerobically grown cells, even though we did not detect any further *nifA/ntrC*-homologous region in the *B. japonicum* genome with the hybridization conditions used. The first *ntrC*-analogous gene in a *Rhizobium* species may have been found recently in *R. meliloti*, and it was reported that *ntrC* mutations had only little effect on symbiotic nitrogen fixation (Ausubel *et al.*, 1985).

The results of Table I have shown that *nif* promoter activation by the *B. japonicum* NifA protein, when tested in an *E. coli* background, is absolutely dependent on the presence of a functional *ntrA* gene. Moreover, *nifA*-mediated activation also occurs in a *B. japonicum* background (Table II) irrespective of whether the *nif* promoter is from *K. pneumoniae (nifH)* or *B. japonicum* (*nifH, nifD*). It appears reasonable, therefore, to postulate the existence of a *ntrA(rpoN)*-like gene in *B. japonicum*, the product of which would serve as a specific σ factor for *nif* promoters similarly to its role in enterobacteria for *ntr*-controlled promoters (Hirschman *et al.*, 1985; Hunt and Magasanik, 1985).

The mutational analysis shown in Figure 3 allows the conclusion that transcription of the *B. japonicum nifA* gene is initiated >1.2 kb upstream of *nifA*, leaving room for at least one more gene to the 5′ side of *nifA*. Thus, *nifA* could be the promoter-distal cistron within an operon of at least two genes. The presumptive operon would terminate after the end of *nifA*, since the adjacent gene to the 3′ side, *fixA*, has previously been shown to be transcribed from its own promoter (Fuhrmann *et al.*, 1985). Thus, the transcriptional organization of the *B. japonicum nifA* gene is cleary different from that in *R. meliloti* in which *nifA* appears to be expressed either from its own promoter immediately to the 5′ side of the gene or also from the promoter ('P2') of a putative *fixABCnifA* operon (Buikema *et al.*, 1985; Kim *et al.*, 1985).

Interestingly, the *B. japonicum nifA* upstream region does not appear to be strictly required for symbiotic Fix activity. This is shown by mutants A11, A7 and A1 which still have Fix activity, despite the fact that the upstream region is mutated, while *nifA* expression is maintained by transcriptional read-through from the *aph* promoter (Figure 3). This situation is reminiscent of the *K. pneumoniae nifLA* operon in which non-polar *nifL* mutations had been reported to have a $NifL^-A^+$ phenotype, i.e. the corresponding mutant strains were Nif^+ (Hill *et al.*, 1981). Under nitrogen-fixing conditions the *K. pneumoniae nifL* gene does not appear to be of functional importance, whereas in response to aeration or high ammonia supply the NifL protein acts as a *nif*-specific repressor (Merick *et al.*, 1982). We have performed an experiment with *B. japonicum* mutant A11 in which a microaerobic culture (at the onset of acetylene reduction) was shifted to heavy aeration, and labelled with L-[^{35}S]methionine to analyse *de novo* cellular protein synthesis. The result (not shown) was that the synthesis of all the identified O_2-repressible proteins did not escape from repression which argues against the existence of a *nifL*-like gene at the position of the A11 mutation. Nevertheless, there is 1-kb space between the A11 mutation and *nifA* so that the existence of a *nifL*-like gene in this region cannot be ruled out at present.

The oxygen partial pressure controls the synthesis of nitrogenase polypeptides and several other proteins: when *B. japonicum* was cultivated in the free-living state these proteins were depressed at low O_2 tension as opposed to aerobiosis which led to complete repression (Scott *et al.*, 1979). Experiments such as those shown in Figure 4 have now demonstrated that many of the oxygen-controlled proteins are also subject to *nifA*-mediated control. Irrespective of whether or not all these proteins are *nif* or *fix* gene products, this result suggests that *nifA*-mediated control and respiratory control by oxygen may either be tied together at some unknown level or they exert their regulatory effects independently on the same target genes. One possibility is that a NifL-type repressor protein inactivates the *nifA* product in response to oxygen; this mechanism was first proposed for *K. pneumoniae* (Buchanan-Wollaston and Cannon, 1984). Alternatively, oxygen could control the transcription and/or translation of the *nifA* gene, or the *B. japonicum* NifA protein itself may be sensitive to oxygen. Our future research will certainly be directed towards a solution of this key problem.

A somewhat unexpected finding was that a functionally intact *B. japonicum nifA* gene also appears to be required for the normal development of a determinate root nodule. First, mutations affecting *nifA* expression lead to a higher frequency of (futile) nodulation events. The corresponding mutants seem to circumvent a plant-internal regulatory phenomenon that has been termed 'autoregulation', in which effective nodulation events inhibit further nodulation on other parts of the roots (Pierce and Bauer, 1983). Autoregulation has been shown to be prevalent even before the onset of symbiotic nitrogen fixation (Pierce and Bauer, 1983; Kosslak and Bohlool, 1984). Thus, a *nifA*-controlled early bacterial function may exist which signals other root tissues to suppress further nodule development. It should be pointed out

Table III. Bacterial strains and plasmids

	Relevant phenotype or genotype[c]	Reference or origin
Strains		
B. japonicum 110*spc4*	Spc^R	Regensburger and Hennecke (1983)
B. japonicum A9	Spc^R *nifA*::*aph*	This work
E. coli RR28	*hsdR*$^-$ *hsdM*$^-$ *recA*$^-$ *endA*$^-$ *pheS12*	Hennecke *et al.* (1982)
E. coli SM10	RP4-2 Km^R *tet*::Mu, integrated in the chromosome	Simon *et al.* (1983)
E. coli 17-1	*hsdR*$^-$, RP4-2 *kan*::Tn*7* *tet*::Mu, integrated in the chromosome	Simon *et al.* (1983)
E. coli MC1061	Δ(*lacIPOZYA*) *X74*, *hsdR*$^-$	Casadaban *et al.* (1983)
E. coli ET8000	*rbs lacZ*::IS*1* *gyrA* $hutC^c_K$	MacNeil *et al.* (1982)
E. coli ET8894[a]	Δ(*rha-ntrC*) 1703::Mu*cts62*	MacNeil *et al.* (1982)
E. coli ET8045[a]	*ntrA208*::Tn*10*	MacNeil *et al.* (1982)
Plasmids		
pBR329	Ap^R Cm^R Tc^R	Covarrubias and Bolivar (1982)
pACYC177	Ap^R Km^R	Chang and Cohen (1978)
pACYC184	Cm^R Tc^R	Chang and Cohen (1978)
pKC7	Ap^R Km^R	Rao and Rogers (1979)
pSUP202	Ap^R Cm^R Tc^R, *oriT* from RP4	Simon *et al.* (1983)
pSUP202 *cam*::Tn*5*	Ap^R Km^R Tc^R	M.Hahn, unpublished
pMC1403	Ap^R *lac'ZYA*	Casadaban *et al.* (1983)
pRK290	Tc^R	Ditta *et al.* (1980)
pRK2013	Km^R *tra*	Figurski and Helinski (1979)
pLAFR1	Tc^R *cos*	Friedmann *et al.* (1982)
pMC71A	Cm^R (pACYC184) Kp-*nifA*^{c}	Buchanan-Wollaston *et al.* (1981)
pMM12	Ap^R (pACYC177) Kp-*ntrC*^{c}	Espin *et al.* (1982)
pRJ1008	Ap^R (pMC1403) Bj-Φ(*nifD'-'lacZ*)*hyb*	Alvarez-Morales and Hennecke (1985)
pRJ1009	Ap^R (pMC1403) Bj-Φ(*nifH'-'lacZ*)*hyb*	Alvarez-Morales and Hennecke (1985)
pMB1	Ap^R (pMC1403) Kp-Φ(*nifH'-'lacZ*)*hyb*	Buck *et al.* (1985)
pRD532	Ap^R (pMC1403) Kp-Φ(*nifL'-'lacZ*)*hyb*	Drummond *et al.* (1983)
pRJ1024	Tc^R (pRK290) Bj-Φ(*nifH'-'lacZ*)*hyb*	This work
pRJ1025	Tc^R (pRK290) Bj-Φ(*nifD'-'lacZ*)*hyb*	A.Alvarez-Morales, unpublished
pRJ1039	Tc^R (pRK290) Bj-Δ(promoter)-Φ(*nifD'-'lacZ*)*hyb*	A.Alvarez-Morales, unpublished
pKP1026	Tc^R (pRK290) Kp-Φ(*nifH'-'lacZ*)*hyb*	This work
pKP1027	Tc^R (pRK290) Kp-Φ(*nifL'-'lacZ*)*hyb*	This work
pL48-11G	Tc^R (pLAFR1) Bj-*nodC nifA fixA*	Lamb and Hennecke (1986)
pRJ7515[b]	Km^R (pBR329 *tet*::*aph*) Bj-*nifA*^{c}	This work
pRJ7523	Km^R (pACYC177) Bj-*nifA*^{c}	This work

[a]Strains derived from *E. coli* ET8000.
[b]This plasmid and derivatives of it are depicted in Figure 2.
[c]Abbrevations: Kp = *K. pneumoniae*; Bj = *B. japonicum*.

that this particular NifA phenotype cannot be an indirect consequence of nitrogen starvation in the soybean plant (due to lack of symbiotic N_2 fixation), since mutations in other *B. japonicum nif* and *fix* genes (*nifD, nifK, nifH, nifE, nifB, fixA, fixB, fixC*, Hahn and Hennecke, 1984; Hahn *et al.*, 1984; S.Ebeling and M.Gubler, unpublished results) do not lead to such a phenotype. Recently, Carroll *et al.* (1985) provided evidence for a soybean (*G. max*) gene that could also be involved in autoregulation of nodule formation.

Second, mutations affecting *nifA* also lead to dramatic disturbances in the development of infected nodule plant cells. From light and electron microscopic investigations it appears as if NifA$^-$ mutants, once released from the infection thread, are treated like plant pathogens. We could observe symptoms of nodule tissue degradation (Figure 5) and the typical signs of severe, premature bacteroid degradation (Werner *et al.*, 1984). Thus, *nifA* may also control functions that help the bacterial symbiont to overcome plant defence reactions and to persist as nitrogen-fixing bacteroids. Again, it is important to note that nitrogen non-fixing soybean nodules infected by other *B. japonicum nif* and *fix* mutants do not show such peculiar phenotypes.

In conclusion, it is surprising to learn how many processes in the *B. japonicum*–soybean symbiosis may be pleiotropically controlled by the bacterial *nifA* gene. At the molecular level, however, these processes may perhaps be determined by just a few key genes. Consequently, it will be of interest for future work to identify all those genes which, besides the nitrogenase genes, are subject to *nifA*-mediated control.

Materials and methods

Bacterial strains and plasmids

These are listed in Table III. *B. japonicum* 110*spc4* is called the 'wild-type' throughout this paper. *E. coli* strains Sm10 and 17-1 served as donor strains for the mobilization of pSUP202 derivatives. *E. coli* MC1061, ET8000, ET8894, ET8045, and *B. japonicum* A9 were recipients for testing *nif::lac* fusions. *E. coli* RR28 was used for transformations in cloning experiments. Cosmid pL48-11G has previously been shown to link the *B. japonicum nifA/fixA* region to *nodABC* (Lamb and Hennecke, 1986). Transfer of pRK290-derived plasmids into *B. japonicum* was achieved by triparental matings using pRK2013 as helper plasmid (Ditta *et al.*, 1980).

Media and growth of cells

B. japonicum strains were grown aerobically in PSY medium (Regensburger and Hennecke, 1983). For selections, antibiotics were used at the following concentrations (μg/ml): spectinomycin 200, kanamycin 200, tetracycline 150. Microaerobic growth of *B. japonicum* strains, protein labelling with L-[^{35}S]methionine,

preparation of crude extracts and two-dimensional gel electrophoresis was done as described (Scott *et al.*, 1979; O'Farrell, 1975) with modifications given by Regensburger *et al.* (1986). For routine growth of *E. coli* cells LB medium was used. Nitrogen-free medium (NFDM, Dixon *et al.*, 1977) was used for growth of *E. coli* cells to be tested for β-gal activity. Additional supplements were glutamine (200 μg/ml) for *E. coli* ET8000 and glutamine, casamino acids and yeast extract (each at 200 μg/ml; filter-sterilized) for *E. coli* MC1061. Antibiotics were used at the following concentrations (μg/ml): ampicillin 100, chloramphenicol 20, kanamycin 30, spectinomycin 200, tetracycline 10.

Recombinant DNA work

Cloning, restriction endonuclease site mapping, nick translation, Southern blotting and transformation were performed using established protocols (Maniatis *et al.*, 1982). Conditions for interspecies hybridizations were described by Fuhrmann *et al.* (1985). *NifA*-internal DNA sequences were established by chemical degradation of 5′ and 3′ end-labelled DNA at the *nifA*-internal *Eco*RI site (Figure 1) using the method of Maxam and Gilbert (1980).

DNA probes for interspecies hybridizations

The *K. pneumoniae ntrC* probe was obtained as follows. A 1.3-kb *Eco*RI/*Pvu*I fragment from plasmid pMM12 (Espin *et al.*, 1982) carrying *ntrC* plus the 3′ end of *ntrB* was subcloned into vector pBR322 (Bolivar *et al.*, 1977). A 890-bp *Sal*I/*Pvu*I fragment (Buikema *et al.*, 1985) was purified thereof; it contained exclusively *ntrC*-internal DNA. The *K. pneumoniae nifA* probe was a purified 1737-bp *Pst*I/*Sma*I fragment from plasmid pMC71A (Buchanan-Wollaston *et al.*, 1981); it contained 1231 bp of the *nifA* structural gene, 287 bp of *nifA*–*nifB* intergenic DNA and 219 bp of the 5′ end of *nifB* (Buikema *et al.*, 1985; M.Drummond, W.Arnold and A.Pühler, personal communication).

Construction of B. japonicum mutants

For site-directed insertions or replacements we used Tn*5*-derived fragments carrying the kanamycin resistance gene. Plasmids pSUP202*cam*::Tn*5* and pKC7 served as DNA sources for the following three fragments: a 3424-bp *Hin*dIII fragment, a 2347-bp *Xho*I fragment and a 1637-bp *Hin*dIII/*Xho*I fragment. The *Xho*I-site on one end of the latter two fragments is located within the streptomycin resistance gene of the *kan bleo str* operon of Tn*5* (Mazodier *et al.*, 1985). These fragments were inserted into specific sites of plasmid clones from the *B. japonicum nifA* region. The mutagenized *B. japonicum* DNA fragment was then cloned into pSUP202 and transferred into *E. coli* 17-1 or Sm10 (Simon *et al.*, 1983). Matings to *B. japonicum* 110*spc4* were done essentially as described by Hahn and Hennecke (1984). *B. japonicum* exconjugants were selected on PSY agar containing 200 μg/ml kanamycin and 20 μg/ml chloramphenicol or 200 μg/ml spectinomycin to counterselect against the 17-1 or Sm10 donors, respectively. Co-integrate-containing strains (resulting from single cross-over) were distinguished from true marker exchange mutants (resulting from double cross-over) by their tetracycline resistance provided by the vector pSUP202. To confirm the genomic DNA structure, total DNA from putative mutants was analyzed by appropriate Southern blot hybridizations. Thus, all mutations shown in Figure 3 were verified with regard to their positions and the orientations of the inserted Tn*5* fragments.

β-Galactosidase assays

E. coli cells harbouring *nifD*::*lacZ* fusion plasmids were grown overnight at 28°C in 7 ml screw-capped plastic vials containing 5 ml of supplemented NFDM medium. Measurement of β-gal activity in microaerobically grown *B. japonicum* was done by taking 260 μl samples of the culture (7 days after inoculation) and using 100 μl aliquots for duplicate assays. 50 μl of the remaining sample were diluted and used for cell density measurement at 600 nm. Permeabilization of cell samples, β-gal assays and calculation of enzyme units were done identically for *E. coli* and *B. japonicum* as described (Miller, 1972). β-Gal assays were routinely done in duplicate.

Plant infection tests

Surface sterilization of soybean seeds (*G. max* L.Merr *cv.* Williams), inoculation, plant cultivation and acetylene reduction assays were done as described previously (Hahn and Hennecke, 1984; Lamb and Hennecke, 1986). For determination of specific acetylene reduction activity (μmol ethylene formed/h/g nodule dry weight) all nodules from each plant were collected, dried overnight at 80°C and weighed. Specimen preparation for electron microscopy was done as described by Hahn *et al.* (1984).

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References

Alvarez-Morales,A. and Hennecke,H. (1985) *Mol. Gen. Genet.*, **199**, 306–314.
Ausubel,F.M., Buikema,W.J., Earl,C.D., Klingensmith,J.A., Nixon,B.T. and Szeto,W.W. (1985) In Evans,H.J., Bottomley,P.J. and Newton,W.E. (eds), *Nitrogen Fixation Research Progress*. Martinus Nijhoff Publishers, Dordrecht, pp. 165–171.
Beynon,J., Cannon,M., Buchanan-Wollaston,V. and Cannon,F (1983) *Cell*, **35**, 665–671.
Bolivar,F., Rodriguez,R.L., Greene,P.J., Betlach,M.C., Heyneker,H.L. and Boyer,H.W. (1977) *Gene*, **2**, 95–113.
Buchanan-Wollaston,V. and Cannon,F. (1984) In Veeger,C. and Newton,W.E. (eds), *Advances in Nitrogen Fixation Research*. Nijhoff/Junk Publishers, The Hague, p. 732.
Buchanan-Wollaston,V., Cannon,M.C., Beynon,J.L. and Cannon,F.C. (1981) *Nature*, **294**, 776–778.
Buck,M., Khan,H. and Dixon,R. (1985) *Nucleic Acids Res.*, **13**, 7621–7638.
Buikema,W.J., Szeto,W.W., Lemley,P.V., Orme-Johnson,W.H. and Ausubel,F.M. (1985) *Nucleic Acids Res.*, **13**, 4539–4555.
Carroll,B.J., McNeil,D.L. and Gresshoff,P.M. (1985) *Proc Natl. Acad. Sci. USA*, **82**, 4162–4166.
Casadaban,M.J., Martinez-Arias,A., Shapira,S.K. and Chou,J. (1983) *Methods Enzymol.*, **100**, 293–308.
Chang,A.C.Y. and Cohen,S.N. (1978) *J. Bacteriol.*, **134**, 1141–1156.
Chelm,B.K., Carlson,T.A. and Adams,T.H. (1985) In Evans,H.J., Bottomley,P.J. and Newton,W.E. (eds), *Nitrogen Fixation Research Progress*. Martinus Nijhoff Publishers, Dordrecht, p. 217.
Covarrubias,L. and Bolivar,F. (1982) *Gene*, **17**, 79–89.
Ditta,G., Stanfield,S., Corbin,D. and Helinski,D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7347–7351.
Dixon,R.A. (1984) *J. Gen. Microbiol.*, **130**, 2745–2755.
Dixon,R., Kennedy,C., Kondorosi,A., Krishnapillai,V. and Merrick,M. (1977) *Mol. Gen. Genet.*, **157**, 189–198.
Drummond,M., Clements,J., Merrick,M. and Dixon,R. (1983) *Nature*, **301**, 302–307.
Espin,G., Alvarez-Morales,A., Cannon,F., Dixon,R. and Merrick,M. (1982) *Mol. Gen. Genet.*, **186**, 518–524.
Figurski,D.H. and Helinski,D.R. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1648–1652.
Friedman,A.M., Long,S.R., Brown,S.E., Buikema,W.J. and Ausubel,F.M. (1982) *Gene*, **18**, 289–296.
Fuhrmann,M. and Hennecke,H. (1984) *J. Bacteriol.*, **158**, 1005–1011.
Fuhrmann,M., Fischer,H.M. and Hennecke,H. (1985) *Mol. Gen. Genet.*, **199**, 315–322.
Hahn,M. and Hennecke,H. (1984) *Mol. Gen. Genet.*, **193**, 46–52.
Hahn,M., Meyer,L., Studer,D., Regensburger,B. and Hennecke,H. (1984) *Plant Mol. Biol.*, **3**, 159–168.
Hennecke,H., Günther,I. and Binder,F. (1982) *Gene*, **19**, 231–234.
Hill,S., Kennedy,C., Kavanagh,E., Goldberg,R.B. and Hanau,R. (1981) *Nature*, **290**, 424–426.
Hirschman,J., Wong,P.-K., Sei,K., Keener,J. and Kustu,S. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7525–7529.
Hunt,T.P. and Magasanik,B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8453–8457.
Kaluza,K. and Hennecke,H. (1984) *Mol. Gen. Genet.*, **196**, 35–42.
Kim,C.-H., Ditta,G. and Helinski,D.R. (1985) In Evans,H.J. Bottomley,P.J. and Newton,W.E. (eds), *Nitrogen Fixation Research Progress*. Martinus Nijhoff Publishers, Dordrecht, p. 186.
Kosslak,R.M. and Bohlool,B.B. (1984) *Plant Physiol.*, **75**, 125–130.
Lamb,J.W. and Hennecke,H. (1986) *Mol. Gen. Genet.*, **202**, 512–517.
MacNeil,T., Roberts,G.P., MacNeil,D. and Tyler,B. (1982) *Mol. Gen. Genet.*, **188**, 325–333.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY.
Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–561.
Mazodier,P., Cossart,P., Giraud,E. and Gasser,F. (1985) *Nucleic Acids Res.*, **13**, 195–205.
Merrick,M. (1983) *EMBO J.*, **2**, 39–44.
Merrick,M., Hill,S., Hennecke,H., Hahn,M., Dixon,R. and Kennedy,C. (1982) *Mol. Gen. Genet.*, **185**, 75–81.
Miflin,B.J. and Cullimore,J.V. (1984) In Verma,D.P.S. and Hohn,T. (eds) *Genes Involved in Microbe–Plant Interactions*. Springer-Verlag, Wien/NY, pp. 129–178.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, NY.
Mulligan,J.T. and Long,S.R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6609–6613.
O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.

Ow,D.W. and Ausubel,F.M. (1983) *Nature*, **301**, 307–313.
Pierce,M. and Bauer,W.D. (1983) *Plant Physiol.*, **73**, 286–290.
Rao,R.N. and Rogers,S.G. (1979) *Gene*, **7**, 79–82.
Regensburger,B. and Hennecke,H. (1983) *Arch. Microbiol.*, **135**, 103–109.
Regensburger,B., Meyer,L., Filser,M., Weber,J., Studer,D., Lamb,J.W., Fischer,H.-M., Hahn,M. and Hennecke,H. (1986) *Arch. Microbiol.*, **144**, in press.
Schetgens,R.M.P., Hontelez,J.G.J., van den Bos,R.C. and van Kammen,A. (1985) *Mol. Gen. Genet.*, **200**, 368–374.
Scott,D.B., Hennecke,H. and Lim,S.T. (1979) *Biochim. Biophys. Acta*, **565**, 365–378.
Simon,R., Priefer,U. and Pühler,A. (1983) In Pühler,A. (ed.), *Molecular Genetics of the Bacteria–Plant Interaction*. Springer, Berlin, pp. 98–106.
Sundaresan,V., Ow,D.W. and Ausubel,F.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4030–4034.
Szeto,W.W., Zimmerman,J.L., Sundaresan,V. and Ausubel,F.M. (1984) *Cell*, **36**, 1035–1043.
Weber,G., Reiländer,H. and Pühler,A. (1985) *EMBO J.*, **4**, 2751–2756.
Werner,D., Mörschel,E., Kort,R., Mellor,R.B. and Bassarab,S. (1984) *Planta*, **162**, 8–16.

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