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Reiteration of Genes Involved in Symbiotic Nitrogen Fixation by Fast-Growing *Rhizobium japonicum*†

R. K. PRAKASH AND A. G. ATHERLY*

Department of Genetics, Iowa State University, Ames, Iowa 50011

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By using cloned *Rhizobium meliloti* nodulation (*nod*) genes and nitrogen fixation (*nif*) genes, we found that the genes for both nodulation and nitrogen fixation were on a plasmid present in fast-growing *Rhizobium japonicum* strains. Two *Eco*RI restriction fragments from a plasmid of fast-growing *R. japonicum* hybridized with *nif* structural genes of *R. meliloti*, and three *Eco*RI restriction fragments hybridized with the *nod* clone of *R. meliloti*. Cross-hybridization between the hybridizing fragments revealed a reiteration of *nod* and *nif* DNA sequences in fast-growing *R. japonicum*. Both *nif* structural genes *D* and *H* were present on 4.2- and 4.9-kilobase *Eco*RI fragments, whereas *nifK* was present only on the 4.2-kilobase *Eco*R2 fragment. These results suggest that the *nif* gene organizations in fast-growing and in slow-growing *R. japonicum* strains are different.

Rhizobium japonicum forms root nodules and fixes atmospheric nitrogen in association with economically and agriculturally important soybeans. Considerable progress has been made in understanding the genes of fast-growing *Rhizobium* spp. that are involved in symbiotic nitrogen fixation since the genes for nodulation and nitrogen fixation are linked on a large plasmid (15). Although the organization of structural genes for nitrogen fixation (*nif*) is understood in slow-growing *R. japonicum* (4), very little is known about other genes involved in symbiotic nitrogen fixation. The recent discovery of fast-growing *R. japonicum* strains (5) allows a rapid analysis of the organization of symbiotic nitrogen fixation genes as they are present on a large plasmid (7). In this paper, we report the presence of at least two copies of nodulation and nitrogen fixation genes on a large plasmid of fast-growing *R. japonicum* strains.

The fast-growing *R. japonicum* strains USDA 193 and USDA 205 used in this study nodulate both Peking and American cultivars but do not fix nitrogen with American cultivars. Hybridization studies with the *nif* structural genes *D* and *H* of *Rhizobium meliloti* and with intact plasmid DNAs from several fast-growing *R. japonicum* strains provided evidence for the presence of *nif* genes on 186- and 112-megadalton plasmids of strains USDA 193 and USDA 205, respectively (7). To identify the region on the plasmid that is homologous to *nif* structural genes of *R. meliloti*, the plasmid DNA from *R. japonicum*, which was isolated by the method described earlier (8), was digested with the restriction endonuclease *Eco*RI and separated by electrophoresis on 0.7% agarose gels. The DNA was then transferred to nitrocellulose by the method of Southern (14) and annealed with 1×10^6 to 2×10^6 cpm of ^{32}P-labeled pRmR2 DNA, which contains *nif* structural genes *D* and *H* of *R. meliloti* (11). Hybridization was carried out for 40 h at 37°C in a mixture of 10 × Denhardt solution (2), 3× SSC, 0.5% sodium dodecyl sulfate, and 100 μg of salmon sperm DNA per ml, followed by two 5-min washes with 2× SSC and two 30-min washes with 3× SSC–1% sodium dodecyl sulfate–0.1% PP_i. Autoradiography in the presence of an intensifying screen was carried out at −70°C for 16 h. Two *Eco*RI fragments of 4.9 and 4.2 kilobases (kb) from plasmid DNAs of *R. japonicum* USDA 193 and USDA 205 hybridized with the *nif* structural genes of *R. meliloti*. (Fig. 1A).

Analysis of the cosmid clone bank constructed by partial digestion of *R. japonicum* plasmid DNA showed that the 4.9- and 4.2-kb *Eco*RI fragments homologous to the *nif* genes of *R. meliloti* were located on separate clones (data not shown). This finding gave a clue that the observed hybridization of two *Eco*RI fragments with the *nif* structural genes of *R. meliloti* may not be due to an *Eco*RI site within the *nif* structural genes but could be due to the presence of two copies of *nif* sequences. To test this possibility, we identified the 4.2-kb *Eco*RI fragment that was homologous to the *nif* genes of *R. meliloti* from a clone bank, isolated it by using agarose gel elution techniques (16), labeled it with [^{32}P]dCTP, and hybridized it to blots containing *Eco*RI digests of *R. japonicum* USDA 193 and USDA 205 plasmid DNAs. The 4.2-kb *Eco*RI fragment hybridized strongly with the 4.9-kb *Eco*RI fragment and to its own sequences (Fig. 1B). These results thus established the presence of two copies of *nif* sequences on the plasmid of fast-growing *R. japonicum*. The weak hybridization with a third band of 5.8 kb could be caused by another copy of part of the *nif* structural genes or by hybridization with *nif* promoter sequences (1).

The probe that we used for the original hybridization was pRmR2, which has a DNA fragment coding for *nif* structural genes *D* and *H* (11). It is clear that both *nifD* and *nifH* or either one might be present in at least two copies. Hybridization of probes carrying *R. meliloti nifD* (*Eco*RI-*Hin*dIII fragment of pRmR2) or *nifH* (*Xho*I fragment of pRmR2) with *Eco*RI-digested *R. japonicum* plasmid DNA suggested that both *nifD* and *nifH* are located in 4.2- and 4.9-kb *Eco*RI fragments (data not shown). To determine whether *nifK* was also duplicated, the 1.7-kb *Eco*RI fragment of plasmid pRmR8L (12), which contains the *nif* structural gene *K*, was isolated (16) and used as a labeled probe. The *nifK* sequence of *R. meliloti* hybridized only to the 4.2-kb *Eco*RI fragment, which implied that *nifD* and *nifH* but not *nifK* were reiterated (Fig. 1C). Further, the results showed that the *nif* gene organization of fast-growing *R. japonicum* is different from that of slow-growing *R. japonicum* (4).

* Corresponding author.
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To see whether the *nod* genes were also reiterated in fast-growing *R. japonicum* strains, we extended the studies by using a *nod* gene probe of *R. meliloti*. By complementation studies, several *nod* genes in *R. meliloti* have been found to be within an 8.7-kb *Eco*RI restriction fragment (6). Furthermore, by transposon mutagenesis analysis of this *Eco*RI fragment, the nod genes of *R. meliloti* have been identified in a 3.5-kb *Eco*RI-*Bam*HI restriction fragment (S. Long, personal communication). Therefore, we subcloned the 3.5-kb *Eco*RI-*Bam*HI fragment from plasmid pRmSL26 into pBR322 and then used it as a ^{32}P-labeled *nod* probe. Three *Eco*RI fragments from a plasmid of fast-growing *R. japonicum* strains hybridized with the 3.5-kb *nod* probe of *R. meliloti* (Fig. 2B). The hybridizing bands, with lengths of 5.3 and 2.8 kb, were common to both *R. japonicum* USDA 193 and USDA 205. The third band from a plasmid of *R. japonicum* USDA 193 was 11.3 kb, whereas that from USDA 205 was 9.4 kb. The hybridizing *Eco*RI fragment of *R. japonicum* from a plasmid of *R. japonicum* USDA 193 was identified from a cosmid bank constructed from this plasmid and subcloned in vector pACYC184. The fragments were then cross-hybridized to determine whether the DNA sequences homologous to *nod* genes of *R. meliloti* were repeated. When the 11.3-kb *Eco*RI fragment of *R. japonicum* USDA 193 plasmid homologous to the *nod* genes of *R. meliloti* was used as a probe, it hybridized only to its own sequences and to a 9.4-kb *Eco*RI fragment of the *R. japonicum* USDA 205 plasmid (Fig. 2C). However, after longer exposure (2 days) of the blot to X-ray film, a weak signal was observed with 2.5- and 3-kb *Eco*RI fragments (data not shown). On the other hand, the 2.8-kb *Eco*RI fragment, besides hybridizing to its own sequences, also showed homology with a 5.3-kb *Eco*RI fragment of a plasmid from *R. japonicum* USDA 193 and USDA 205 (Fig. 2D). No hybridization was detected between the 2.8- and 11.3-kb *Eco*RI fragments. Site-specific transposon mutagenesis analysis of the 2.8- and 11.3-kb *Eco*RI fragments showed that both fragments carried genes involved in different functions of the nodulation process (R. K. Prakash, N. M. DuTeau, and A. G. Atherly, unpublished data). The results presented here further suggested that the 5.3-kb *Eco*RI fragment homologous to the *nod* genes of *R. meliloti* was the duplication of at least part of the *nod* gene sequence present on the 2.8-kb *Eco*RI fragment of the plasmid from fast-growing *R. japonicum*.

The data presented here thus demonstrated that both *nif*

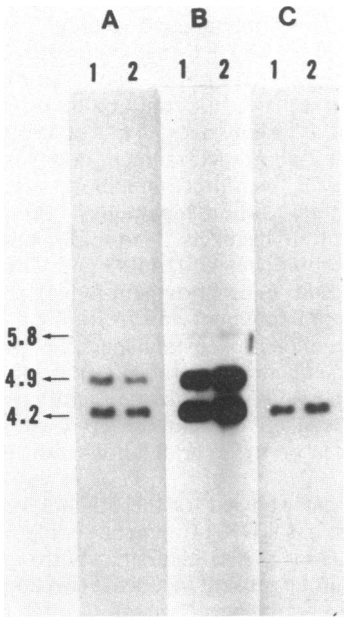

FIG. 1. Hybridization of heterologous and homologous probes of *nif* genes with plasmid DNAs from fast-growing *R. japonicum* USDA 193 (lane 1) and USDA 205 (lane 2). Approximately 1 μg each of plasmid DNA from *R. japonicum* USDA 193 and USDA 205 was digested with restriction enzyme *Eco*RI and separated on 0.7% agarose gels. The restriction pattern of the plasmid DNA is shown in Fig. 2. (A) Autoradiogram of the *Eco*RI digestion pattern of *R. japonicum* plasmid DNA obtained after hybridization against ^{32}P-labeled pRmR2, which contains *nif* structural genes *D* and *H* of *R. meliloti*. (B) Hybridization pattern of plasmid DNA when the 4.2-kb *Eco*RI fragment of *R. japonicum* USDA 193 plasmid DNA was used as a probe. (C) Autoradiogram of *R. japonicum* plasmid DNA after hybridization with the *nifK* probe of *R. meliloti*.

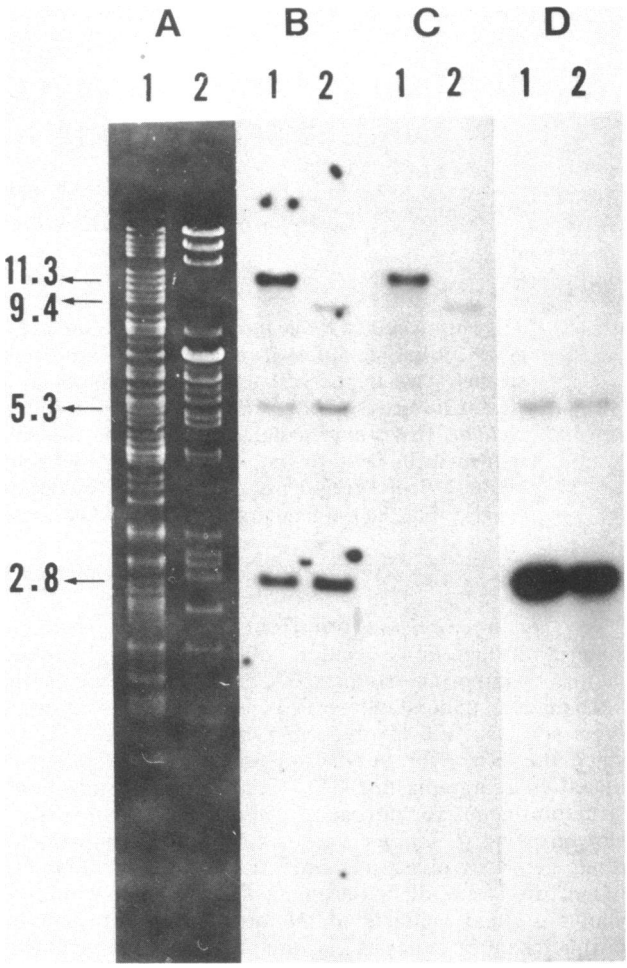

FIG. 2. Hybridization of a DNA fragment of *R. meliloti nod* genes with the plasmid DNA from fast-growing *R. japonicum*. (A) Restriction endonuclease *Eco*RI digest of plasmid DNAs from USDA strains 193 (lanes 1) and 205 (lanes 2). (B) Autoradiogram of the *Eco*RI digests of *R. japonicum* plasmid DNA after hybridization against the ^{32}P-labeled 3.5-kb fragment of *R. meliloti* containing the *nod* genes. (C and D) Autoradiogram of *Eco*RI-digested *R. japonicum* plasmid DNA obtained after hybridization with the 11.3-kb (C) and 2.8-kb (D) *Eco*RI fragments of plasmid DNA from *R. japonicum* USDA 193.

and *nod* gene sequences were present in more than one copy in *R. japonicum* USDA 193 and USDA 205. This phenomenon could be common in all of the fast-growing *R. japonicum* strains inasmuch as the *nif* and *nod* hybridization patterns are identical within these species (R. V. Masterson, R. K. Prakash, and A. G. Atherly, unpublished data). Further, both the *nif* and *nod* DNAs of *R. meliloti* hybridized to the same bands whether plasmid DNA or chromosomal DNA was used, which suggests that the reiteration of symbiotic nitrogen fixation genes are within the plasmid DNA of fast-growing *R. japonicum*. Reiteration of *nif* sequences has been reported for *Rhizobium phaseoli* (9), the cyanobacteria *Anabaena* spp. (10) and *Calothrix* spp. (3), and the purple, nonsulfur-utilizing bacterium *Rhodopseudomonas capsulata* (13). *Anabaena* spp. and *Calothrix* spp. have more than one copy of the *nifH* gene; *Rhodopseudomonas capsulata* has multiple copies of *nifH*, *D*, and *K*. This work showed that in fast-growing *R. japonicum*, *nifD* and *nifH* were present in two copies and that the functional *nifHDK* operon was on the 4.2-kb *Eco*RI fragment of the plasmid from fast-growing *R. japonicum*. This is in contrast to that observed for slow-growing *R. japonicum*, in which each of the *nifH*, *D*, and *K* genes is present in one copy per genome and *nifH* and *nifDK* are on separate operons (4). The role of extra copies of *nif* and *nod* genes in fast-growing *R. japonicum* is not yet clear, but recent results with *Rhodopseudomonas capsulata* show that the extra *nif* gene sequence can be functionally activated (13).

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