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In *Rhizobium japonicum* the Nitrogenase Genes *nifH* and *nifDK* Are Separated

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In contrast to *Klebsiella pneumoniae* or fast-growing *Rhizobium* species, such as *R. meliloti*, where the nitrogenase structural genes are clustered in one operon (*nifHDK*), in slow-growing *Rhizobium japonicum* 110, *nifH* and *nifDK* are on separate operons.

In *Klebsiella pneumoniae* the three constituent polypeptides of the nitrogenase complex are encoded by the genes *nifH* (for component 2) and *nifD*/*nifK* (for component 1) (2, 11). These genes, together with a fourth gene *nifY* (17) of unknown function, are organized in one operon *nifHDKY* and transcribed into a single polycistronic mRNA in that order (2, 10, 11, 17). The *nifH* and *nifD* genes, in particular, were found to hybridize to the DNA of a wide variety of N_2-fixing bacteria (19). This has led to the detection and subsequent cloning of nitrogenase structural genes from several *Rhizobium* species (1, 3, 4, 7, 9, 12, 18, 19). More detailed analysis has revealed that in *R. meliloti* (3, 20, 25) and *R. trifolii* (K. F. Scott, Abstr. Twelfth Int. Union Biochem. Congr. 1982, p. 397) the *nifHDK* genes are organized and transcribed in the same way as in *K. pneumoniae*.

We had previously cloned and characterized nitrogenase genes from *R. japonicum*, the nitrogen-fixing soybean symbiont (5, 7, 9). Strong interspecies homology was found only with the *K. pneumoniae nifD* gene (9). No such homology was found with *nifK*, and little, but inconclusive, hybridization was found with *nifH* (7, 9). Initially, it was believed that *nifH* was linked to *nifD* (7), but the expression of the homologous region in minicells of *Escherichia coli* yielded only the β and α subunits of components 1 (Rj1), suggesting the existence of a *nifDK* operon (5). Its location is depicted in Fig. 1. The question of whether or not the *R. japonicum nifH* gene is located immediately upstream of *nifDK* is now addressed in this report. The answer clearly is that *nifH* is unlinked and located on a separate operon. The results of the following experiments support this conclusion.

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(i) Sequencing of *nifDK*. Additional evidence for the clustering of *nifDK* has now been obtained by DNA sequence analysis. Short DNA segments from within the *nifD* and *nifK* genes were cloned into M13 phages (14, 15) and sequenced by the chain termination method (21). It was possible to compare *nifD* sequences with those from *K. pneumoniae* (22) and *nifK* sequences with those from the blue-green alga (cyanobacterium) *Anabaena* sp. 7120 (13). In both cases, homologous regions were found (Fig. 1).

Attempts to express cloned DNA regions on the right (upstream) of *nifDK* in *E. coli* minicells were unsuccessful, i.e., no *nifH*-analogous coding region for component 2 protein was found (5). Negative results obtained by heterologous expression experiments, however, cannot be taken as complete confirmation of the absence of a gene in question. It seemed mandatory, therefore, to disprove (or prove) the presence of the *nifH* gene by further experiments.

(ii) Interspecies hybridization. The DNA regions around *nifDK* were tested for *nifH*-specific interspecies hybridization. As radioactive probes (Fig. 1), we used an *Eco*RI-*Kpn*I DNA fragment containing part of the *K. pneumoniae nifH* region (22, 24) and a *Hin*dIII fragment containing all of *R. meliloti nifH* plus 42 base pairs of *nifD* (1, 25). No hybridization was found to any *R. japonicum* DNA region right or left of *nifDK* in either Southern blot (23) or colony hybridization (6) experiments (data not shown). We also performed a reverse kind of experiment in which the *Xho*I fragment of pRJ4025 (cf. Fig. 1) was transcribed in vitro by purified RNA polymerase from *R. japonicum*. The radioactive transcript(s) were then used to probe *K. pneumoniae* and *R. meliloti nifH* DNA and controls in a dot-blot hybridization experiment (15). Again, no *nifH*-specific interspecies hybridization was found (data not shown).

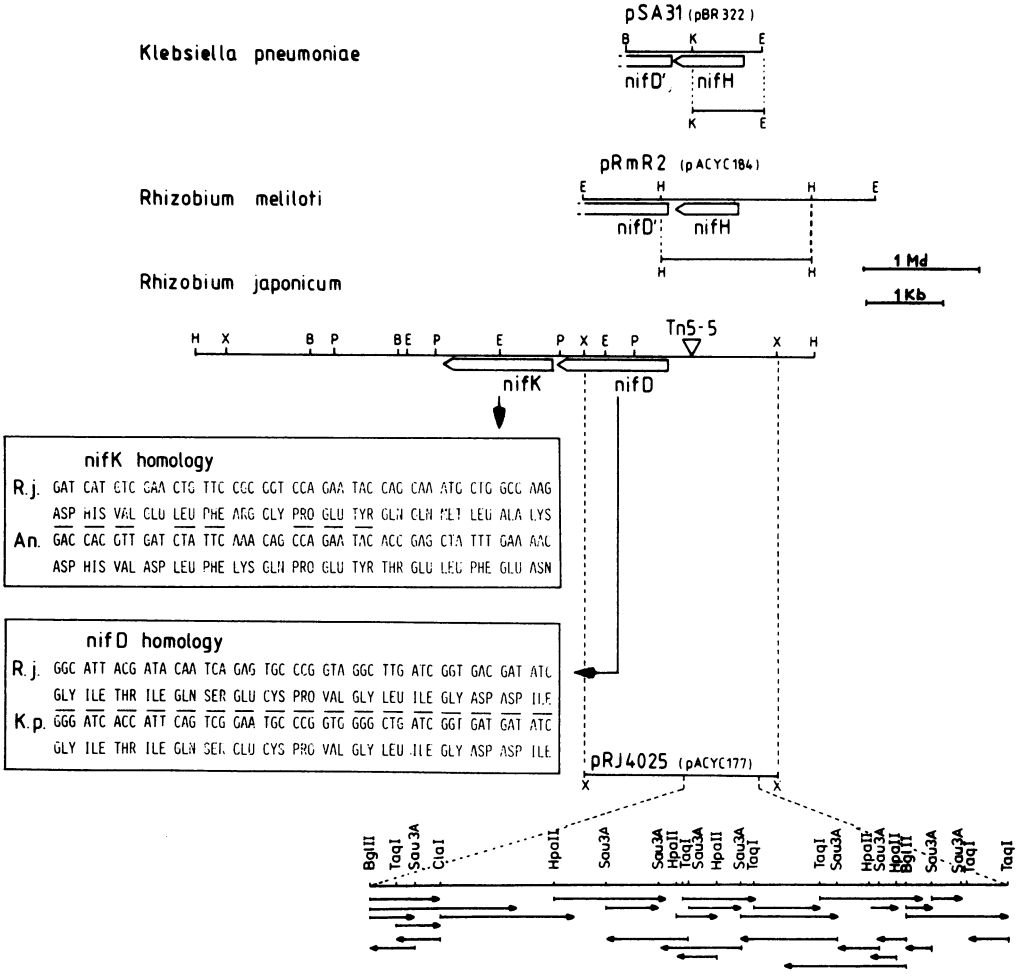

FIG. 1. Recombinant plasmids or DNA fragments or both used in this work which contain nitrogenase structural genes from *K. pneumoniae* (11), *R. meliloti* (19), and *R. japonicum*. Vector DNA is not drawn, but the vectors are indicated in parentheses. The scale is given in Md and kilobase pairs (kb). The map positions of *nifH*, *nifD*, and *nifK* are indicated. The lower part of the figure shows the regions sequenced by the chain termination method. The lengths and directions of sequencing are indicated by the horizontal arrows. The *nifD* sequence originates within the central *Eco*RI-*Pst*I region of *nifD*. The *nifK* sequence originates within the rightward *Eco*RI-*Pst*I region of *nifK*. For comparison, we have shown the homologous sequences, together with the deduced amino acid sequences, of *nifD* from *K. pneumoniae* (K.p.) (22) and *nifK* from *Anabaena* sp. 7120 (An.) (13). Directly homologous amino acids are underlined.

(iii) DNA sequence analysis. We have sequenced 1,082 base pairs upstream of *nifD*. The sequencing strategy is depicted in Fig. 1. The complete nucleotide sequence is not shown here but is available on request. The computer-aided comparison of these sequences with those from *nifH* of *K. pneumoniae* (22, 24), *R. meliloti* (25), and *Anabaena* sp. 7120 (16) failed to reveal any significant homology, either on the nucleotide sequence level or on the amino acid sequence level, in all possible reading frames. Possible reading frames exist which would code for two 15-kilodalton polypeptides or, in the case of a frameshift sequence error, for one 30-kilodalton polypeptide.

(iv) Tn*5* mutation. A mutant strain of *R. japonicum* was obtained after site-directed mutagenesis (M. Hahn and H. Hennecke, manuscript in preparation); a Tn*5* insertion located about 250 base pairs upstream of *nifD* (Fig. 1) did not affect nodulation on soybeans or symbiotic nitrogen fixation (Nod^+ Fix^+). It is unlikely that an outward reading promoter of Tn*5* would produce this phenotype, since this Tn*5* mutation has been shown to prevent the synthesis of the α and β subunits of Rj1 by polarity (5).

(v) **Restriction endonuclease digestion.** To determine whether any *nifH*-homologous sequence occurs elsewhere within the *R. japonicum* genome, we performed the experiment shown in Fig. 2. Total genomic DNA was digested with *Eco*RI, *Pst*I, *Bam*HI, or *Hin*dIII, separated on agarose gels, and blotted onto nitrocellulose filters. These blots were then hybridized either with the ^{32}P-labeled *K. pneumoniae Eco*RI-*Kpn*I *nifH* fragment or with the ^{32}P-labeled *R. meliloti Hin*dIII *nifH* fragment (Fig. 1). In both cases the same *R. japonicum* restriction fragments hybridized a 1.9-megadalton (Md) *Eco*RI fragment, a 9.5-Md *Pst*I fragment, a 1.75-Md *Bam*HI fragment and, very faintly, a 0.5-Md *Hin*dIII fragment (Fig. 2). We have cloned the 9.5-Md *Pst*I fragment into vector pHE3 (8). Restriction analysis of this fragment has revealed the *Eco*RI, *Bam*HI, and *Hin*dIII fragments mentioned before. None of these fragments, however, is contained within or overlaps with the regions around *nifDK*.

The fact that the small *Hin*dIII fragment almost escapes detection by the weak interspecies homology would be an explanation as to why we were previously unable to find the *R. japonicum nifH* gene by colony hybridization (9): our colony bank consisted of cloned *Hin*dIII fragments.

In summary, these results show that the cloned *R. japonicum* region drawn in Fig. 1 does not contain a *nifH*-analogous gene as part of a *nifHDK* operon. The *nifH* region is not in the vicinity of *nifDK*, but elsewhere on the *R. japonicum* genome. From the so-far-identified DNA regions on either side of *nifDK*, we infer that, in *R. japonicum*, the *nifH* gene must be located at least 12 kilobase pairs away from the beginning of *nifD* or at least 5 kilobase pairs away from the end of *nifK* (unpublished data).

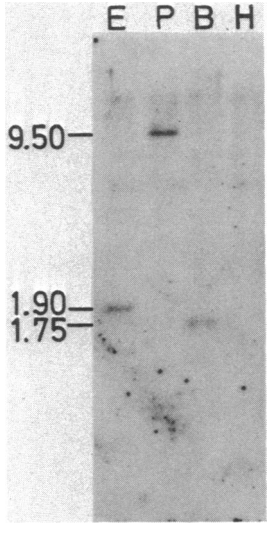

FIG. 2. Detection of *nifH*-specific sequences in total genomic *R. japonicum* DNA by Southern blot hybridization. The restriction digests of total DNA were done with *Eco*RI (E), *Pst*I (P), *Bam*HI (B), and *Hin*dIII (H). In this experiment, the *nifH*-containing *Hin*dIII fragment of pRmR2 (Fig. 1) was used as a radioactive hybridization probe. The molecular weights ($\times 10^6$) of the more strongly hybridizing bands are indicated on the left.

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