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Biochemical and genetic analysis of the *nifHDKE* region of *Rhizobium* ORS571

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Summary. Deletions and Tn*5* insertions were obtained in a cloned 10 kb *Bam*HI–*Bgl*II fragment carrying the *nifHDKE* region of *Rhizobium* ORS571 and were recombined into the host genome. Genetic analysis of the mutants, comparison of polypeptides synthesized under conditions of repression and depression of N_2 fixation, and biochemical complementation of crude extracts were performed. All Nif$^-$ mutants were also Fix$^-$. Three transcription units were identified, *nifHDK, nifE* and a new *nif* locus adjacent to *nifE*; no *nif* locus was found in the immediate vicinity upstream of *nifH*. Fifteen polypeptides synthesized under conditions of N_2 fixation were characterized by two-dimensional gel electrophoresis. Ten of them are likely to be *nif* products and polypeptides encoded by *nifH, nifD, nifK* and tentatively *nifE* were identified. Physiological and biochemical evidence for the functioning of the second copy of *nifH* is reported. Nitrogenase component 2 synthesized by this copy could not be differentiated from component 2 synthesized in the wild-type strain. When the function of *nifH* copy 1 was abolished, the amount of component 2 synthesized was about 30% of that synthesized in the wild-type strain.

Key words: Rhizobium – Nitrogen fixation – *Nif* products – Tn*5* mutagenesis

Introduction

In *Klebsiella pneumoniae*, 17 genes involved in nitrogen fixation have been identified (Dixon 1984; Elmerich 1984). The structural genes for nitrogenase are highly conserved in diazotrophs and they have been cloned from several diazotrophs using homology to *K. pneumoniae* probes. The fast-growing *Rhizobium* strain ORS571, isolated from stem nodules of the tropical legume *Sesbania rostrata*, can grow in the free-living state at the expense of molecular nitrogen (Elmerich et al. 1982; Dreyfus et al. 1983). The organization of the *nif* genes of *Rhizobium* ORS571 has been analysed by hybridization using *K. pneumoniae nif* DNA probes. A cluster containing *nifHDKE* has been identified, and a second copy of *nifH* (referred to as *nifH* copy 2) detected (Norel et al. 1985a), and recently cloned (Norel et al. 1985b; Donald et al. 1986). Nitrogenase from the *Rhizobium* strain ORS571 was purified to homogeneity from free-living diazotrophically grown organisms (Kush et al. 1985). Component 1, a Mo–Fe protein had a M_r of 219,000 and consisted of two types of subunits of M_r 56,000 and 59,000. Component 2, a Fe-protein had a M_r of 74,000 with a single type of subunit of M_r 36,000. Nitrogenase activity was subject to "switch-off" when ammonia was added to a N_2-fixing culture (Kush et al. 1985). Switch-off resulted specifically from a decrease in activity of component 2: however, no change in the electrophoretic mobility in SDS polyacrylamide gel electrophoresis (PAGE) of component 2 subunit was observed (Kush et al. 1985). Mutants impaired in nitrogenase activity were isolated on nitrogen-free solid medium after ethyl methanesulfonate (EMS) mutagenesis, but except for mutant 5740 (Elmerich et al. 1982) and some glutamine auxotrophs (Donald and Ludwig 1984), they were not characterized.

We report here the isolation and study of Nif$^-$ mutants obtained by Tn*5* site-directed mutagenesis of a cloned DNA region containing the *nifHDKE* cluster and the adjacent regions. Our results provide evidence for the genetic organization of the *nifHDKE* cluster, and are essentially in agreement with the recent report by Donald et al. (1986). A new *nif* region was detected adjacent to *nifE* and no other *nif* gene was found in the immediate vicinity of *nifH*. Physiological and biochemical evidence for the functioning of *nifH* copy 2 is reported. In addition, we present data on the characterization of the *nif* products and function, using biochemical complementation and polypeptide analysis by one and two-dimensional polyacrylamide gel electrophoresis.

Materials and methods

Bacterial strains and plasmids. The wild-type *Rhizobium* strain was ORS571 (Dreyfus and Dommergues 1981). Mutants 5740, 5751 and 5795 were Nif$^-$ derivatives of ORS571 obtained after EMS mutagenesis. Mutant 5740 has been previously described and is impaired in nitrogenase protein 1 activity (Elmerich et al. 1982); mutant 5751 is a prototroph whereas 5795 requires glutamine for growth. Other mutants carried insertions or deletions shown in Fig. 1. *Escherichia coli* strains were S17.1 (*pro thi recA* RP4-2, Tc::Mu–Km::Tn*7* Tra$^+$ IncP-1) (Simon et al. 1984), C600 (*trp his recA rif*) and HB101 (*pro leu recA*) (our labo-

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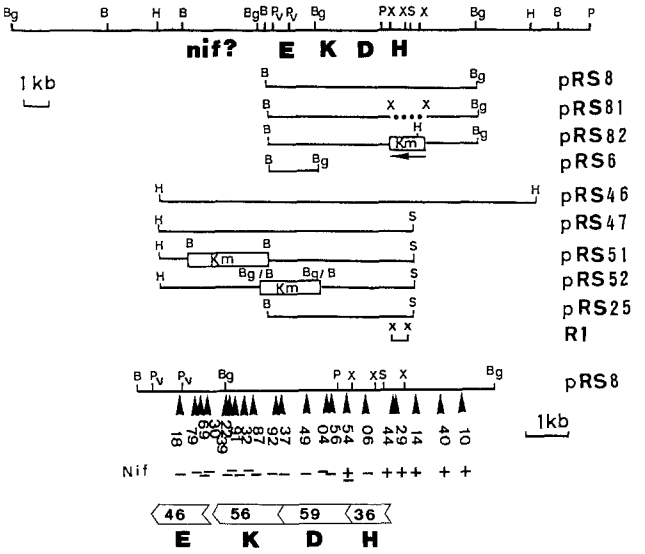

Fig. 1. Localisation of Tn*5* insertions and of in vitro generated deletions in the *nifHDKE* region. Restriction sites: B, *Bam*H1; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; P_v, *Pvu*II; S, *Sal*I; Sm, *Sma*I; X, *Xho*I. *Arrowheads* show the location of Tn*5* insertions; *open boxes* the Km^r cartridge and a *dotted line* the position of the deletion. The *question mark* indicates a putative new *nif* region. Localization of the *nifHDK* genes and their direction of transcription is shown by *boxed arrows*, the molecular weight of the *nif* products are indicated in kDa. The vector for pRS8, pRS81, pRS82, and pRS6 was pRK290. The vector for pRS25, pRS46, pRS47, pRS51, and pRS52 was pSUP202. The R1 fragment corresponds to a 0.6 kb *Xho*I fragment, purified from pRS25 which was used as an intragenic probe for *nifH*. Plasmid pRS8 has been previously described (Norel et al. 1985a). The Km^r cartridge in pRS82 was a 2.2 kb *Xho*I fragment from Tn*5*. The Km^r cartridge in pRS51 and pRS52 was a 1.5 kb *Bam*HI fragment from pUC4K

ratory collection). Plasmid vectors were pRK290 (Mob^+ Tc^r IncP) (Ditta et al. 1980) and pSUP202 (Amp^r Cm^r Tc^r Mob^+) (Simon et al. 1984). Plasmid pRS1, a pRK290 derivative that carries a 13.5 kb *Bam*HI DNA fragment of ORS571 homologous to a *K. pneumoniae nifHDK* probe, has been previously described (Elmerich et al. 1982). Plasmids pRK2013 (Km^r IncP, Ditta et al. 1980) and pPH1JI ($Gent^r$ IncP, Hirsch and Beringer 1984) were used in conjugation experiments. Plasmids pUC4K (Messing and Vieira 1982) and pBR322::Tn*5* (provided by Dr. Mazodier) were used to purify the kanamycin-resistance cartridges. Other plasmids are schematized in Fig. 1; they were maintained in *E. coli* HB101.

Media, growth conditions, nitrogenase assay, and plant tests. Media and growth conditions for *Rhizobium* and *E. coli* and the nitrogenase assay with whole cells were performed as previously reported (Elmerich et al. 1982). One unit (U) of activity was defined as 1 nmol ethylene produced/min. Crude extracts were obtained according to Kush et al. (1985). The Nif^- mutants were grown in nitrogen free medium LSO (Elmerich et al. 1982) containing 1 mg/ml glutamate, and collected 10 h after the end of growth. Under these conditions, nitrogenase activity was completely derepressed in the wild type. Biochemical complementation was performed with pure nitrogenase components 1 and 2 from strain ORS571 as described (Kush et al. 1985). Plant tests were performed as described (Elmerich et al. 1982). Nitrogenase activity of the nodules was measured on excised roots, after three weeks of incubation. Plantlets inoculated with the wild type ORS571 had an activity of 0.6 U/mg of nodules. Fix^- mutants had less than 0.03 U/mg.

Conjugation and site-directed mutagenesis. Plasmids in *E. coli* S17.1 were transferred to *Rhizobium* according to Simon et al. (1984). In other cases, tri-parental matings were performed, using pRK2013 as a mobilizing agent. For Tn*5* mutagenesis in *E. coli*, plasmids derived either from pSUP202 or pRK290 were introduced by transformation into *E. coli* S17.1 – Tn*5*, (a S17.1 derivative containing Tn*5* in the chromosome, provided by Dr. Mazodier). Independent transformants were mated with *E. coli* C600. After conjugation on solid LB medium for 2 h and reisolation on selective LB Rif Km medium, Tn*5* was localized by restriction mapping. The mutations were subsequently transferred into the strain ORS571 genome by homologous recombination according to Ruvkun et al. (1982) and Simon et al. (1984). Plasmid pPH1JI was used to cure strain ORS571 of pRK290 derivatives.

DNA techniques. Plasmid purification and construction were performed using conventional techniques previously described (Elmerich et al. 1982). Details on plasmid construction are reported in the legend of Fig. 1.

Physical localisation of Tn5 or of the kanamycin cartridge in the ORS571 genome. Correct homogenotization was checked in deletion mutants 57151, 57152, 57182 (Table 1) and in all Tn*5* mutants shown in Fig. 1, except mutant carrying insertion 44 which was only used as a Nif^+ control. Total DNA of the mutants was extracted according to Elmerich et al. (1982) from a 10 ml culture in LSN medium. Restriction, Southern blotting and hybridization were performed as described (Elmerich et al. 1982). In each case the restricted sample was loaded on two different gels to perform hybridization with a *nif* probe and with an insertion probe. DNA from the Tn*5* mutant carrying insertion 54 (Fig. 2) and from the deletion strain 57182 was digested with *Bam*HI, *Bgl*II, *Hin*dIII and *Sma*I. Restricted DNA was probed with the R1 fragment (see Fig. 1) and the 2.2 kb *Xho*I fragment from Tn*5* carrying the Km^r gene. DNA from the deletion strains 57151 and 57152 was digested with *Hin*dIII, since the Km cartridge from pUC4K which was substituted into the deleted fragment contained a single *Hin*dIII site. Restricted DNA was probed with the *Hin*dIII fragment from pRS46 and with pUC4K. DNA from the other insertion mutants was digested with *Bgl*II and probed with the *Bam*HI – *Sal*I fragment purified from pRS25 and with DNA of pBR322::Tn*5*.

Polypeptide analysis. Cells grown in complete medium were centrifuged, washed and transferred, at OD_{560} 0.3, into 25 ml flasks containing 5 ml LSO medium, under an atmosphere of Ar/O_2 (97/3, v/v). After three hours incubation at 37° C, with shaking, 10 µCi ^{35}S-methionine (SMM31b CEA, 1,075 Ci/mmole) were added. After 30 min, 5 mg methionine was added. Cells were centrifuged anaerobically, washed with LSO medium containing methionine, and resuspended in 100 µl Tris-HCl 100 mM, pH 7.5 buffer. The suspension was sonicated twice for 30 s with a Branson B-12 sonifier, treated with 1 µl of 5 mg/ml DNAse. At this step, samples could be kept frozen at −80° C or subjected directly to electrophoresis. The isoelectric focusing was per-

Table 1. Phenotype of various *Rhizobium* ORS571 mutants

Strain	Mutation	Nitrogenase activity with whole cells (%)					*nif* polypeptides			Plant phenotype	
		No plasmid	pRS8	pRS81	pRS6	pRS818 *nif18*-Tn*5*	H	D	K	Nod	Fix U/mg nodules
ORS571	wild type	100	76	71	70	76	+	+	+	+	0.6
5740	EMS	<0.1	10	<0.1	<0.1	<0.1	+	+	+	+	<0.03
5751	EMS	<0.1	<0.1	NT	NT	NT	−	−	−	+	<0.03
5795	EMS	<0.1	<0.1	NT	NT	NT	−	−	−	+	<0.03
57044	44-Tn*5*, Nif$^+$	53	39	71	NT	51	+	+	+	+	0.55
57006	*nif06*-Tn*5*	<0.1	8	<0.1	<0.1	0.9	+	−	−	+	<0.03
57004	*nif04*-Tn*5*	<0.1	6	<0.1	<0.1	0.7	+	−	−	+	<0.03
57049[a]	*nif49*-Tn*5*	<0.1	NT	NT	NT	NT	+	−	−	+	<0.03
57087	*nif87*-Tn*5*	<0.1	2	<0.1	<0.1	0.8	+	−	−	+	<0.03
57032[a]	*nif32*-Tn*5*	<0.1	NT	NT	NT	NT	+	w	−	+	<0.03
57022	*nif22*-Tn*5*	<0.1	NT	NT	NT	NT	+	−	−	+	<0.03
57039	*nif39*-Tn*5*	<0.1	2	<0.1	<0.1	0.5	+	−	−	+	<0.03
57030[a]	*nif30*-Tn*5*	<0.1	NT	NT	NT	NT	+	−	−	+	<0.03
57069[a+]	*nif69*-Tn*5*	0.1	NT	NT	NT	NT	+	+	+	+	<0.03
57079[a]	*nif79*-Tn*5*	0.1	NT	NT	NT	NT	+	+	+	+	<0.03
57018	*nif18*-Tn*5*	<0.1	4	5.2	6	<0.1	+	−	−	+	<0.03
57182	*nif* deletion	30	NT	NT	NT	NT	+	+	+	+	0.5
57151	*nif* deletion	<0.1	NT	NT	NT	NT	+	+	+	+	<0.03
57152	*nif* deletion	<0.1	NT	NT	NT	NT	+	−	−	+	<0.03

NT, not tested; +, polypeptide band detected; −, not detected; w, weak

[a] Contains pPH1JI, no complementation could be performed

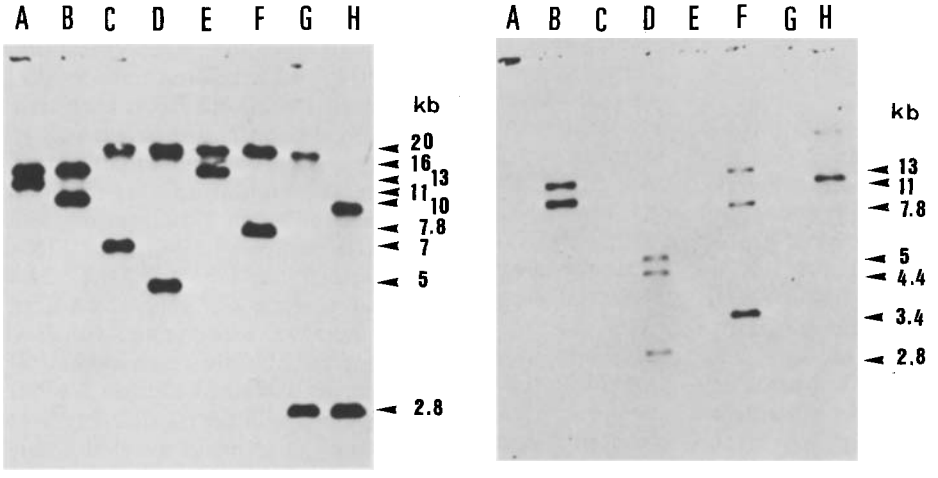

Fig. 2. Physical mapping of the Tn*5* insertion in the mutant strain 57054. A, C, E, G, total DNA from ORS571; B, D, F, H, total DNA from 57054; A, B, digested with *Bam*HI; C, D, *Bgl*II; E, F, *Hin*dIII; G, H, *Sma*I. *Left*, hybridization with the R1 fragment containing *nifH*; *right*, hybridization with the 2.2 kb *Xho*I fragment from Tn*5*

formed with a LKB Multiphor apparatus, according to the manufacturer's protocol and as previously reported (Houmard et al. 1980). Several pH gradients were used, the best resolution was obtained with Ampholine pH 5–8 from Pharmacia. SDS electrophoresis in the second dimension was performed using a polyacrylamide gradient of 5%–15%. One-dimensional SDS slab gel electrophoresis was performed according to Laemmli and Favre (1973) using 7.5% or 10% polyacrylamide. SDS was from Koch-Light. Scintillation autoradiography was as reported (Houmard et al. 1980). Labelling of cells under conditions of repression of nitrogen fixation was performed according to the same protocol, except that ammonia at 15 mM final concentration was added to the medium before inoculation.

Results

Isolation of Nif$^-$ mutants by site-directed mutagenesis

A series of Tn*5* insertions were isolated in pRS8 and two methods were used to introduce the mutations into the genome of *Rhizobium* ORS571.

In the first series of experiments, ORS571 transconjugants containing pRS8-Tn*5* were super-infected with pPH1JI and Nif$^-$ mutants containing pPH1JI were obtained. However, as pPH1JI was stable in strain ORS571, it was not possible to perform complementation tests by reintroducing pRK290 derivatives into the mutants. Nor did we succeed in observing a recombination event after spontaneous segregation of pRS8, as described with

pRK290 derivatives in *Bradyrhizobium japonicum* (Haugland et al. 1984). Therefore, insertions in pRS8 were recombined in pRS46 or in pRS47, which are derivatives of the suicide vehicle pSUP202 and cannot replicate in *Rhizobia* (Simon et al. 1984). Among the Km^r mutants obtained for the same insertion some were Nif^- and others were Nif^+. The Nif^+ mutants were also Cm^r and probably resulted from an integration of the plasmid into the host genome. The same type of results have been reported in *B. japonicum* (Hahn and Hennecke 1984). However after successive reisolations, Nif^- Cm^s derivatives were obtained. The same technique was used to recombine deletions carried by plasmids pRS82, pRS51 and pRS52. The nomenclature of recombinant mutants was as follows: 570 followed by the number of Tn*5* insertion (e.g., 57018 for insertion number 18) and 571 followed by the number of the pRS carrying a deletion (e.g., 57182 for deletion carried by plasmid pRS82).

Physical mapping of the insertion or deletion in ORS571 Km^r mutants

Figure 1 shows the location of the Tn*5* insertions and the extent of the deletions. Hybridization with an insertion probe and with a probe containing the appropriate DNA region was used to check that Tn*5* or the Km^r cartridge was inserted at the correct location after recombination into the host genome.

Figure 2 shows an example of the results obtained in the case of insertion 54, which is located between *nifH* and *nifD*. DNA isolated from strain 57054 was digested with four enzymes and hybridization was performed using the R1 fragment as the *nif* probe (see Fig. 1) and the 2.2 kb *Xho*I fragment of Tn*5*. As shown in Fig. 2, the R1 fragment revealed two fragments in the wild type due to *nifH* reiteration (Norel et al. 1985) and as expected no fragment was visible with the Tn*5* probe. In the mutant, the size of the fragment carrying the *nifH* copy 1 was modified. For example *Bgl*II restriction revealed a 5 kb fragment (which was also revealed with the Tn*5* probe) instead of a 7 kb fragment in the wild type. In Tn*5* the two *Bgl*II sites are located 1.5 kb from each end. Thus the Tn*5* insertion in the mutant was localized about 3.5 kb from the *Bgl*II site upstream of *nifH*. With the Tn*5* probe, since the probe contains part of the inverted repeat of the transposon, DNA located both on the right and the left of the insertion was revealed. This allowed the insertion to be mapped about 2.9 kb from the *Bgl*II site downstream of *nifH*.

In the case of the deletion strain 57182 (deleted of *nifH* copy 1) hybridization was performed with DNA extracted from free-living cells and from bacteroids. The same fragments were revealed with the two DNA preparations. Hybridization with the R1 fragment still revealed the second *nifH* copy, but fragments carrying copy 1 were not detected (data not shown). Hybridization with the Km^r cartridge revealed only fragments corresponding to *nifH* copy 1. Thus, recombination of the *nifH* deletion occurred at the correct location and no rearrangement occurred in bacteroids.

Mutant phenotype and genetic complementation

The Nif phenotype of Tn*5* insertion mutants is shown in Fig. 1 and data on the complementation of some of these mutants and of deletion mutants are given in Table 1.

As expected, insertion mutants in DNA regions homologous to *nifD*, *nifK*, or *nifE* (e.g., 57004, 57049, 57018) and the *nifE* deletion mutant 57152 were totally devoid of nitrogenase activity. In addition, they were Nod^+ Fix^-. Mutant 57151 that carried a deletion in the region downstream of *nifE* was also Nif^- Nod^+ Fix^-. Insertion mutants in the region upstream of *nifH* copy 1 (e.g., 57044) had a nitrogenase activity ranging from 20%–50% of the wild-type activity. As strain ORS571 harbouring pPH1JI did not display more than 50% nitrogenase activity, it was not clear whether the lowered in vitro nitrogenase activity displayed by mutants such as 57044 was due to the presence of Tn*5* in the host genome, to the presence of pPH1JI or to a leaky Nif phenotype. However, since these mutants were also Nod^+ Fix^+, we assumed that they were not impaired in a *nif* gene.

Mutant 57182, which carries a deletion of *nifH* copy 1, had 30% of the wild-type nitrogenase activity and was considered as Nif^+. It was also Nod^+ Fix^+. This observation strongly suggested that *nifH* copy 2 was functional in ORS571.

With regard to the transcriptional organization of the *nifHDKE* cluster, it appears that, in spite of the functioning of *nifH* copy 2, insertions in copy 1 (e.g., 57006) led to a Nif^- phenotype. The simplest explanation accounting for this observation is that the insertion was polar on the expression of *nifD* and *nifK*. However, insertion mutant 57054 displayed 10% nitrogenase activity and was considered as Nif^+. Genetic complementation of mutations in *nifHDKE* was not very efficient and even pRS8, which carries a wild-type *nifHDKE* cluster, restored less than 10% of nitrogenase activity when introduced into various mutants (Table 1). We have previously observed that complementation of EMS mutant 5740 by pRS1 was low (Elmerich et al. 1982) and the same observation was made by Donald et al. (1986) with insertion mutants. In addition, none of the plasmids containing a Tn*5* insertion in DNA regions homologous to *nifHDKE* complemented mutant 5740, possibly due to a transdominant effect as suggested by Donald et al. (1986). As reported in Table 1, partial diploids, containing the *nif06* (*nifH*) insertion in the chromosome and the *nif18* (*nifE*) insertion in pRS8, (pRS818), exhibit less than 1% nitrogenase activity: however, this value was significantly higher than the nitrogenase activity of mutants 57006 and 57018. Moreover, diploids containing mutation *nif06* in the chromosome and *nif04* (*nifD*), *nif49* (*nifD*) or *nif87* (*nifK*) in pRS8 had no detectable activity (data not shown). Plasmid pRS81 restored about 5% nitrogenase activity to insertion *nif18*, which is located in the *nifE* homology region (Table 1). This suggested that *nifE* was transcribed independently of *nifHDK*. Moreover, pRS6 which contained a 2.3 kb *Bgl*II – *Bam*HI fragment covering the *nifE* homology region also complemented insertion *nif18* (Table 1). This reinforced the assumption that *nifE* was transcribed independently from *nifHDK* and also suggested that *nifE* cannot extend beyond the *Bgl*II or *Bam*HI sites. Therefore, we assumed that the Nif^- phenotype observed with strain 57151, which carries a deletion of the 4 kb *Bam*HI fragment adjacent to *nifE*, indicated the presence of a new *nif* region in the vicinity of *nifE*. In addition, the putative new gene (s) in this region were likely to be transcribed separately from *nifE*.

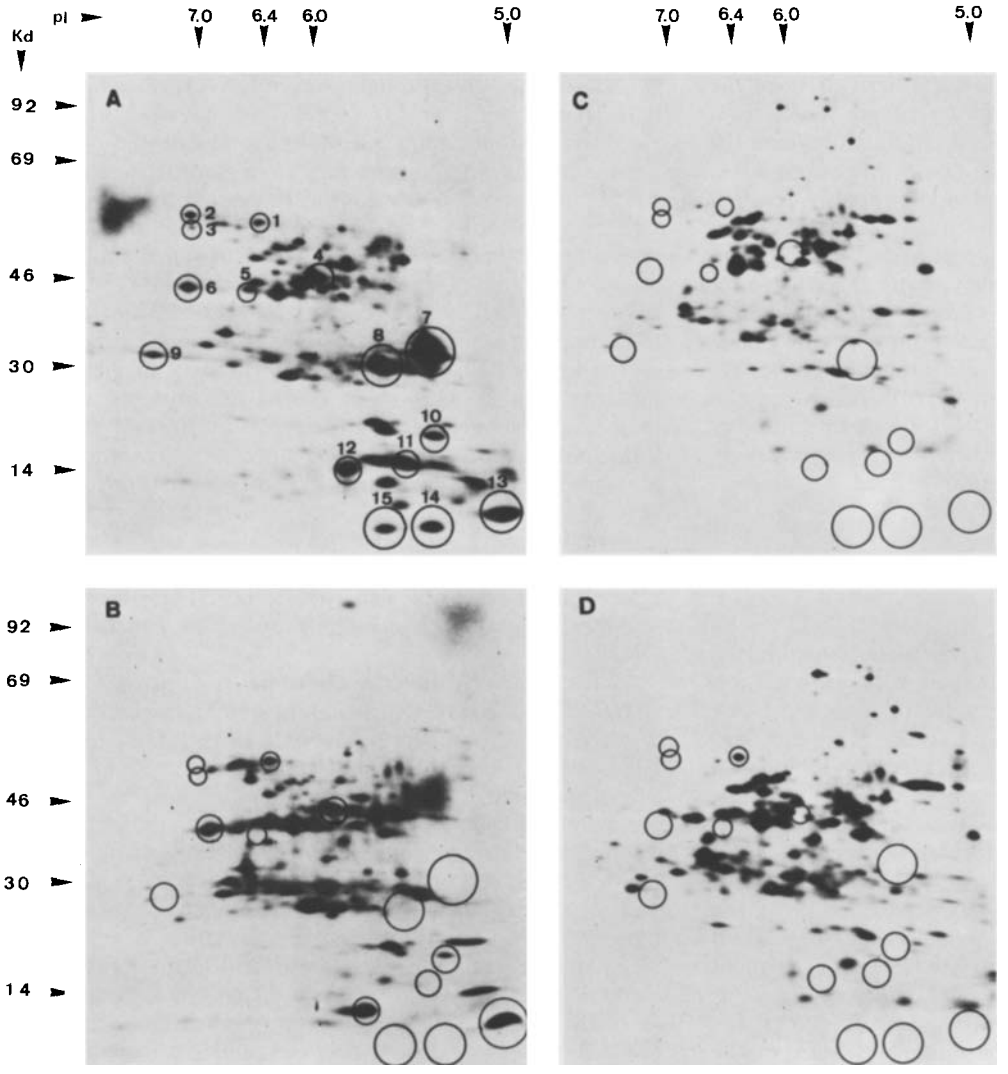

Fig. 3A–D. Two-dimensional gel electrophoresis of ^{35}S-methionine labelled proteins synthesized. **A, C** by strain ORS571; **B, D** by mutant 5751, **C, D** in the presence of ammonia; **A, B** in the absence of ammonia

Polypeptide analysis

Identification of nif specific polypeptides. Autoradiograms of two-dimensional gels of proteins synthesized by the wild type strain ORS571, in the presence or in the absence of ammonia are shown in Fig. 3 and a list of 15 polypeptides synthesized only in the absence of ammonia is given in Table 2. Knowing the M_r, pI and relative electrophoretic mobility of purified nitrogenase subunits (Kush et al. 1985), polypeptides 2 and 3 were assigned to component 1 subunits, and polypeptide 7 to the subunit of component 2. From migration in an SDS one-dimensional gel (Fig. 4), three major bands of M_r 59,000, 56,000 and 36,000 corresponding to the nitrogenase subunits and polypeptide bands of M_r 70,000, 46,000 were detected. The polypeptide of 70,000 was rarely detected in two-dimensional gels. Moreover, as shown in Fig. 3 and Table 2, many of the polypeptides that could be separated according to their pI had a very close M_r and were probably unresolved and appeared as a single band in one-dimensional SDS gels (e.g., p1 and p2; p5 and p6; p8 and p9; p13, p14 and p15). The absence in two-dimensional gels of polypeptides of M_r higher than 59 kDa was probably due to inefficient protein extraction under non-denaturing conditions.

Several EMS mutants were analysed by one and two-dimensional gel electrophoresis. For most of them, all the polypeptides detected in the wild type were present as expected for point mutants (e.g., 5740, data not shown). Two mutants had an interesting phenotype. Strain 5795, isolated as a Nif$^-$ Nod$^+$ Fix$^-$ mutant, required glutamine for growth at a normal rate in ammonia minimal medium. This mutant was also impaired in the utilization of other nitrogen sources such as glutamate, arginine, proline, and ni-

Table 2. Polypeptide synthesized under conditions of derepression of nitrogen fixation

Polypeptide	Two-dimensional gel		Polypeptide	Two-dimensional gel	
(p)	$M_r \times 10^{-3}$	pI	(p)	$M_r \times 10^{-3}$	pI
1	59	6.3	8	33	5.6
2	59	6.9	9	33	7.2
3	56	6.9	10	28	5.3
4	50	5.8	11	20	5.6
5	46	6.3	12	18	5.8
6	46	6.9	13	10	5.0
7	36	5.3	14	10	5.4
			15	10	5.6

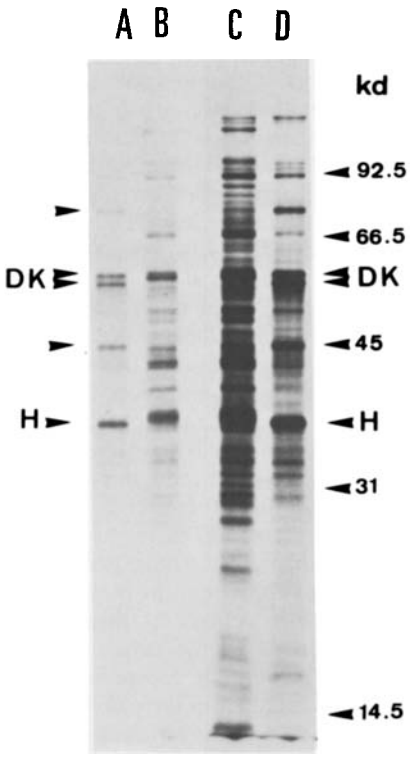

Fig. 4. One-dimensional gel electrophoresis (10% polyacrylamide) of ^{35}S-methionine labelled proteins synthesized by strain ORS571. Lanes B, C, in the presence of ammonia; lanes A, D, in the absence of ammonia. The time of exposure for autoradiography was 1 day for samples corresponding to lanes A and B and 6 days for lanes C and D. The position of polypeptides corresponding to nitrogenase subunits (H, D, K) is indicated in the margin. In *Rhizobium* ORS571 subunit D is larger than subunit K (see text)

trate. The mutant did not produce any of the 15 polypeptides listed in Table 2 (data not shown), suggesting it was impaired in regulation of nitrogen fixation and that the 15 polypeptides were specifically produced under derepression conditions. Mutant 5751, was prototrophic and Nif$^-$ Nod$^+$ Fix$^-$. It also appeared as a regulatory mutant of nitrogen fixation, since 10 of the 15 polypeptides detected were not synthesized, including the nitrogenase components (Fig. 3). It is thus likely that the majority but not all of the polypeptides detected under conditions of derepression of nitrogen fixation are specific *nif* products. In addition, mutant 5751 produced a polypeptide with migration properties analogous to polypeptide 1 in the presence of ammonia (Fig. 3).

Polypeptides produced by the insertion and deletion mutants. Mutants carrying Tn*5* in the region homologous to *nifD* (57004, 57049) or to *nifK* (57087, 57037) produced the polypeptide of M_r 36,000 corresponding to the component 2 subunit, but the 59,000 and 56,000 polypeptide bands corresponding to component 1 subunits were absent. However in *nifK* mutant 57032, the 59,000 polypeptide band was visible on one-dimensional SDS gel, suggesting that this polypeptide was the *nifD* product. By hybridization, it was not possible to determine the end of *nifK* precisely (Norel et al. 1985a). Since in the insertion mutants 57022, 57039 and 57030 we could not detect the component 1 subunits, we assumed that *nifK* extended until insertion *nif30* (data not shown).

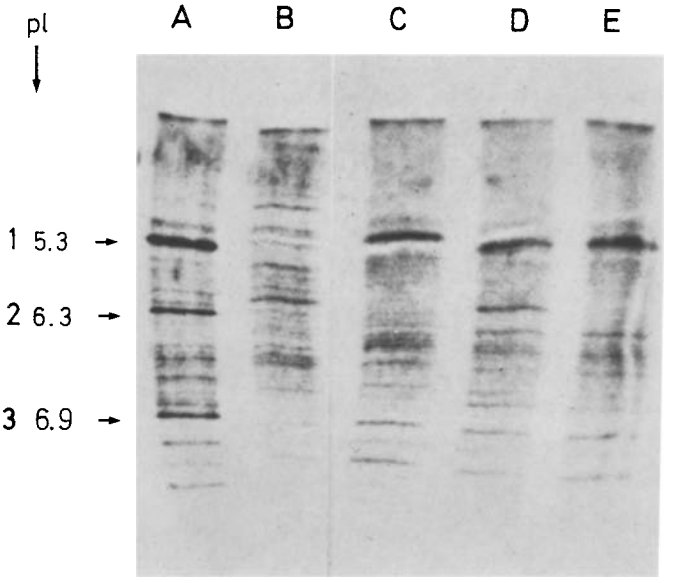

Fig. 5. Isoelectric focusing gel of ^{35}S-methionine labelled proteins synthesized by the wild type and the Nif$^-$ mutants under conditions of nitrogen fixation (lanes A, C, D, E) or ammonia repression (lane B). A and B, ORS571; C, 57018, D, 57151; E, 57152

In *nifH* copy 1 deletion mutant 57182, in agreement with its Nif$^+$ phenotype, the three polypeptides were detected. In the Nif$^-$ mutant 57006, which carried a Tn*5* insertion in *nifH* copy 1, the 59,000 and 56,000 polypeptide bands were absent but the 36,000 polypeptide band was present. This was in agreement with a polar effect of *nifH* copy 1 on the transcription of *nifDK* and with *nifH* copy 2 being functional.

Analysis of mutants localized in the *nifE* homology region revealed two phenotypes. The 56,000 and 59,000 polypeptide bands were found in mutants 57069, and 57079. Strain 57018 and also the deletion strain 57152 lacked the 56,000 and 59,000 polypeptides. This suggests that, in the case of strain 57018, the component 1 subunits were not stable when immature. On two-dimensional gels, it was not possible to determine which polypeptide should be assigned to the *nifE* product, though we observed a decrease in the intensity of spot 5, in strains 57018 and 57152. The two strains lacked a polypeptide of pI around 6.3 on isoelectric focusing gels (Fig. 5). In addition, using 40 cm slab gels of 7.5% polyacrylamide, a band of M_r 44,000 with low intensity, present in the wild type, was absent in extracts of mutants 57018, 57079 and 57152. This band was detected in extracts of mutants 57022, 57032, 57030, 57069 and 57151. Thus, the *nifE* product was tentatively assigned to polypeptide 5 (Table 2) of pI 6.3 and M_r 46,000. On two-dimensional gels, the polypeptide region of M_r around 46,000 was overloaded and likely corresponded to several products. No polypeptide could be assigned to the *nif* region adjacent to *nifE*.

Biochemical complementation

Biochemical complementation of crude extracts from deletion mutants was performed with purified components of the nitrogenase of ORS571. It was found previously that *K. pneumoniae* component 1 could restore nitrogenase activity of an extract of strain 5740. Indeed a crude extract

Table 3. In vitro biochemical complementation of crude extracts of mutants by ORS571 nitrogenase purified components

Strain	Nitrogenase specific activity[a] Addition to crude extract		
	None	+RS1 20 µg	+RS2 200 µg
ORS571	70	68	71
5740	<0.1	48	<0.1
57182	24	8	32
57151	<0.1	35	<0.1
57152	<0.1	7	<0.1

RS1, component 1; RS2, component 2

[a] Nitrogenase activity is expressed in nmol/min per mg protein

of the mutant was also complemented by pure component 1 of ORS571 nitrogenase (Table 3). Crude extract of *nifH* deletion mutant 57182 had a nitrogenase activity which was decreased by addition of component 1 and slightly increased by addition of pure component 2, indicating a deficiency of the extract in component 2. It was also observed that the in vivo nitrogenase activity of mutant 57182 was sensitive to switch-off by ammonia and glutamine (data not shown). Nitrogenase activity in crude extracts of deletion mutants 57152 and 57151 was restored by addition of component 1, but not by component 2. This result was expected in the case of strain 57152, whose deletion is likely to extend from *nifE* beyond the 3′ end of *nifK* and which did not produce the *nifDK* polypeptides. Results obtained with mutant 57151 suggested that the region adjacent to *nifE* plays a role in the maturation of *nifDK* products.

Discussion

Study of Tn*5* insertion and deletion mutants of the *nifHDKE* region of *Rhizobium* ORS571 with a Nif$^-$ Fix$^-$ phenotype, confirms the existence of functional *nif* genes, which were previously identified by hybridization to heterologous probes (Norel et al. 1985a) and which are required for nitrogen fixation both ex and in planta. By random mutagenesis using the plasmid pVP2021, Donald et al. (1985) isolated insertion mutants with a Nif$^-$ phenotype. Mapping of the mutants in the *nifHDKE* region is in agreement with our data (Donald et al. 1986). Though genetic complementation values observed are low, our results are in agreement with the existence of three transcription units: *nifHDK, nifE* and a new *nif* region adjacent to *nifE*. These three transcription units were also described by Donald et al. (1986). The existence of a new *nif* locus adjacent to *nifE* is based on strain 57151, which carries a deletion of the 4 kb *Bam*HI fragment adjacent to *nifE*. The deleted fragment in 57151 does not overlap the *nifE* homology region (Norel et al. 1985a) and pRS6 complements *nifE-Tn5* insertion mutants. It is likely that strain 57151 is deleted for at least one new *nif* gene which cannot be *nifE* and which is probably transcribed independently of *nifE*. Insertions *nif79* and *nif69* produced nitrogenase component 1 subunits and thus do not map in *nifK*. It is not clear whether insertion *nif69* is localized in the *nifE* cistron since it produced the 44,000 polypeptide band. However, the existence of another gene between *nifK* and *nifE* is quite unlikely. Mutant 57054, which carried a Tn*5* insertion between *nifH* and *nifD*, retained 10% of the wild-type nitrogenase activity. Considering a previous report of the non-polar Tn*5* effect in the *lac* operon (Berg et al. 1980), it was assumed that in this mutant, Tn*5* was located at the *nifH/nifD* intercistronic junction and that *nifH* and *nifDK* might be transcribed separately from a Tn*5* promoter or from a promoter-like sequence created by the Tn*5* insertion. The isolation of insertions between *nifH* and *nifD* with no polar effect on the transcription of *nifDK* (Nif$^+$ phenotype) has been described in *Rhizobium meliloti* (Corbin et al. 1983) and *Azospirillum brasilense* (Perroud et al. 1985).

A crude extract of *nifH* deletion mutant 57182 retained about 30% of the wild-type activity. In this strain, the Km^r gene is in the same orientation as *nifDK*. The Nif$^+$ phenotype of the mutant could be explained by assuming that the *nifDK* products are transcribed from the Km promoter and that the strain contains another functional copy of *nifH*. It is likely that transcription of *nifDK* is not as efficient as in the wild type. It appears however from data in Table 3 that the nitrogenase of this mutant is still deficient in component 2. Although the curves of titration of one of the nitrogenase component by the other are not linear (Kush et al. 1985), the amount of component 1 in the mutant can be roughly estimated from data in Table 3, as 32/70, i.e. 45% of the wild type and the amount of component 2 as 45% × 24/32, i.e. 33%. By several criteria, component 2 of the mutant cannot be differentiated from component 2 of the wild type. In particular, the corresponding polypeptides had exactly the same electrophoretic mobility in SDS gels and the nitrogenase of the mutant, like that of the wild-type strain, was subject to switch-off by ammonia. The structural gene coding for the component 2 of the mutant is most likely *nifH* copy 2 previously identified by hybridization (Norel et al. 1985a). The copy 2 is functional in the absence of copy 1: however, in the absence of a mutant of copy 2, one cannot be sure that copy 1 is functional. In addition, one may also wonder whether both copies function simultaneously in the wild type. According to the organization of the *nifHDK* operon. it is most likely that *nifH* copy 1 is functional in the wild type. If both copies are functional, the first one might account for about $^2/_3$ of the amount of component 2 synthesized, and the second one for $^1/_3$. The determination of the nucleotide sequence of the two *nifH* copies is in progress. This should give valuable information on the possible expression of the two genes and on the features of their products. In other diazotrophs containing several *nifH* copies it has been shown that they were functional in *Rhizobium phaseoli* (Quinto et al. 1985) and in *Rhodopseudomonas capsulata* activation of extra *nifH* copies was found only after mutagenesis (Scolnik and Haselkorn 1984).

Comparison of proteins synthesized by the wild type in the presence and in the absence of ammonia revealed 15 supplementary polypeptides detectable under conditions of nitrogen fixation. The two mutants, 5795 and 5751, which were impaired in the synthesis of these polypeptides are probably regulatory mutants. Since strain 5795 displays a better growth in the presence of glutamine and since 5751 is a prototroph, it is tempting to speculate that these mutants are impaired in a *ntr-nifA*-like control, well documented in *K. pneumoniae* (Dixon et al. 1984) and also discovered in *Rhizobium* (Buikema et al. 1985). Mutants probably similar to 5795, with a Gln$^-$ Nif$^-$ Fix$^-$ phenotype have been

isolated in ORS571 (Donald and Ludwig 1984). No complementation of strain 5751 and 5795 by plasmids containing *K. pneumoniae nifA* or *nitrBC* was observed (data not reported). Thus, it is too early to draw conclusions about the nature of the mutation carried by these strains. However, the polypeptide phenotype of the mutants encouraged us to consider that at least some of the products detected in the wild type might be *nif* specific. This was indeed the case for the nitrogenase polypeptides and for the polypeptide assigned to *nifE* which, with a pI of 6.3 and a M_r of 46,000, seems to be close to that found in *K. pneumoniae* (Roberts and Brill 1980). In *K. pneumoniae*, the polypeptide encoded by *nifD* is smaller than the one encoded by *nifK* (Roberts and Brill 1980). Interestingly, our results suggest that the polypeptide corresponding to *nifD* (59,000) has an M_r higher than that of the *nifK* polypeptide (56,000). In *B. japonicum* similar data were obtained previously by maxicell analysis of clones carrying *nifDK* (Fuhrmann and Hennecke 1982) and DNA sequencing data (Kaluza and Hennecke 1984). With regard to the *nif* region adjacent to *nifE*, it should contain at least one new *nif* gene. No homology to various probes covering of all the *nif* genes of *K. pneumoniae* was found (data not shown). Though no polypeptide could be assigned to this new *nif* region, biochemical complementation suggested that this region codes for a function involved in maturation of nitrogenase component 1.

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