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Short communication

Characterization of DNA sequences homologous to *Klebsiella pneumoniae nifH, D, K* and *E* in the tropical *Rhizobium* ORS571

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Summary. The fast-growing *Rhizobium* strain ORS571 isolated from the tropical legume *Sesbania rostrata* can grow in the free living state utilizing molecular nitrogen. The organization of the *nif* genes was analyzed by hybridization using *Klebsiella pneumoniae nif* DNA probes. Homology was limited to *nifHDK*, the structural genes for the nitrogenase, *nifE*, which is involved in formation of the iron-molybdenum cofactor and *nifJ*, which is involved in electron transport. This is the first report of homology in another diazotroph to *K. pneumoniae nifE*. A cluster containing *nifHDKE* was identified. The four genes are contiguous on a 6.3 kb *Sal*I-*Bam*HI fragment. They are all in the same orientation and in the same order as in *K. pneumoniae*. A second copy of *nifH* unlinked to this cluster was also identified.

The *Rhizobium* strain ORS571, isolated from stem nodules of the tropical legume *Sesbania rostrata* (Dreyfus and Dommergues 1981) has the property of fixing molecular nitrogen in the free living state (Elmerich et al. 1982; Dreyfus et al. 1983). We previously cloned a 13 kb *Bam*HI fragment and a 16 kb *Hin*dIII fragment from *Rhizobium* ORS571 total DNA, and identified them by hybridization with the plasmid pSA30 which carries *Klebsiella pneumoniae* nitrogenase structural genes (*nifHDK*), *nifY* and part of *nifE* (Elmerich et al. 1982). The characterization of the cloned *nif* genes was not explored fully, though it was likely that the fragments contained at least sequences homologous to *K. pneumoniae nifH* and *nifK*.

Seventeen genes involved in N_2 fixation are clustered on the chromosome of *K. pneumoniae* (for review see: Elmerich 1984). In addition to the structural genes for the nitrogenase (*nifHDK*), seven genes (*nifQBMVSNE*) are thought to code for proteins that are necessary for a functional enzyme. The other genes are involved in regulation (*nifLA*) and electron transport (*nifFJ*). It was first shown by hybridization with *K. pneumoniae nif* probes (Ruvkun and Ausubel 1980) that *nifHDK* were highly conserved among different genera of diazotrophs. It can be assumed that genes coding for proteins with functions similar to those required for a functional nitrogenase in *K. pneumoniae*, could be present in other diazotrophs, and that consequently some homology of DNA sequences might exist between these genes. This led us to examine in more detail the homology of *Rhizobium* ORS571 DNA with *K. pneumoniae* probes covering the entire *nif* cluster. Hybridization was performed with the plasmids shown in Fig. 1. No hybridization was found with pPC936 and pPC937, nor with the vectors pBR322 and pACYC184. Hybridization was observed with pGR113, pSA30 and pPC880. In the case of *nifJ* the results were complicated since we observed repeatedly, for a given enzyme, multiple fragments which hybridized with pPC880 (data not shown).

Hybridization with specific nifH, nifD, nifK and nif"YEN" probes

Nine different probes, specific for the *nif* genes carried by pSA30 and pGR113, were used. Six of the probes were the *K. pneumoniae* DNA fragments K1 to K6 shown in Fig. 1. We also used *Anabaena* 7120 specific *nifD* and *nifK* probes, and an internal part of the *Rhizobium phaseoli nifH* gene. A typical autoradiogram obtained with *nifH* (K2) is shown in Fig. 2 and results are summarized in Table 1.

Restriction fragments of the same size were found to hybridize with the nine different specific probes and with pSA30. This strongly suggested that sequences homologous to *nifH, nifD, nifK* and *nif"YEN"* were clustered in *Rhizobium* ORS571. In particular, the nine probes hybridized with a 13 kb *Bam*HI, and a 16 kb *Hin*dIII fragment, which were cloned in pBR322 (see Fig. 3). With *nifH* probes (K1, K2 and Rp) additional *Bam*HI, *Bgl*II, *Hin*dIII, *Eco*RI, *Sma*I fragments were observed. These fragments could not be due to partial digests, since they were not detected when the same restriction samples were hybridized with the other probes (see Table 1). This suggested the existence of an extra copy of *nifH*.

Localization of nifH and DNA reiteration

Localization and orientation of *nifH* in pRS2 and pRS21 was established by using K1 and K2 probes (see Fig. 3). Homology was limited to a 1 kb *Sal*I-*Pst*I fragment. For K1 weak homology was found with the 0.75 kb *Xho*I (R4) fragment and strong homology with the adjacent 0.6 kb *Xho*I (R3) fragment, whereas K2, which did not contain the 5′-end of *nifH*, hybridized only with R3. Thus, R4 likely contained the 5′-end of *nifH*.

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Dedicated to Professor Georg Melchers to celebrate his 50-year association with the journal

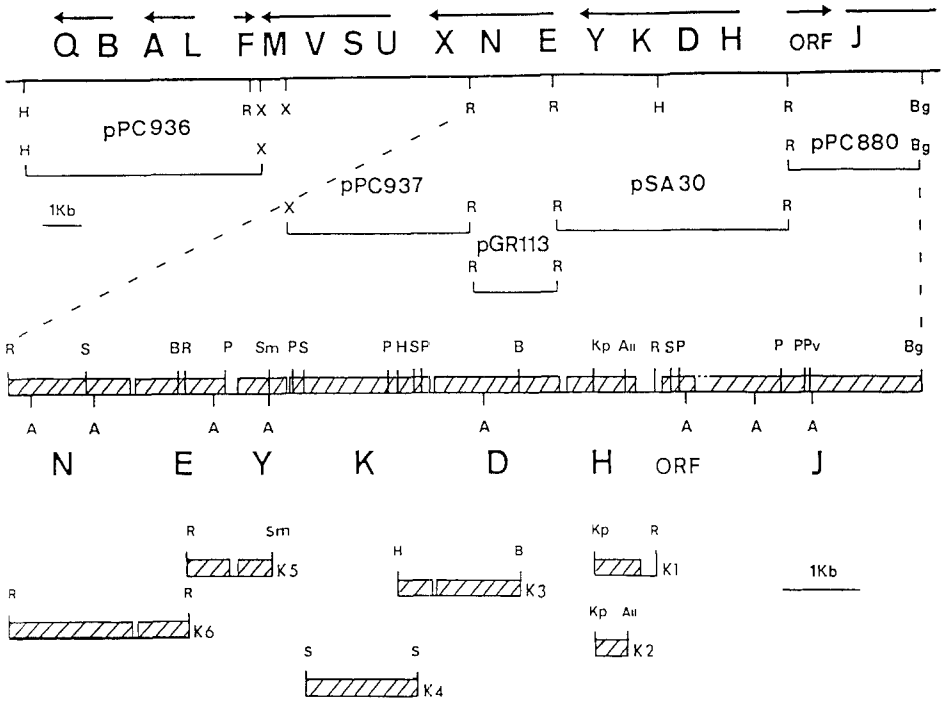

Fig. 1. Physical map of the *nif* cluster of *K. pneumoniae* and of the *nif* probes used. From the top to the bottom: the *nif* cluster of *K. pneumoniae*, with the direction of transcription indicated by the arrows; the DNA fragments cloned in pPC936 (Sibold et al. 1983), pPC937 (this work), pGR113 (Riedel et al. 1983), pSA30 (Cannon et al. 1979), pPC880 (this work); the detailed restriction map of the *nif* DNA from *nifN* to *nifJ*; and the DNA fragments K1 to K6 used as probes. K1, K2, K3 and K4 were purified from subclones of pSA30 constructed by Dr. Sibold. K5 was purified from pSA30 and K6 from pGR113. Restriction sites: B: *Bam*HI; Bg: *Bgl*II; H: *Hin*dIII; R: *Eco*RI; S: *Sal*I; Sm: *Sma*I; P: *Pst*I; X: *Xho*I; Pv: *Pvu*I; A: *Ava*I; AII: *Ava*II; Kp: *Kpn*I. ▨: coding regions according to data of DNA sequencing (Sundaresan and Ausubel 1981), promoter mapping (Beynon et al. 1983) and size of the *nif* polypeptides (see Robson et al. 1983). ORF stands for open reading frame (Shen et al. 1983)

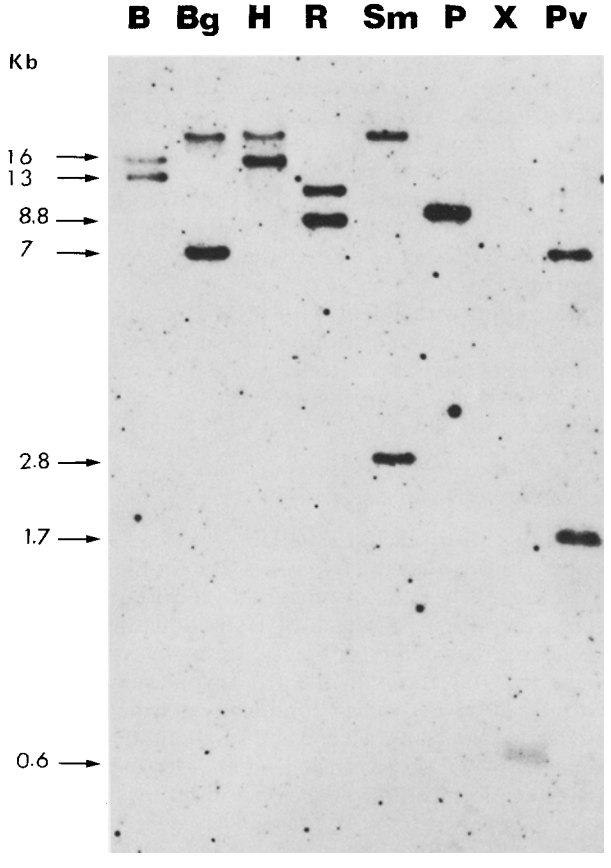

Fig. 2. Hybridization of K2 to restriction fragments of *Rhizobium* ORS571 total DNA. Same abbrevations as in Fig. 1

Table 1. Size of the DNA fragments hybridizing with the *nif* probes

Restriction enzyme	Hybridizing fragments (size in kb)	Probes					
		pSA30	K1	K2, Rp, R3	R4	K3, K4, A1, A2, R2	K5, K6
*Bam*HI	16	+	+	+	+	–	–
	13	+	+	+	+	+	+
*Bgl*II	>20	+	+	+	+	–	–
	7	+	+	+	+	+	–
	2.8	(+)	–	–	–	–	+
*Hin*dIII	>20	+	+	+	+	–	–
	16	+	+	+	+	+	+
*Sma*I	>20	+	+	+	+	+	+
	2.8	+	+	+	+	–	–
*Xho*I	11	+	–	–	–	+	+
	0.85	–	(+)	–	+	–	–
	0.7	–	(+)	–	+	–	–
	0.6	+	+	+	–	–	–

Probes: pSA30 and K1 to K6, see Fig. 1; R2, R3, and R4 see Fig. 3; A1: 1.2 kb *Eco*RI-*Hin*dIII fragment of the 5′-end of *Anabaena* 7120 *nifD* (Rice et al. 1982); A2: 0.7 kb *Hin*dIII fragment of the 5′-end of *Anabaena nifK* (Rice et al. 1982); Rp: 0.3 kb *Sal*I internal fragment of *nifH* or *R. phaseoli* (Quinto et al. 1982). DNA extraction, restriction digests and hybridization were as previously described (Elmerich et al. 1982). Band intensity was recorded after 2 days exposure to the X-Ray film for R2, R3, R4 probes, after 2 or 3 days for K1, K2, Rp and pSA30 probes and after 4 or 5 days for the other probes: – no band visible; (+): weak homology; + strong homology

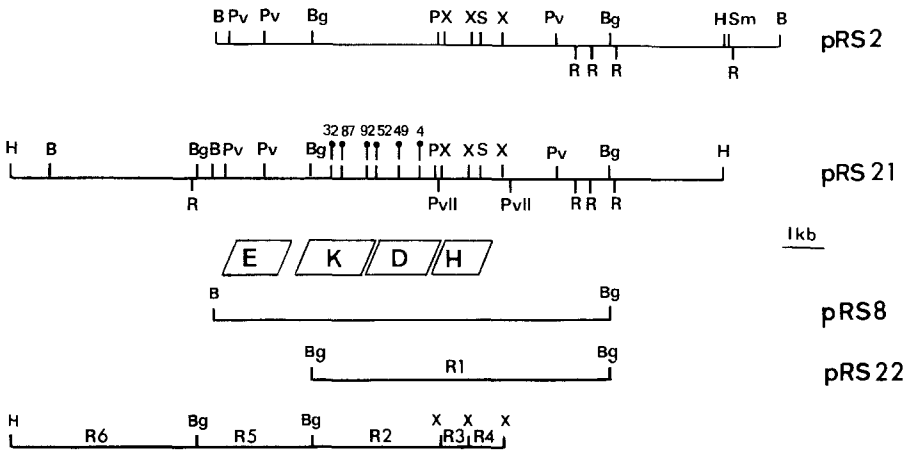

Fig. 3. Physical map of the *Rhizobium* ORS571 DNA fragments carrying the *nifHDKE* cluster. From the top to the bottom: physical map of the 13 kb *Bam*HI and the 16 kb *Hin*dIII fragments cloned in pRS2 and pRS21, localization of the *nifHDKE* genes, DNA fragments R1 to R6 used as probes as well as the DNA fragments cloned in pRS8 and pRS22. Plasmid pRS2 was previously described (Elmerich et al. 1982). Plasmids pRS21 and pRS22 were constructed in pBR322 and pRS8 in pRK290 (for refs. see Elmerich et al. 1982). R1 and R2 were purified from pRS2; R3 and R4 from pRS22; R5 and R6 from pRS21. Restriction sites as in Fig. 1. The *Pvu*II sites (PvII) are indicated only in the *nifH* region. ↑: position of Tn*5* insertions used to localize *nifD* and *nifK*. Tn*5* insertions in pRS8 were isolated in *E. coli*. The *nifHDK* genes are drawn according to the molecular weight of the purified polypeptides (36000, 56000 and 59000 daltons, Kush et al. 1985). The *nifE* gene is drawn according to the size of *K. pneumoniae nifE* contained in the probes used

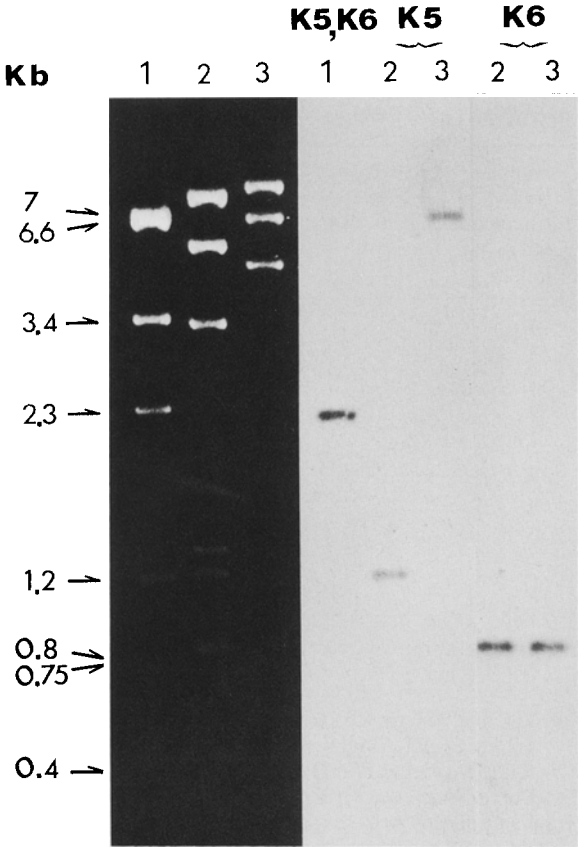

Fig. 4. Hybridization of K5 and K6 to restriction fragments of pRS21. Left part: restriction pattern of pRS21 hydrolyzed by: *Bam*HI + *Bgl*II (lane 1); *Pvu*I + *Bgl*II (lane 2) and *Pvu*I (lane 3). Right part: corresponding autoradiogram after hybridization with the K5 or K6 probe

To demonstrate *nifH* reiteration, R3 and R4 were used as probes against *Rhizobium* ORS571 total DNA. The R2 fragment was chosen as negative control because it did not share homology with *nifH*. As reported in Table 1, hybridization with R3 and R4 revealed the same *Bam*HI, *Bgl*II, *Hin*dIII fragments as the heterologous *nifH* probes, indicating the existence of two *nifH* copies. Copy I was referred to as the *nifH* copy located near the R2 fragment. The extra *nifH* copy was referred to as copy II and was located in a 2.8 kb *Sma*I fragment. Copy II likely overlapped two *Xho*I fragments, one of 0.6 kb possibly identical to R3 found in copy I, another of 0.85 kb that likely corresponded to R4 in copy I.

Hybridization with R1 or with pRS22 revealed all the fragments that were found with R2, R3, and R4, but additional fragments were also detected (data not shown). This suggested that DNA fragments different from those carrying *nifH* were also reiterated.

Localization of nifD and nifK

Hybridizations with specific *nifD* (A1) and *nifK* (K4 and A2) probes showed that the two genes were in the R2 fragment (see Fig. 3). It was not possible to localize the genes more precisely, due to the lack of restriction sites in this region. To overcome this difficulty, derivatives of pRS8 containing Tn*5* insertions in the R2 region were isolated and hydrolyzed by *Bgl*II. Tn*5* contains two symetric *Bgl*II sites located 1.5 kb from each end and surrounding an internal fragment of 2.7 kb (Rothstein et al. 1980). Thus, depending of the localization of the Tn*5* insertion in the R2 fragment, one or two *Bgl*II fragments were expected to hybridize with the *nifD* or the *nifK* probe. Insertions 4 and 49 were found in *nifD*, whereas insertions 87 and 32 were found in *nifK* (see Fig. 3). Thus it was concluded that *nifD*

was proximal to *nifH*. Insertion 4 was located in the 5′-end of *nifD*, and insertion 32 was located in the 3′-end of *nifK*. The junction between *nifD* and *nifK* could not be precisely located. According to the size of the *nifD* and *nifK* products (59,000 and 56,000 daltons) (Kush et al. 1985), it is likely that the junction is located near the Tn*5* insertions 52 and 92.

Localization and identification of a DNA sequence homologous to nifE

Homology with the *nif"YEN"* probes was expected to be in the 2.8 kb *Bgl*II R5 fragment (Fig. 3). Figure 4 shows that homology with K5 was limited to the 1.2 kb *Bgl*II-*Pvu*I fragment (lane 2), whereas homology with K6 was found within the adjacent 0.8 kb *Pvu*I fragment (lanes 2 and 3). The R5 fragment was used in turn as a probe against plasmids pSA30 and pGR113 restricted by various enzymes (data not shown). In the case of pGR113, hybridization was observed with the 1.1 kb *Eco*RI-*Ava*I fragment containing the 3′-end of *nifE* (see Fig. 1), and no homology was found with *nifN*. In the case of pSA30, homology was found only with the 0.5 kb *Eco*RI-*Pst*I fragment containing the 5′-end of *nifE*. No hybridization was observed with the adjacent 0.8 kb *Pst*I fragment which likely contained *nifY*. R6 did not hybridize with pGR113 or pSA30. Thus, homology to *nif"YEN"* sequences was limited to *nifE* and the presence of *nifN* or *nifY* was not detected on the adjacent fragment.

Two Tn*5* insertions, located in the 0.8 kb *Pvu*I fragment sharing homology to K6, were isolated in pRS8. The insertions were introduced in *Rhizobium* ORS571 genome after marker exchange according to the protocol of Ruvkun and Ausubel (1981). The resulting mutants were Nif$^-$ Nod$^+$ Fix$^-$ (data not shown).

Discussion

Homology was observed between cloned *nif* sequences of *K. pneumoniae, Anabaena* and *R. phaseoli* and *Rhizobium* ORS571 total DNA. Our results establish the existence of a *nifHDKE* cluster in *Rhizobium* ORS571 as well as the presence of a second copy of *nifH*.

Until recently, homology to *K. pneumoniae nif* DNA was mainly limited to *nifHDK* (Ruvukun and Ausubel 1980). The three genes are organized in a cluster in several diazotrophs, e.g. *Azospirillum* (Quiviger et al. 1982), *Azotobacter* (Jones et al. 1984) and fast growing *Rhizobia* (see e.g. Ruvkun et al. 1982). In other cases, such as *Anabaena*, *nifHD* are separated from *nifK* (Rice et al. 1982) and in slow growing *R. japonicum*, *nifH* is separated from *nifDK* (Kaluza et al. 1983).

The presence of an extra copy of *nifH* is not a feature limited to *Rhizobium* ORS571. It was described in *Anabaena* (Rice et al. 1982) *Azotobacter* (Jones et al. 1984) and in *R. phaseoli*, where at least 3 copies were found (Quinto et al. 1982). In *Rhodopseudomonas capsulata*, the entire *nifHDK* cluster is reiterated three times (Scolnick and Haselkorn 1984) and in fast growing strains of *R. japonicum* two copies of *nifH* and *nifD* were found (Prakash and Atherly 1984).

Homology to genes other than *nifHDK* has been described in a few cases, as for example for the *nifV/S* region (Rice et al. 1982; Jones et al. 1984), *nifA* (Nair et al. 1983; Szeto et al. 1984; Scott et al. 1984) and *nifB* (Rossen et al. 1984). Homology to *nifE* found in *Rhizobium* ORS571, is to our knowledge, the only example described at the moment. Concerning the hybridization detected with *K. pneumoniae nifJ*, it cannot be totally ruled out that the *nifJ* probe shared homology to a repetitive sequence of *Rhizobium* ORS571 genome. Further experiments are required to establish if a gene equivalent to *nifJ* exists in this strain.

In *Rhizobium* ORS571, the *nifHDKE* cluster is contained in a 6.3 kb *Sal*I-*Bam*HI fragment. It is worth noting that *nifE* is located near *nifK*, a position similar to that one found in *K. pneumoniae*, and that the four genes are in the same relative order. The four genes are in the same orientation as deduced from hybridization with the various probes, since some of them overlapped (e.g. K1, K2) and others were specific for DNA regions in the 5′-end (e.g. K5, A1, A2) or in the 3′-end (e.g. K6). Their orientation is similar that one found in *K. pneumoniae*. The second copy of *nifH* is not located in the immediate neighbourhood of the *nifHDKE* cluster.

Tn*5* insertions in the sequence homologous to *K. pneumoniae nifE* abolish nitrogen fixation of *Rhizobium* ORS571 *in planta* and *ex planta*. Thus, this sequence corresponds to a *nif* gene. In *K. pneumoniae nifE* is involved in the formation or the insertion of the iron-molybdenum-cofactor in the nitrogenase Mo-Fe protein (Roberts et al. 1978). It is likely that a similar gene is conserved in ORS571. The transcriptional organization of the *nifHDKE* cluster is under study and experiments are in progress to establish if the two copies of *nifH* are functional.

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