Nucleotide Sequence and Functional Analysis of the Two *nifH* Copies of *Rhizobium* ORS571

By FRANÇOISE NOREL AND CLAUDINE ELMERICH*

Unité de Physiologie Cellulaire, Département des Biotechnologies, Institut Pasteur,
28 Rue du Dr Roux, 75724 Paris Cedex 15, France

(Received 25 September 1986; revised 24 November 1986)

In *Rhizobium* ORS571, which is able to form both root and stem nodules on the tropical plant *Sesbania rostrata* and to grow in the free-living state at the expense of N₂, two copies of the *nifH* gene, which codes for the nitrogenase Fe-protein, were characterized. One copy, *nifH1*, was localized in the *nifHDK* operon; the other, *nifH2*, was localized elsewhere. *nifH1* and *nifH2* differed in only six nucleotides and both encoded a polypeptide of 296 amino acids with a single change at position 282, a serine in *nifH1* and a threonine in *nifH2*. At position 102, arginine in the sequence Gly-Arg-Gly-Val-Ile-Thr might, as in *Rhodospirillum rubrum*, be the site of 'switch-off' inactivation. Compared with other *nifH* genes, the highest S_{AB} was found with *Bradyrhizobium japonicum* and *Rhizobium* sp. (*Parasponia*). A sequence identical (*nifH1*) or very similar (*nifH2*) to the *nif* consensus sequence was found upstream from the initiation codon. Homology with sequences upstream from *Klebsiella pneumoniae nif* promoters, which affect *nifA*-mediated activation, was also found in both cases. From mutants carrying either a single *nifH1* or *nifH2* deletion, or a double *nifH1*–*nifH2* deletion, it appeared that both genes were functional *ex planta* and *in planta* and that no other *nifH* copy seemed to be present in ORS571. The optimal expression of *nifH1* and of *nifH2* might depend on different physiological conditions.

INTRODUCTION

The fast-growing *Rhizobium* strain ORS571, isolated from stem nodules of the tropical legume *Sesbania rostrata*, forms nodules on both stems and roots of the host plant (Dreyfus & Dommergues, 1981) and can grow in the free-living state at the expense of N₂ as the sole nitrogen source (Elmerich *et al.*, 1982; Dreyfus *et al.*, 1983). The nitrogenase complex that catalyses the reduction of N₂ to NH₃ has been isolated from a variety of diazotrophs (Eady & Smith, 1979) including *Rhizobium* ORS571 (Kush *et al.*, 1985). The nitrogenase of *Rhizobium* ORS571 was subject to 'switch-off' when ammonia was added to a N₂-fixing culture (Kush *et al.*, 1985). In all cases, nitrogenase was shown to be composed of two protein components, a MoFe-protein (component I) and an Fe-protein (component II). The MoFe-protein is a tetramer composed of two non-identical subunits (α and β) and the Fe-protein is a dimer composed of two identical subunits (Eady & Smith, 1979). In a few cases, the complete or partial amino acid sequence of both proteins has been determined (Tanaka *et al.*, 1977; Hausinger & Howard, 1982; Hase *et al.*, 1984). Moreover, nitrogenase isolated from various organisms shows a remarkable similarity in component composition and the formation of active heterologous complexes has been reported (Elmerich & Burris, 1978). The genes coding for the nitrogenase polypeptides (*nifHDK*) were initially characterized in *Klebsiella pneumoniae*, where they are organized in a single transcription unit (Elmerich *et al.*, 1978; Merrick *et al.*, 1978). The *nifH* gene encodes the two identical subunits of component II whereas *nifD* and *nifK* encode the α and β subunits of component I, respectively. As shown by hybridization with *K. pneumoniae nif*

Abbreviation: ORF, open reading frame.

probes, the *nifHDK* genes, in particular *nifH*, are highly conserved among different genera of diazotrophs (Ruvkun & Ausubel, 1980; Mazur *et al.*, 1980). This facilitated identification and cloning of the structural genes for nitrogenase from a variety of N₂ fixers including bacteria of the genus *Rhizobium* (Hennecke, 1981; Elmerich *et al.*, 1982; Quinto *et al.*, 1982; Scott *et al.*, 1982; Downie *et al.*, 1983; Scott *et al.*, 1983*a, b*; Fuhrmann & Hennecke, 1984). In *Rhizobium* ORS571, identification and cloning of DNA fragments homologous to *K. pneumoniae nifH, D, K* and *E* were previously reported (Elmerich *et al.*, 1982; Norel *et al.*, 1985*a*; Donald *et al.*, 1986). By site-directed mutagenesis, it was shown that these genes are organized in two independent transcription units, *nifHDK* and *nifE* (Donald *et al.*, 1986; Denèfle *et al.*, 1987). The nucleotide sequence of *nifH* from ten eubacteria and from a methanogenic archaebacterium is now established: *Anabaena* 7120 (Mevarech *et al.*, 1980), *K. pneumoniae* (Scott *et al.*, 1981; Sundaresan & Ausubel, 1981), *R. meliloti* (Torök & Kondorosi, 1981), *Rhizobium* sp. (*Parasponia*) (Scott *et al.*, 1983*a*), *R. trifolii* (Scott *et al.*, 1983*b*), *Bradyrhizobium japonicum* (Fuhrmann & Hennecke, 1984), *Azotobacter vinelandii* (Brigle *et al.*, 1985), *Rhizobium phaseoli* (Quinto *et al.*, 1985), *Clostridium pasteurianum* (Chen *et al.*, 1986), *Azotobacter chroococcum* (Robson *et al.*, 1986) and *Methanococcus voltae* (Souillard & Sibold, 1986). From these results, *nifH* appears as the most conserved translated gene in bacteria. Another interesting feature of *nifH* is the presence of multiple copies of the gene in several diazotrophs including *R. phaseoli* (Quinto *et al.*, 1982), *Anabaena* 7120 (Rice *et al.*, 1982), *B. japonicum* (Prakash & Atherly, 1984), *Calothrix* sp. (Kallas *et al.*, 1983), *Rhodospseudomonas capsulata* (Scolnik & Haselkorn, 1984), *Azotobacter chroococcum* (Robson *et al.*, 1986), *C. pasteurianum* (Chen *et al.*, 1986), and *Rhizobium* ORS571 (Norel *et al.*, 1985*a, b*; Donald *et al.*, 1986). A functional analysis of the reiterated copies was done in *Rps. capsulata* (Scolnik & Haselkorn, 1984), *R. phaseoli* (Quinto *et al.*, 1985), and *Azotobacter chroococcum* (Robson *et al.*, 1986).

Because of the unusual properties of strain ORS571, forming both root and stem nodules, fixing N₂ under both free-living and symbiotic conditions and having a nitrogenase subject to 'switch-off' by inactivation of component II, we decided to determine the nucleotide sequence of the two *nifH* copies and to study their expression. Results reported here show that the two copies are almost identical, closely related to *nifH* of *B. japonicum* (Fuhrmann & Hennecke, 1984) and of *Rhizobium* sp. (*Parasponia*) (Scott *et al.*, 1983*a*), and that they are functional both *ex planta* and *in planta*.

METHODS

Bacterial strains, bacteriophages, plasmids and growth conditions. Bacterial strains and bacteriophages are listed in Table 1. *Rhizobium* strains were grown as described by Elmerich *et al.* (1982). *Escherichia coli* strains were grown in Luria Broth medium (Maniatis *et al.*, 1982). The final concentrations of antibiotics for *Rhizobium* are indicated in Table 5; those for *E. coli* were ($\mu\text{g ml}^{-1}$): carbenicillin, 100; chloramphenicol, 25; tetracycline, 5; kanamycin, 40; gentamicin, 20; spectinomycin, 25. Carbenicillin was used to select the Amp^R phenotype. Plasmids are listed in Table 1 or described in Fig. 1. Plasmids containing *K. pneumoniae nif* genes were previously described (Norel *et al.*, 1985*a*) and are not listed in Table 1. Plasmid pRK2013 was used to mobilize IncP Tra⁻ plasmids. Marker exchange in ORS571 was done using pPH1J1 and R751 as described by Denèfle *et al.* (1987). The cloning vector pHE3 and the methods for direct selection of recombinant DNA in *E. coli* RR28 were previously described (Hennecke *et al.*, 1982).

Molecular biology techniques. DNA isolation, endonuclease digestion, ligation, alkaline phosphatase and *Bal*31 treatment, filling-in of protruding ends, DNA transformation, nick translation, nitrocellulose filter hybridization, and colony hybridization were done essentially as described by Maniatis *et al.* (1982). DNA sequencing was done by the method of Sanger *et al.* (1977) with phage M13 derivatives (Messing & Vieira, 1982) that were propagated in *E. coli* TG1. Sequence data were compiled by computer analysis by using the program devised by Staden (1983). To prepare a Sp^R cartridge with *Sal*I-*Sma*I extremities, a 3.4 kb *Bam*HI fragment from pGV764 containing the Sp^R gene was cloned into pUC9 to yield pPC942. From this plasmid, the *Sal*I-*Sma*I fragment containing the Sp^R gene was recovered.

Plant test and nitrogenase assay. Root inoculation of *Sesbania rostrata* plantlets and nitrogenase assays were done as described by Elmerich *et al.* (1982).

Table 1. Bacterial strains, bacteriophages and plasmids

Relevant properties		Reference	
<i>Escherichia coli</i>			
RR28	<i>leu thi pro pheS12 recA r⁻ m⁻</i>	Hennecke <i>et al.</i> (1982)	
MC1061	<i>araD139 Δ(ara-leu)7697 rpsL galU galK Δ(lacIPOZY)X74</i>	Casadaban <i>et al.</i> (1980)	
TG1	<i>Δ(lac-pro) supE thi hsdD5/F' traD36 lacI^Q ΔlacZM15</i>	Wain-Hobson <i>et al.</i> (1985)	
HB101	<i>leu thi pro hsdR recA rpsL</i>	Elmerich <i>et al.</i> (1982)	
<i>Rhizobium</i> ORS571			
Wild strain		Dreyfus & Dommergues (1981)	
57182	<i>ΔnifH1 Km^R</i> (pPH1J1)	Denéfle <i>et al.</i> (1987)	
57171	<i>ΔnifH2 Sp^R</i> (R751)	This work	
57172	<i>ΔnifH2 Sp^R ΔnifH1 Km^R</i> (pPH1J1)	This work	
Bacteriophages			
M13mp8	Lac ⁺	Vieira & Messing (1982)	
M13mp9	Lac ⁺	Vieira & Messing (1982)	
Plasmids	Vector or replicon	Relevant properties	References
pUC8	pBR322	Ap ^R Lac ⁺	Vieira & Messing (1982)
pUC9	pBR322	Ap ^R Lac ⁺	Vieira & Messing (1982)
pHE3	pACYC184	Cm ^R <i>pheS</i>	Hennecke <i>et al.</i> (1982)
pBR322::Tn5	pBR322	Ap ^R Tc ^R Km ^R	This laboratory
pLA29-27	RK2	IncP Tc ^R Km ^R Tra ⁻	Allen & Hanson (1985)
pRK2013	ColE1	IncP Km ^R Tra ⁺	Figurski & Helinski (1979)
pPH1J1		IncP Gm ^R Tra ⁺	Hirsch & Beringer (1984)
R751		IncP Tp ^R Tra ⁺	Jobanputra & Datta (1974)
pGV764	pBR325	Source of the Sp ^R gene	R. Deblaere, Bruxelles
pPC942	pUC9	Source of the Sp ^R cartridge	This work
pRS2	pBR322	Ap ^R (see Fig. 1)	Elmerich <i>et al.</i> (1982)
pRS82	pRK290	Tc ^R Km ^R (see Fig. 1)	Denéfle <i>et al.</i> (1987)

RESULTS AND DISCUSSION

Cloning of the second copy of nifH

Hybridization, with a *nifH* intragenic probe prepared from *K. pneumoniae* or *R. phaseoli*, of *Rhizobium* ORS571 total DNA digested with several restriction endonucleases revealed two fragments in most cases, except with *Pst*I where a single 9.5 kb fragment was detected (Norel *et al.*, 1985a). The same result was observed when the R1 fragment (Fig. 1), was used as a probe (Norel *et al.*, 1985a). This suggested that in ORS571 the two *nifH* copies were carried by two *Pst*I fragments of about the same size. The *nifH* copy adjacent to *nifDK* will be referred to as *nifH1* and the second *nifH* copy as *nifH2*. To clone the *Pst*I fragments containing *nifH1* and *nifH2*, size-fractionated fragments (between 7 and 12 kb) of ORS571 total DNA were ligated at the unique *Pst*I site of pHE3. Transformants were screened by colony hybridization using the R1 fragment as probe and recombinant plasmids containing a 9.5 kb *Pst*I DNA insert were analysed. The physical map of pRS68, which contains *nifH1* is shown in Fig. 1. Another plasmid, termed pRS54 (Fig. 1), appeared to carry *nifH2*. The localization of *nifH2* in the insert was established by using the R1 fragment as a probe. Homology to R1 was limited to the 1.65 kb *Pst*I–*Sma*I fragment and a strong homology was found within the 0.6 kb *Xho*I fragment (R2 in Fig. 1). The R2 fragment was used as a probe against pRS2 hydrolysed by *Xho*I and *Bam*HI + *Xho*I. Only the R1 fragment hybridized with R2. Moreover, R1 and R2, when used as probes against ORS571 total DNA restricted by several endonucleases, revealed hybridizing fragments of similar size (e.g. 13 and 16 kb *Bam*HI fragments, 7 and 20 kb *Bgl*II fragments, 2.8 and 20 kb *Sma*I fragments, a 0.6 kb *Xho*I fragment, and a 9.5 kb *Pst*I fragment) (data not shown). The restriction map of the insert carried by pRS54 (Fig. 1) was presented in a preliminary report (Norel *et al.*, 1985b). The entire 9.5 kb *Pst*I fragment from pRS54 was used as a probe against plasmids carrying several *nif* genes of *K. pneumoniae*. No homology was detected except with *nifH*. We

Fig. 1. Restriction map of *Rhizobium* ORS571 DNA fragments carrying the first copy of *nifH* (*nifH1*) in the *nifHDKE* cluster and the second copy of *nifH* (*nifH2*). (a) Physical map of the DNA region carrying the *nifHDKE* cluster. The localization of the *nifHDKE* genes and the direction of transcription (Norel *et al.*, 1985a) are indicated by boxes and arrows, respectively. The localization of the adjacent *nif* region is indicated by --- (Denèfle *et al.*, 1987). Plasmids pRS2 and pRS82 were previously described (Elmerich *et al.*, 1982; Denèfle *et al.*, 1987). Plasmid pRS68 contained the 9.5 kb *Pst*I fragment cloned into pHE3. Plasmid pRS72, used as a source of DNA for sequencing, was constructed as follows. The 1 kb *Pst*I-*Sal*I fragment carrying *nifH1* was cloned into pUC8 to yield pRS67 (not shown). The adjacent 0.5 kb *Sal*I-*Xho*I fragment was subsequently cloned at the *Sal*I site of pRS67 in the correct orientation to yield pRS72. The R1 fragment was purified from pRS72. (b) Physical map of the DNA region containing *nifH2* whose localization is indicated by 5-pointed stars. The 9.5 kb *Pst*I fragment was cloned into pHE3 and pLA29-17 to yield pRS54 and pRS55, respectively. The 1.65 kb *Pst*I-*Sma*I fragment was cloned into pUC9 and pUC8 to yield pRS56 and pRS57 (not shown), respectively, which were used for sequencing. The R2 fragment was purified from pRS54. Plasmid pRS71 was constructed as follows. The 1.1 kb *Sal*I-*Sma*I fragment of pRS54, which contained *nifH2*, was substituted by the 3.4 kb *Sal*I-*Sma*I fragment of pPC942, which contained a *Sp*^R gene, to yield pRS66 (not shown). The deletion was subsequently transferred into pRS55, by homologous recombination between pRS55 and pRS66 in *E. coli* MC1061. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; P, *Pst*I; Pv, *Pvu*I; R, *Eco*RI; S, *Sal*I; Sph, *Sph*I; Sm, *Sma*I; X, *Xho*I.

therefore assumed that no DNA sequence homologous to any *nif* gene of *K. pneumoniae* was located in the vicinity of *nifH2*. Plasmids pRS68 and pRS54 were both used as probes against ORS571 total DNA. This allowed us to establish or to corroborate the physical map of DNA fragments surrounding the probes (Fig. 1). Our data are in agreement with restriction maps of the two *nifH* regions of ORS571 also reported by Donald *et al.* (1986).

DNA sequence analysis of the two *nifH* coding regions

The complete nucleotide sequence of ORS571 *nifH1* and *nifH2* was established. Fig. 2 gives an outline of the sequencing strategy employed. About 1500 nucleotides were sequenced in each region.

Nucleotide sequence of *nifH1*. The orientation of *nifH1* was deduced from hybridization experiments (Norel *et al.*, 1985a). Computer analysis of the three possible reading frames in both strands revealed a single frame large enough in the correct orientation to encode a *nifH*

Fig. 2. Strategy used for sequencing *Rhizobium* ORS571 *nifH* genes. Arrows indicate direction and length of sequenced fragments. A, Sequences obtained after cloning *nifH1* restriction fragments from pRS2 and pRS72 and *nifH2* restriction fragments from pRS54 and pRS56 into M13 derivatives. B, Fragments obtained after *Bal*31 treatment of pRS72 (*nifH1*) and pRS56(*nifH2*) and subcloning into M13 derivatives. Restriction sites as in Fig. 1. H, *Hae*III. Stars indicate the extent and direction of transcription of the *nifH* genes.

polypeptide. The nucleotide sequence of *nifH1* and the predicted amino acid sequence are shown in Fig. 3. From the presumptive ATG start codon at position +1 to the TGA stop codon at position 889, an open reading frame (ORF) accounts for a polypeptide of 296 amino acids. Purified nitrogenase component II had an estimated M_r of 36000 and a pI of 5.3 (Kush *et al.*, 1985). The M_r of the polypeptide deduced from the sequence is 31951, thus slightly different from that of purified component II. The discrepancy between the two estimations may be due to the acidic nature of the polypeptide, which in SDS-PAGE migrates as a polypeptide with a higher apparent M_r . As no other in-frame ATG was found upstream from the presumptive initiation codon and as the M_r matches with other *nifH* products (Eady & Smith, 1979), we assumed that the identified ORF was the correct one. The sequence upstream from the start codon was determined for about 450 bp. A 5'-AGGAG-3' sequence, which is a potential ribosome-binding site (Shine & Dalgarno, 1974), is located seven nucleotides upstream from the suggested initiation codon.

A second ORF, located downstream from *nifH1*, was found. This ORF starts with an ATG located in position 959 and is preceded by a 5'-AGGA-3' sequence eight nucleotides upstream from the potential initiation codon. The predicted amino acid sequence of this ORF is presented

[illegible]

in Fig. 3. On the basis of amino acid sequence homology with the *nifD* gene from *B. japonicum* (Kaluza & Hennecke, 1984), this ORF has been assigned to the ORS571 *nifD* gene. It is well established that in *K. pneumoniae* *nifH* is the first gene in a polycistronic operon, followed by *nifD*, *nifK* and *nifY*. A similar arrangement (*nifHDK*) was found in two fast-growing rhizobia, *R. meliloti* (Ruvkun *et al.*, 1982) and *R. leguminosarum* (Schetgens *et al.*, 1984). In two members of the slow-growing rhizobia, *B. japonicum* and *Rhizobium* sp. (*Parasponia*), *nifD* and *nifK* form one operon and *nifH* is located elsewhere (Kaluza *et al.*, 1983; Scott *et al.*, 1982). A similar organization (i.e. *nifH* and *nifDK* transcribed separately) was found in strain IRc78 which is representative of the tropical cowpea *Rhizobium* miscellany (Yun & Szalay, 1984). In the region flanking the 3' end of ORS571 *nifH1* coding region, an inverted repeat was found 11 nucleotides downstream from the *nifH1* termination codon (Fig. 3). The mRNA transcribed from this region could potentially form a stem characteristic of a loop terminator structure. The potential stem is formed by 10 consecutive GC pairs. The structure, with a free energy of $\Delta G = -38.2 \text{ kcal mol}^{-1}$ ($-159.8 \text{ kJ mol}^{-1}$) at 25 °C as estimated by the method of Tinoco *et al.* (1973), should be stable. Such a GC-rich inverted repeat structure was already described in *B. japonicum* in the 3' end flanking region of *nifH* and *nifK*, and it was suggested that these sequences could function as transcription terminators (Fuhrmann & Hennecke, 1984; Thöny *et al.*, 1985). By using Tn5 mutagenesis in the ORS571 *nifHDK* homologous sequences, we showed that the ORS571 nitrogenase structural genes were organized into a single transcription unit (Denéfle *et al.*, 1987). However, the potential inverted repeat structure preceding *nifD* might have a regulatory effect on ORS571 *nifDK* transcription. Similar inverted repeat structures have also been described downstream from each of the nitrogenase structural genes in *A. vinelandii* and in *A. chroococcum* (Brigle *et al.*, 1985; Kennedy *et al.*, 1985). In *A. vinelandii*, the *nifHDK* genes are part of the same operon (Brigle *et al.*, 1985) and in *A. chroococcum*, four transcripts were found to hybridize to a *nifH* probe during nitrogenase derepression (Jones *et al.*, 1984; Kennedy *et al.*, 1985). The size of the transcripts was compatible with a termination near the stop codons of *nifH*, *nifD* and *nifK* within the *nifHDK* intergenic regions (Kennedy *et al.*, 1985). Thus a transcriptional regulatory function of the inverted repeat structures could not be ruled out.

Nucleotide sequence of *nifH2*. Computer analysis of the sequenced 1.65 kb *Pst*I–*Sma*I fragment revealed a single ORF large enough to encode a *nifH* product. Moreover the ORF, which would code for a product of 296 amino acids, shows a high degree of homology, both at the nucleotide and at the amino acid sequence level, with the *nifH1* coding region. The nucleotide and amino acid sequences of the *nifH2* coding region are shown in Fig. 3. No in-frame ATG was found upstream from the potential start codon at position +1. Only six nucleotides were changed between *nifH1* and *nifH2*. Five of these changes affect the third nucleotide of the corresponding codon and have no effect at the amino acid level. The only changed amino acid between the two genes is residue 282, a serine in *nifH1* and a threonine in *nifH2*, two amino acids which are chemically related.

Reiterated *nifH* copies have been found in other N_2 -fixing organisms. In *C. pasteurianum* (Chen *et al.*, 1986), one of the three *nifH* sequences that were identified (namely *nifH1*) encodes a 273 amino acid sequence identical to that determined with the isolated Fe-protein. A second

Fig. 3. Complete nucleotide sequence of *Rhizobium* ORS571 *nifH1* and *nifH2* coding and flanking regions. The nucleotide sequence of the *nifH1* non-coding strand is shown in lines with 1 in the margin. In the ORFs, the derived amino acids are printed above the corresponding codons. Numbering of both nucleotides and amino acids starts at the beginning of the *nifH1* coding sequence. The nucleotide sequence of *nifH2* non-coding strand is shown in lines with 2 in the margin. Nucleotides in the *nifH2* coding region identical to *nifH1* are indicated by dots. Changes in nucleotides or amino acids are mentioned in the text. Downstream from *nifH1*, the first 42 amino acid residues of *nifD* are presented. Amino acids identical to the *B. japonicum* *nifD* gene (Kaluza & Hennecke, 1984) are indicated by +. Structural features in the nucleotide sequence of *nifH1* and *nifH2* flanking regions are marked as follows: boxed region, consensus *nif* promoter sequence (Ausubel, 1984); underlined sequences, ribosome-binding site and consensus for upstream activator sequence of *nif* promoters (Buck *et al.*, 1986); arrows, inverted repeat. *, Nucleotides also found in *B. japonicum* *nifH* 5' non-coding region (Fuhrmann & Hennecke, 1984).

Table 2. Comparison of regions upstream from ORS571 *nifH1* and *nifH2* coding sequences with other *nif* promoters

• indicates homology with *nif* promoter consensus sequence.

Origin	Position	Sequence
<i>nif</i> consensus†	—	5'-CTGGCACNNNNNTTGCA-3'
<i>K. pneumoniae nifH</i> ‡	—57 to —41	5'-CTGGTATGTTCCCTGCA-3'
<i>B. japonicum nifH</i> §	—178 to —162	5'-TTGGCACGGCTGTTGCT-3'
ORS571 <i>nifH1</i>	—196 to —180	5'-CTGGCACACCCGTTGCA-3'
ORS571 <i>nifH2</i>	—169 to —153	5'-ATGGCACGGTCGTTGCT-3'

† Ausubel (1984). ‡ Beynon *et al.* (1983). § Fuhrmann & Hennecke (1984).

copy was sequenced and, in the 272 amino acids that it probably encodes, 23 residues differed from the *nifH1*-encoded Fe-protein. The third *nifH*-related sequence was found to be less homologous to *nifH1* and *nifH2*. On the other hand, the coding regions of the three *nifH* copies found in *R. phaseoli* are identical (Quinto *et al.*, 1985).

The flanking regions of the *nifH2* coding sequence were determined for about 500 nucleotides upstream from the initiation codon and about 250 nucleotides downstream from the stop codon. A 5'-AGGA-3' potential ribosome binding site was found nine nucleotides upstream from the *nifH2* potential start codon. From data reported in Fig. 3, it appeared that nucleotide sequences upstream from *nifH1* and *nifH2* are different. This situation differs from that found in *R. phaseoli* (Quinto *et al.*, 1985) where the sequences are identical up to nucleotide 96 upstream from the presumptive initiation codon. Beyond, one of the *nifH* regions diverges while the two others remain identical. The *R. phaseoli nifH* potential transcriptional regulatory signals are located within the region which is conserved upstream from the three *nifH* genes. In the region downstream from the *nifH2* coding sequence (Fig. 3) an inverted repeat structure is located seven nucleotides after the stop codon. A potential stem is formed by nine consecutive basepairs, eight of which are GC pairs. It would be slightly less stable than that found in the case of *nifH1*. No ORF was detected downstream from *nifH2*.

Potential promoter regions in the two nifH sequences. The two *nifH* promoter regions were compared to other known *nif* promoters and to the *nif* promoter consensus sequence which was shown to be located 11 bp upstream from the transcription start of *nif* genes or of other genes subject to *ntrC/nifA* control (Ausubel, 1984). Striking similarities with the *nif* consensus sequence were found, in particular *nifH1* (Table 2). No sequence reminiscent of a typical *E. coli* RNA polymerase binding site (5'-TTGACA-N₁₇-TATAAT) (Rosenberg & Court, 1979) was found in either of the two *nifH* promoter regions. This was in agreement with the fact that *nifH*, *D* and *K* products were not expressed when plasmid pRS2 was introduced into *E. coli* minicells.

The sequences upstream from *K. pneumoniae nif* promoters, which affect *nifA*-mediated activation, contain a 5'-^AG-N₇-TGT-N₄-T-N₅-ACA-3' consensus sequence (Buck *et al.*, 1986). Two regions similar to this consensus were found in the *nifH2* promoter region between nucleotides —253 to —238 and between nucleotides —282 to —267 (Fig. 3). In the *nifH1* promoter region, only the sequence between nucleotides —280 and —265 was identical to the consensus (Fig. 3). However, since no specific deletions were constructed in these sequences, we do not know if they are required for a fully efficient transcription of the *nifH* gene as was the case in *B. japonicum* (Alvarez-Morales *et al.*, 1986). It also remains to be determined whether the slight difference between the two ORS571 *nifH* genes consensus sequences might account for a different regulation of their expression.

The two *nifH* non-coding regions were compared to the *nifH* promoters from various rhizobia. Little homology was found to the *nifH1* non-coding region. However, the *nifH2* sequence located downstream from the potential promoter region, between nucleotides —152 and —76, exhibited

Table 3. Binary matching coefficients (S_{AB}) calculated for *Rhizobium* ORS571 *nifH1* and *nifH* from different N_2 -fixing organisms at the DNA and deduced amino acid sequence levels

S_{AB} for two organisms A and B was calculated as follows: $S_{AB} = 2 \times (\text{no. of identical nucleotides or amino acids between A and B}) / [(\text{total no. of nucleotides or amino acids in A}) + (\text{total no. of nucleotides or amino acids in B})]$. References corresponding to the different bacteria are mentioned in the Introduction.

Organism	S_{AB}	
	Nucleotide sequence	Amino acid sequence
<i>Anabaena</i> 7120	0.63	0.72
<i>Klebsiella pneumoniae</i>	0.72	0.75
<i>Azotobacter vinelandii</i>	0.72	0.73
<i>A. chroococcum</i>	0.72	0.71
<i>Rhizobium meliloti</i>	0.72	0.79
<i>R. phaseoli</i>	0.74	0.80
<i>R. trifolii</i>	0.71	0.77
<i>Bradyrhizobium japonicum</i>	0.78	0.92
<i>Rhizobium</i> sp. (<i>Parasponia</i>)	0.80	0.93
<i>Clostridium pasteurianum</i>		
<i>nifH1</i>	0.51	0.61
<i>nifH2</i>	0.50	0.62
<i>Methanococcus voltae</i>	0.44	0.49

a strong homology to sequences located downstream from the *nifH* promoter of *B. japonicum* (Fig. 3) and *Rhizobium* sp. (*Parasponia*) (data not shown).

The significance of this conserved region is not yet known. In *B. japonicum* this presumably untranslated leader region which could potentially form secondary structures is not required for *nifA*-mediated expression of *nifH* promoter in *E. coli*. (Kaluza *et al.*, 1985). However, it cannot be ruled out that this region is required for optimal *nifH* expression in the homologous host (Kaluza *et al.*, 1985).

Comparison of amino acid and DNA sequences of nifH1 and nifH from other organisms. The sequence of the Fe-protein in ORS571 shows similarities to the Fe-protein sequences from other organisms. In particular, the protein contains the five cysteine residues which are invariant in all species and which are supposed to be involved in the binding of the iron acid-labile sulphur cluster to the apoprotein. The sequence conservation is particularly clustered around the cysteine residues and in the N-terminal region which, according to Robson (1984), might contribute to an adenine-nucleotide-binding domain. Based on the nucleotide and amino acid sequences, similarity coefficients (S_{AB}) (Fox *et al.*, 1977) were calculated between the ORS571 *nifH1* gene and other *nifH* genes (Table 3). The highest degree of homology was found with two members of the slow-growing rhizobia, *Rhizobium* sp. (*Parasponia*) and *B. japonicum*. It was previously suggested that *nifH* genes and 16S rRNA within a given bacterium may have evolved in parallel (Hennecke *et al.*, 1985). Moreover, from analysis of both 16S rRNA and *nifH* homology between rhizobia it was found that fast- and slow-growing rhizobia fall into two groups (Hennecke *et al.*, 1985). Based on *nifH* S_{AB} comparison, it appears that ORS571 is much more related to rhizobia belonging to the slow growers, in agreement with taxonomic studies.

In ORS571, nitrogenase is subject to 'switch-off' which, as in photosynthetic bacteria, results from a specific inactivation of the Fe-protein (Kush *et al.*, 1985). This phenomenon, which was discovered with free-living bacteria, was also observed in bacteroids (this laboratory, unpublished). In *Rhodospirillum rubrum*, inactivation was shown to be due to a covalent modification of the Fe-protein. The modifying group is adenosine diphosphoribose linked through the terminal ribose to a guanidino nitrogen of arginine. The modified site in the protein is Gly-Arg(ADP-ribose)-Gly-Val-Ile-Thr (Pope *et al.*, 1985). This amino acid sequence, which is located around residues 100 and 106, is highly conserved in all *nifH* products whose sequence has been established or deduced from the nucleotide sequence, with the exception of *Anabaena* 7120 (Mevarech *et al.*, 1980) and *C. pasteurianum* (Chen *et al.*, 1986) where Val is replaced by Ile. In

Table 4. *Codon usage in the ORS571 nifH gene*

Residue and codon	No. of codons	Residue and codon	No. of codons	Residue and codon	No. of codons
Phe UUU	1	Pro CCU	0	Lys AAA	0
UUC	3	CCC	4	AAG	14
Leu UUA	0	CCA	0	Asp GAU	0
UUG	0	CCG	4	GAC	15
CUU	0	Thr ACU	0	Glu GAA	5
CUC	15	ACC	8	GAG	20
CUA	0	ACA	0	Cys UGU	0
CUG	11	ACG	5	UGC	5
Ile AUU	1	Ala GCU	1	Trp UGG	0
AUC	22	GCC	17	Arg CGU	0
AUA	0	GCA	1	CGC	13
Met AUG	13	GCG	11	CGA	0
Val GUU	1	Tyr UAU	5	CGG	0
GUC	10	UAC	6	AGA	1
GUA	0	His CAU	1	AGG	0
GUG	9	CAC	5	Gly GGU	1
Ser UCU	0	Gln CAA	0	GGC	26
UCC	8	CAG	9	GGA	0
UCA	2	Asn AAU	3	GGG	3
UCG	3	AAC	11		
AGU	0			Total	296
AGC	3				

ORS571 *nifH1* and *nifH2* products, the Gly-Arg-Gly-Val-Ile-Thr sequence is found between residues 101 and 107 (Fig. 3). Although some features of the 'switch off/on' phenomenon in ORS571 seemed to be different from that observed in photosynthetic bacteria (Kush *et al.*, 1985), it is possible that Arg in position 102 is the site of the 'switch-off' modification.

G + C content and codon usage in ORS571 nifH genes. Codon usage in ORS571 *nifH* genes is very biased (Table 4). Four amino acids are coded by a single coding triplet (glutamine, lysine, aspartate and cysteine). An asymmetrical codon usage occurs for isoleucine, glutamate, arginine and glycine. In total, 21 of the 60 coding triplets are not used. The absence of tryptophan, which is another feature of most *nifH* genes, should be noted. The G + C content in the *nifH* coding region is 64% and a very high G + C content was found in the third position of codons, 92% as compared to 61% in the first position and to 39% in the second. This probably reflects the high G + C content of the ORS571 genome, which is 66% (Dreyfus, 1982).

Functional analysis of the two nifH copies

We previously reported that *nifH1* deletion mutant 57182 had a Nif⁺ Nod⁺ Fix⁺ phenotype (Denèfle *et al.*, 1987). The plasmid used to construct this mutant was pRS82 (Fig. 1) in which the two *Xho*I fragments carrying *nifH1* were replaced by a Km^R cartridge from Tn5. It is likely that in pRS82 the *nifDK* transcription is initiated from the Km^R gene promoter. The Nif⁺ Fix⁺ phenotype of mutant 57182 led us to conclude that a second copy of *nifH* was functional (Denèfle *et al.*, 1987). However, we had no proof that this second *nifH* gene was the *nifH2* copy detected by hybridization and studied here. Thus, it was of interest to obtain both a *nifH2* mutant and a double *nifH1-nifH2* mutant.

Table 5 indicates the procedure followed to obtain strain 57171 that carries a deletion of *nifH2* and strain 57172 that carries a double *nifH1-nifH2* deletion. The method, similar to that described by Ruvkun & Ausubel (1981), was based on the incompatibility between pRK290 and pPH1JI and between pLA29-17 and R751. It was checked by hybridization that deletions in strains 57171 and 57172 had occurred at the correct location (data not shown). The mutants were used to inoculate *Sesbania rostrata* plantlets. Nitrogenase activity both *ex planta* and *in planta* was determined. The double deletion mutant 57172 had a Nif⁻ Nod⁺ Fix⁻ phenotype, indicating that no functional *nifH* gene other than *nifH1* and *nifH2* was present in ORS571. Moreover,

Table 5. Site-directed mutagenesis of the two *nifH* copies

Selection was performed on minimal LSN solid medium (Elmerich *et al.*, 1982), containing appropriate antibiotics: gentamicin (Gm), 10 µg ml⁻¹; kanamycin (Km), 100 µg ml⁻¹; spectinomycin (Sp), 50 µg ml⁻¹; tetracycline (Tc), 10 µg ml⁻¹; trimethoprim (Tp), 400 µg ml⁻¹.

Donor strain	Recipient strain	Selected marker	Frequency	Designation of selected strain
HB101(pRK2013)	ORS571	Tc-Sp	10 ⁻²	ORS571(pRS71)
MC1061(pRS71)				
HB101(R751)	ORS571(pRS71)	Tp	10 ⁻³	57171*
		Tp-Sp (100% Tc ^S)	10 ⁻⁷	
		Tp-Tc	10 ⁻⁹	
HB101(pRK2013)	57171	Tc-Sp	10 ⁻⁸	57171(pRS82)
MC1061 (pRS82)				
HB101 (pPH1JI)	57171 (pRS82)	Gm-Km	10 ⁻⁷	57172†

* Strain 57171 contains plasmid R751.

† Strain 57172 contains plasmid pPH1JI.

strains 57171 and 57182 had a nitrogenase specific activity *ex planta* which was 70% and 30%, respectively, of that observed with the wild-type strain. This could suggest that in the wild-type strain *nifH1* accounts for about 70% of N₂ fixation *ex planta* and *nifH2* for 30%, in agreement with the value of 30% of ORS571 total nitrogenase activity assigned to the *nifH2* gene that was previously calculated from data obtained with crude extracts of mutant 57182 (Denéfle *et al.*, 1987). An unexpected result was the divergence obtained *in planta*, since the nitrogenase specific activity of strain 57182 was about 80%, whereas with strain 57171 the nitrogenase activity was only 15% and the inoculated plantlets looked in poor condition. As the amino acid sequences of the two *nifH* genes deduced from the nucleotide sequences are almost identical, it is likely that the two Fe-proteins have similar specific activities. Two hypotheses could account for this observation: (i) the deletion in strain 57171 might have a polar effect on the transcription of a *fix* gene located downstream; (ii) the expression of the two *nifH* genes might be slightly different *ex planta* and *in planta*. The first hypothesis seems unlikely since the entire 1.65 kb *Pst*I-*Sma*I fragment, deleted in strain 57171, was sequenced and no other ORF was detected downstream from *nifH2*. In *R. phaseoli*, it was shown that at least two of the three *nifH* genes can be functionally expressed (Quinto *et al.*, 1985). In *Rps. capsulata* only one *nifH* gene is functional in the wild-type strain but a second copy can be activated when the first one is mutated (Scolnik & Haselkorn, 1984). In *A. chroococcum*, the second *nifH* copy was transcribed under molybdenum deficiency conditions (Robson *et al.*, 1986). The significance of these reiterated *nifH* sequences whether functional or not under defined physiological conditions is not yet known but their existence raises a puzzling and interesting question which might be solved by further investigations based, in particular, on the study of *nifH-lacZ* fusions.

The authors wish to thank Dr J.-P. Aubert for discussions, Dr D. Dean for reading the manuscript, Dr R. Deblaere for the gift of pGV764, Ms N. Desnoues and A. Paquelin for skilful technical assistance, and the personnel of the Centre d'Informatique Scientifique at the Pasteur Institute for computer facilities. This work was supported by a grant (no. GBI-5-028F) from the Commission of the European Communities and by research funds from the University Paris 7.

REFERENCES

- ALLEN, L. L. & HANSON, R. S. (1985). Construction of broad-host-range-cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. *Journal of Bacteriology* **161**, 955-962.
- ALVAREZ-MORALES, A., BETANCOURT-ALVAREZ, M., KALUZA, K. & HENNECKE, H. (1986). Activation of the *Bradyrhizobium japonicum* *nifH* and *nifDK* operons is dependent on promoter-upstream DNA sequences. *Nucleic Acids Research* **14**, 4207-4227.

- AUSUBEL, F. M. (1984). Regulation of nitrogen fixation genes. *Cell* **37**, 5–6.
- BEYNON, J. L., CANNON, M., BUCHANAN-WOLLASTON, V. & CANNON, F. M. (1983). The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. *Cell* **34**, 665–671.
- BRIGLE, K. E., NEWTON, W. E. & DEAN, D. R. (1985). Complete nucleotide sequence of the *Azotobacter vinelandii* nitrogenase structural gene cluster. *Gene* **37**, 37–44.
- BUCK, M., MILLER, S., DRUMMOND, M. & DIXON, R. (1986). Upstream activator sequences are present in the promoters of nitrogen fixation genes. *Nature, London* **320**, 374–378.
- CASADABAN, M., CHOU, J. & COHEN, S. (1980). *In vitro* gene fusions that join an enzymatically active β -galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. *Journal of Bacteriology* **143**, 971–980.
- CHEN, K. C. K., CHEN, J. S. & JOHNSON, J. L. (1986). Structural features of multiple *nifH*-like sequences and very biased codon usage in nitrogenase genes of *Clostridium pasteurianum*. *Journal of Bacteriology* **166**, 162–172.
- DENÈFLE, P., KUSH, A., NOREL, F., PAQUELIN, A. & ELMERICH, C. (1987). Biochemical and genetic analysis of the *nifHDKE* region of *Rhizobium* ORS571. *Molecular and General Genetics* **206**, (in the Press).
- DONALD, R. G. K., NEES, D. W., RAYMOND, C. K., LOROCH, A. I. & LUDWIG, R. A. (1986). Characterization of three genomic loci encoding *Rhizobium* sp. strain ORS571 N_2 fixation genes. *Journal of Bacteriology* **165**, 72–81.
- DOWNIE, J. A., MA, Q. S., KNIGHT, C. D., HOMBRECHER, G. & JOHNSTON, A. W. B. (1983). Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between the nitrogenase genes and a *nifA* like gene. *EMBO Journal* **2**, 947–952.
- DREYFUS, B. L. (1982). *La symbiose entre Rhizobium et Sesbania rostrata, légumineuse à nodules caulinaires*. Doctorat-ès-Sciences thesis, University of Paris 7.
- DREYFUS, B. L. & DOMMERGUES, Y. R. (1981). Nitrogen-fixing nodules induced by *Rhizobium* on the stem nodules of the tropical legume *Sesbania rostrata*. *FEMS Microbiology Letters* **10**, 313–317.
- DREYFUS, B. L., ELMERICH, C. & DOMMERGUES, Y. R. (1983). Free-living *Rhizobium* strain able to grow on N_2 as sole nitrogen source. *Applied and Environmental Microbiology* **45**, 711–713.
- EADY, R. & SMITH, B. E. (1979). Physico-chemical properties of nitrogenase and its components. In *A Treatise on Dinitrogen Fixation*, sections I and II, pp. 765–798. Edited by R. W. F. Hardy, F. Bottomley & R. C. Burns. New York: Wiley.
- ELMERICH, C., HOUMARD, J., SIBOLD, L., MANHEIMER, I. & CHARPIN, N. (1978). Genetic and biochemical analysis of mutants induced by bacteriophage Mu DNA integration into *Klebsiella pneumoniae* nitrogen fixation genes. *Molecular and General Genetics* **165**, 181–189.
- ELMERICH, C., DREYFUS, B. L., REYSSET, G. & AUBERT, J.-P. (1982). Genetic analysis of nitrogen fixation in a fast-growing *Rhizobium*. *EMBO Journal* **1**, 499–503.
- EMERICH, D. W. & BURRIS, R. H. (1978). Complementary functioning of the component proteins of nitrogenase from several bacteria. *Journal of Bacteriology* **134**, 936–943.
- FIGURSKI, D. H. & HELINSKI, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 1648–1652.
- FOX, G. E., PECHMANN, K. R. & WOESE, C. R. (1977). Comparative cataloguing of 16S rRNA; molecular approach to prokaryotic systematics. *International Journal of Systematic Bacteriology* **27**, 44–57.
- FUHRMANN, M. & HENNECKE, H. (1984). *Rhizobium japonicum* nitrogenase Fe protein gene (*nifH*). *Journal of Bacteriology* **158**, 1005–1011.
- HASE, T., WAKABAYASHI, S., NAKANE, T., ZUMFT, W. G. & MATSUBARA, H. (1984). Structural homologies between the amino acid sequence of *Clostridium pasteurianum* MoFe-protein and the DNA sequences of *nifD* and *nifK* genes of phylogenetically diverse bacteria. *FEBS Letters* **166**, 39–43.
- HAUSINGER, R. P. & HOWARD, J. B. (1982). The amino acid sequence of the nitrogenase iron protein from *Azotobacter vinelandii*. *Journal of Biological Chemistry* **257**, 2483–2490.
- HENNECKE, H. (1981). Recombinant plasmids carrying nitrogen fixation genes of *Rhizobium japonicum*. *Nature, London* **291**, 354–355.
- HENNECKE, H., BINDER, F. & GUNTHER, I. (1982). A novel cloning vector for the direct selection of recombinant DNA in *Escherichia coli*. *Gene* **19**, 231–234.
- HENNECKE, H., KALUZA, K., THONY, B., FUHRMANN, M., LUDWIG, W. & STACKEBRANDT, E. (1985). Concurrent evolution of nitrogenase genes and 16S rRNA in *Rhizobium* species and other nitrogen-fixing bacteria. *Archives for Microbiology* **142**, 342–348.
- HIRSCH, P. R. & BERINGER, J. E. (1984). A physical map of pPH1J1 and pJB4J1. *Plasmid* **12**, 139–141.
- JOBANPUTRA, R. S. & DATTA, N. (1974). Trimethoprim R factors in enterobacteria from clinical specimens. *Journal of Medical Microbiology* **7**, 169–178.
- JONES, R., WOODLEY, P. & ROBSON, R. (1984). Cloning and organization of some genes for nitrogen fixation from *Azotobacter chroococcum* and their expression in *Klebsiella pneumoniae*. *Molecular and General Genetics* **197**, 318–327.
- KALLAS, T., REBIERE, M. C., RIPPKA, R. & TANDEAU DE MARSAC, N. (1983). The structural *nif* genes of the cyanobacteria *Gloeotheca* sp. and *Calothrix* sp. share homology with those of *Anabaena* sp. but the *Gloeotheca* genes have a different arrangement. *Journal of Bacteriology* **155**, 427–431.
- KALUZA, K., FUHRMANN, M., HAHN, M., REGENSBURGER, B. & HENNECKE, H. (1983). In *Rhizobium japonicum* the nitrogenase genes *nifH* and *nifDK* are separated. *Journal of Bacteriology* **155**, 915–918.
- KALUZA, K. & HENNECKE, H. (1984). Fine structure analysis of the *nifDK* operon encoding the α and β subunits of dinitrogenase from *Rhizobium japonicum*. *Molecular and General Genetics* **196**, 35–42.
- KALUZA, K., MORALES, A. A. & HENNECKE, H. (1985).

- Oligonucleotide directed mutagenesis of the *Rhizobium japonicum nifH* promoter. *FEBS Letters* **188**, 37–42.
- KENNEDY, C., ROBSON, R., JONES, R., WOODLEY, P., EVANS, D., BISHOP, P., EADY, R., GAMAL, R., HUMPHREY, R., RAMOS, J., DEAN, D., BRIGLE, K., TOUKDARIAN, A. & POSTGATE, J. (1985). Genetic and physical characterization of *nif* and *ntr* genes in *Azotobacter chroococcum* and *A. vinelandii*. In *Nitrogen Fixation Research Progress*, pp. 469–476. Edited by H. J. Evans, P. J. Bottomley & W. E. Newton. Dordrecht & Boston: M. Nijhoff.
- KUSH, A., ELMERICH, C. & AUBERT, J.-P. (1985). Nitrogenase of *Sesbania Rhizobium* strain ORS571: purification, properties, and 'switch-off' by ammonia. *Journal of General Microbiology* **131**, 1765–1777.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MAZUR, B. J., RICE, D. & HASELKORN, R. (1980). Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridization probes. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 186–190.
- MERRICK, M., FILSER, M., KENNEDY, C. & DIXON, R. (1978). Polarity of mutations induced by insertion of transposons Tn5, Tn7 and Tn10 into the *nif* gene cluster of *Klebsiella pneumoniae*. *Molecular and General Genetics* **165**, 103–111.
- MESSING, J. & VIEIRA, J. (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene* **19**, 269–276.
- MEVARECH, M., RICE, D. & HASELKORN, R. (1980). Nucleotide sequence of a cyanobacterial *nifH* gene coding for nitrogenase reductase. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 6476–6480.
- NOREL, F., DESNOUES, N. & ELMERICH, C. (1985a). Characterization of DNA sequences homologous to *Klebsiella pneumoniae nifH*, *D*, *K* and *E* in the tropical *Rhizobium* ORS571. *Molecular and General Genetics* **199**, 352–356.
- NOREL, F., KUSH, A., DENÈFLE, P., SALZANO, G. & ELMERICH, C. (1985b). Genetic analysis in *Sesbania Rhizobium* ORS571. In *Nitrogen Fixation Research Progress*, p. 140. Edited by E. J. Evans, P. J. Bottomley & W. E. Newton. Dordrecht & Boston: M. Nijhoff.
- POPE, M. R., MURRELL, S. A. & LUDDEN, P. W. (1985). Covalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginine residue. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 3173–3177.
- PRAKASH, R. K. & ATHERLY, A. G. (1984). Reiteration of genes involved in symbiotic nitrogen fixation by fast-growing *Rhizobium japonicum*. *Journal of Bacteriology* **160**, 785–787.
- QUINTO, C., DE LA VEGA, H., FLORES, M., FERNANDEZ, L., BALADO, T., SOBERON, G. & PALACIOS, R. (1982). Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. *Nature, London* **22**, 724–726.
- QUINTO, C., DE LA VEGA, H., FLORES, M., LEEMANS, J., CEVALLOS, M. A., PARDO, M. A., AZPIROZ, R., DE LOURDES GIRARD, M., CALVA, E. & PALACIOS, R. (1985). Nitrogenase reductase: a functional multi-gene family in *Rhizobium phaseoli*. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 1170–1174.
- RICE, D., MAZUR, B. J. & HASELKORN, R. (1982). Isolation and physical mapping of nitrogen fixation genes from the cyanobacterium *Anabaena* 7120. *Journal of Biological Chemistry* **257**, 13157–13163.
- ROBSON, R. (1984). Identification of possible adenine nucleotide-binding sites in nitrogenase Fe- and MoFe-proteins by amino acid sequence comparison. *FEBS Letters* **173**, 394–398.
- ROBSON, R., WOODLEY, P. & JONES, R. (1986). Second gene (*nifH**) coding for a nitrogenase iron protein in *Azotobacter chroococcum* is adjacent to a gene coding for a ferredoxin-like protein. *EMBO Journal* **5**, 1159–1163.
- ROSENBERG, M. & COURT, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annual Review of Genetics* **13**, 319–353.
- RUVKUN, G. B. & AUSUBEL, F. M. (1980). Interspecies homology of nitrogenase genes. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 191–195.
- RUVKUN, G. B. & AUSUBEL, F. M. (1981). A general method for site directed mutagenesis in prokaryotes: construction of mutations in symbiotic nitrogen fixation genes of *Rhizobium meliloti*. *Nature, London* **289**, 85–89.
- RUVKUN, G. B., SUNDARESAN, V. & AUSUBEL, F. M. (1982). Directed transposon Tn5 mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. *Cell* **29**, 551–559.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCHETGENS, T. M. P., BAKKEREN, G., VAN DUN, C., HONTELEZ, J. G., VAN KAMMEN, A. & VAN DEN BOS, R. C. (1984). Identification and analysis of the expression of *Rhizobium leguminosarum* symbiotic genes. In *Advances in Nitrogen Fixation Research*, p. 699. Edited by C. Veeger and W. E. Newton. The Hague: M. Nijhoff & Dr W. Junk.
- SCOLNIK, P. A. & HASELKORN, R. (1984). Activation of extra copies of genes coding for nitrogenase in *Rhodopseudomonas capsulata*. *Nature, London* **307**, 289–292.
- SCOTT, K. F., ROLFE, B. G. & SHINE, J. (1981). Biological nitrogen fixation: primary structure of the *Klebsiella pneumoniae nifH* and *nifD* genes. *Journal of Molecular and Applied Genetics* **1**, 71–81.
- SCOTT, K. F., HUGUES, J. E., GRESSHOFF, P. M., BERINGER, J. E., ROLFE, B. G. & SHINE, J. (1982). Molecular cloning of *Rhizobium trifolii* genes involved in symbiotic nitrogen fixation. *Journal of Molecular and Applied Genetics* **1**, 315–326.
- SCOTT, K. F., ROLFE, B. G. & SHINE, J. (1983a). Nitrogenase structural genes are unlinked in the non-legume symbiont *Parasponia Rhizobium*. *DNA* **2**, 141–148.

- SCOTT, K. F., ROLFE, B. G. & SHINE, J. (1983*b*). Biological nitrogen fixation: primary structure of the *Rhizobium trifolii* iron protein gene. *DNA* **2**, 149–155.
- SHINE, J. & DALGARNO, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 1342–1346.
- STADEN, R. (1983). Computer methods for DNA sequencers. In *DNA Sequencing*, pp. 311–368. Edited by J. Hindley, T. S. Worth & R. H. Bivdon. Amsterdam: Elsevier Biochemical Press.
- SOUILLARD, N. & SIBOLD, L. (1986). Primary structure and expression of a gene homologous to *nifH* (nitrogenase Fe-protein) from the archaebacterium *Methanococcus voltae*. *Molecular and General Genetics* **203**, 21–28.
- SUNDARESAN, V. & AUSUBEL, F. M. (1981). Nucleotide sequence of the gene coding for the nitrogenase iron protein from *Klebsiella pneumoniae*. *Journal of Biological Chemistry* **256**, 2808–2812.
- TANAKA, M., HANIU, M., YASUNOBU, T. & MORTENSON, L. (1977). The amino acid sequence of *Clostridium pasteurianum* iron protein, a component of nitrogenase. *Journal of Biological Chemistry* **252**, 7093–7100.
- THONY, B., KALUZA, K. & HENNECKE, H. (1985). Structural and functional homology between the α and β subunits of the nitrogenase MoFe-protein as revealed by sequencing the *Rhizobium japonicum nifK* gene. *Molecular and General Genetics* **198**, 441–448.
- TINOCO, J., BORER, P. N., DENGLER, B., LEVINE, M. D., UHLENBECK, O. C., CROTHERS, D. M. & GRALLA, J. (1973). Improved estimation of secondary structure in ribonucleic acid. *Nature, London* **246**, 40–41.
- TOROK, I. & KONDOROSI, A. (1981). Nucleotide sequence of the *R. meliloti* nitrogenase reductase (*nifH*) gene. *Nucleic Acids Research* **21**, 5711–5723.
- VIEIRA, J. & MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- WAIN-HOBSON, S., SONIGO, P., DANOS, O., COLE, S. & ALIZON, M. (1985). Nucleotide sequence of the AIDS virus. LAV. *Cell* **40**, 9–17.
- YUN, A. C. & SZALAY, A. A. (1984). Structural genes of dinitrogenase and dinitrogenase reductase are transcribed from two separate promoters in the broad host range cowpea *Rhizobium* strain IRc78. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 7358–7362.