Rhizobium meliloti has three functional copies of the nodD symbiotic regulatory gene

(nodulation gene/plant–bacterial interaction/host specificity)

MARY A. HONMA and FREDERICK M. AUSUBEL

Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

Communicated by A. M. Pappenheimer, Jr., August 13, 1987 (received for review July 9, 1987)

ABSTRACT We have identified two Rhizobium meliloti genes (nodD2 and nodD3) that are highly homologous and closely linked to the regulatory gene nodD (nodD1). R. meliloti strains containing mutations in the three nodD genes in all possible combinations were constructed and their nodulation phenotypes were assayed on Medicago sativa (alfalfa) and Melilotus alba (sweet clover). A triple nodD2-nodD3-nodD1 mutant exhibited a nod− phenotype on alfalfa and sweet clover, indicating that nodD1 is an essential nodulation gene in R. meliloti. A nodD2 mutant exhibited no discernible defect in nodulation and nodD3 mutants exhibited a delayed nodulation phenotype of 2–3 days when inoculated onto either host. Alfalfa nodules elicited by a nodD1 mutant appeared 5–6 days after wild-type nodules, and sweet clover nodules elicited by a nodD1 mutant appeared 2–3 days after wild-type nodules. nodD2-nodD3 double mutants formed nodules with the same delay as single nodD2 mutants on both hosts. nodD2-nodD3 double mutants elicited sweet clover nodules at the same rate as single nodD3 mutants, but this same double mutant was slightly more delayed in alfalfa nodule formation than the nodD3 mutant. The nodD1-nodD3 mutant exhibited an extremely delayed nodulation phenotype on alfalfa and elicited no nodules on sweet clover. These experiments indicate that nodD1 and nodD3 have equivalent roles in nodulating sweet clover but that nodD1 plays a more important role than nodD3 in eliciting nodules on alfalfa. The nodD1 gene appears to have some effect on alfalfa nodulation and none on sweet clover. Our results indicate that R. meliloti has three functional nodD genes that modulate the nodulation process in a host-specific manner.

In Rhizobium meliloti, two clusters of nodulation (nod) genes involved in the early stages of symbiotic nodule formation are located on a large symbiotic plasmid (pRmeSU47a) and are closely linked to a cluster of nitrogen fixation (nif) genes (1–4). The so-called “common” nodulation genes (nodA, nodB, nodC, nodD) are conserved structurally and functionally among several Rhizobium and Bradyrhizobium species (5–9). In contrast, the host-specific nodulation genes are involved in determining the range of plant hosts that a particular Rhizobium species will nodulate. For example, the R. meliloti host-specific nodulation genes (nede, nodF, nodG, nodH) allow it to form nodules on plants of the genera Medicago, Melilotus, and Trigonella but not on Trifolium, which is a host of Rhizobium trifolii (2, 4).

In Rhizobium leguminosarum, R. trifolii, and R. meliloti, the nodABC genes are induced only in the presence of specific flavone exudates from the host root (11–16). This induction was shown to be dependent on the nodD gene in R. meliloti and R. leguminosarum (11, 13, 17). In the case of R. leguminosarum, the host specificity genes (nede) are also regulated by nodD (18) and it seems likely that the same true for other Rhizobium species as well. Mutations in the nodABC operon block nodulation in all Rhizobium and Bradyrhizobium species tested so far. Similarly, R. leguminosarum and R. trifolii strains carrying mutations in nodD are Nod−. In contrast, R. meliloti nodD mutants still elicit nodules on alfalfa, suggesting that R. meliloti has more than one functional nodD gene (5–7, 19).

In this report, we describe, two R. meliloti nodD (“nodD1”) homologous genes (“nodD2” and “nodD3”) that are closely linked to nodD1. Nodulation experiments with nodD1, nodD2, and nodD3 mutants on alfalfa and sweet clover indicate that all three nodD genes are functional and suggest that the different nodD genes have host-specific roles in the nodulation process.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids. Bacterial strains, phages, and plasmids used in these experiments are listed in Table 1.

DNA Biochemistry. Isolation of total DNA from R. meliloti (24), agarose gel electrophoresis, and radiolabeling of DNA were performed as described (25). Southern blotting and DNA hybridization using GeneScreen or GeneScreenPlus were carried out according to the manufacturer’s instructions or by substituting 0.4 M NaOH/0.6 M NaCl for 1.5 M NaCl/0.15 M sodium citrate (26) as transfer buffer. T4 DNA ligase was purchased from New England Biolabs or Internation Biotechnologies (New Haven, CT) and restriction enzymes were purchased from Boehringer Mannheim or New England Biolabs.

Cloning of nodD2 and nodD3. DNA from R. meliloti strain Rm3600 (derivative of Rm1021), which is deleted for nodD1 and nodD2, was used to construct a size-selected EcoRI gene library consisting of 6.5- to 7.5-kb fragments cloned in plasmid pRX (S. Canning and B. Seed, personal communication). A recombinant plasmid (pNodD2) carrying the 6.8-kb EcoRI fragment was isolated by colony screening using GeneScreen Colony/PlaqueScreen and the 3.5- and 3.5-kb EcoRI–BamHI fragment containing the R. meliloti nodD2 nodD3 ABCD genes as a hybridization probe. The 15.5-kb EcoRI fragment containing nodD2 (as well as the hns genes) had previously been cloned into plasmid pACYC184 (N. Olszewski and F.M.A., unpublished).

Construction of nodD2, nodD3, nodD2–nodD3, nodD2–nodD2, nodD2–nodD3, and nodD2–nodD3 Mutants. A R. meliloti nodD2 deletion/insertion mutation was constructed as follows. A 0.5-kb Xba I–Cla I restriction fragment (see Fig. 1) containing part of nodD2 was deleted from plasmid pNodD2. Two Cla I sites were present in plasmid pNodD2; however, since the Cla I site that overlaps with one Pvu I site (see Fig. 1) was methylated by dam methylase, only one Cla I site was susceptible to digestion with Cla I. A 3.3-kb BamHI fragment

*To whom reprint requests should be addressed.
All possible combinations of nodD mutants (RmD1D2, RmD1D3, RmD2D3, RmD1D2D3) were made by using phage M12-mediated transduction (28). To verify these constructions, four transductants from each experiment were screened by Southern blotting and hybridization using pNodD1, ColEl::Tn5, pAgRR4 (trimethoprim), and pH45 (spectinomycin) as hybridization probes (data not shown).

Nodulation Assays. Plant tests with alfalfa and sweet clover were done as described in the legend to Fig. 3. To optimize nodulation conditions, the amount of wild-type inoculum (Rm1021) was varied over a range of 5 × 10^2 to 10^6 cells per plant. When 5 × 10^2 to 4 × 10^5 wild-type bacteria were inoculated onto alfalfa, nodules began to appear after 5 days. By 13 days, >90% of the plants had one or more nodules. Sweet clover nodules appeared earliest on plants inoculated with 5 × 10^4 to 10^5 wild-type bacteria.

In an initial experiment, the eight strains carrying the two different nodD^1 alleles (nodD^1::sp/g-1 and nodD^1::sp/g-2) were tested along with wild-type Rm1021 on alfalfa and sweet clover plants. The nodD^2-1 mutation showed a more severe phenotype, whereas the nodD^2-2 mutation showed a partially defective phenotype. The RmD1D2D3-1 mutant was completely Nod^- on both hosts, and the RmD1D2D3-2 mutant still nodulated. Therefore, the nodD^1-1 mutation was used in the experiments that follow.

RESULTS

R. meliloti pSym Contains Two Genes Homologous to nodD.

R. meliloti nodD gene (nodD^1) is directly upstream of nodABC in strains Rm1021 and Rm41 (two independent wild-type isolates) and is located on 8.7- and 8.5-kb EcoRI restriction fragments, respectively (Fig. 1). A 32P-labeled plasmid (pNodD^1) carrying the coding sequences of nodD^1 (6) was used to probe a Southern blot of EcoRI-digested genomic DNA from Rm1021 and Rm41. Two hybridization bands, in addition to the one carrying nodD^1, were observed in each strain. In Rm1021, these were 15.5 and 6.8 kb, whereas the additional hybridization bands in Rm41 were 12 and 14 kb (Fig. 2).

To determine whether the nodD^2-1-homologous sequences in Rm1021 were located in the nod/nif region of pRmSU47a, a nodD^1 probe was hybridized to a Southern blot containing EcoRI-digested DNA from three R. meliloti strains containing deletions in the nod/nif region (Rm3600, RmGM1255, RmGM1963; see Fig. 1). In addition, the nodD^1 probe was hybridized to plasmid pGM1467 DNA, which contains a 30-kb insert of R. meliloti pRmSU47a DNA originating >80 kb upstream of nodD^1 (Fig. 1). The results (shown in Fig. 2 and illustrated in Fig. 1) showed that the 6.8- and 15.5-kb EcoRI nodD homologous fragments in Rm1021 map within 80 kb of nodD^1. We designated the nodD^1 homologous regions in the 6.8-kb and 15.5-kb EcoRI fragments nodD^2 and nodD^3, respectively. In additional hybridization experiments (data not shown), nodD^2 was mapped to a 1.5-kb EcoRI–HindIII segment within the central 6.8-kb EcoRI fragment (Fig. 1); similarly, nodD^3 was mapped 5 kb downstream of nodH within a 2.2-kb Cla I fragment.

Nodulation Phenotypes of Single, Double, and Triple nodD Mutants. To assay the symbiotic phenotypes of the different nodD mutants, alfalfa and sweet clover plants were grown and inoculated as described in the legend to Fig. 3. With Rm1021 (wild type), RmTJ988 (nodD^1), RmD2, RmD3-1, RmD1D2, RmD1D3-1, RmD2D3-1, and RmD1D2D3-1 genotypes of strains are listed in Table 1. Several independent nodulation experiments were carried out and similar results were obtained in each; in total, 3000 plants were inoculated and analyzed. The results of one experiment are shown in Fig. 3.
Alfalfa and sweet clover nodules elicited by wild-type *R. meliloti* (Rm1021) first appeared 5–6 days after inoculation. By 13 days after inoculation, >90% of the plants had one or more nodules. The triple *nodD1*-*nodD2*-*nodD3* mutant exhibited a Nod⁺ phenotype when inoculated onto alfalfa and sweet clover, indicating that *nodD* codes for an essential nodulation function. The *nodD2* mutant elicited nodules at the same rate as wild type. In contrast, inoculation with strains carrying a mutation in either the *nodD1* or the *nodD3* gene resulted in nodule formation that was delayed in comparison to wild type. When alfalfa was inoculated with the *nodD1* mutant or double mutant *nodD1*-*nodD2*, the appearance of nodules was delayed 5–6 days compared to plants inoculated with wild-type *R. meliloti* (Fig. 3A). The *nodD3* mutant elicited alfalfa nodules at a rate that was intermediate to wild-type and *nodD1*-induced nodules, with a delay of 2–3 days. The *nodD2*-*nodD3* double mutant elicited nodules with a slight delay relative to the single *nodD3* mutant. The *nodD1*-*nodD3* double mutant nodulated alfalfa plants but at a very delayed rate. Bumps and swellings began appearing on the roots of plants inoculated with this mutant 12 days after inoculation and nodules appeared 2–10 days later. By 28 days after inoculation, about 50% of the plants and bumps on their roots, and after 35 days, 40% of the plants had one or more nodules.

Interestingly, different results were obtained when the same mutants were used to inoculate sweet clover. The four mutants, *nodD1*, *nodD2*, *nodD3*, and *nodD2*-*nodD3*, all exhibited the same 2- to 3-day delay in nodulation. In contrast to alfalfa, no nodules were formed on sweet clover roots after inoculation with double *nodD2*-*nodD3* mutant, although a "browning response" was observed and 10% of the plants had root bumps after 5 weeks.

**DISCUSSION**

In several *Rhizobium* species, four common nodulation genes (*nodA*, *nodB*, *nodC*, *nodD*) are required for the induction of nodule meristems (5–8). Recently, several laboratories have shown that the *nodD* product is required for activation of the *nodABC* operon in the presence of root exudates or specific flavones or flavanones (11, 13). One possibility is that the *nodD* product interacts with specific root exudate molecules.

As shown in this paper, in contrast to *R. leguminosarum* and *R. trifolií*, *R. meliloti* has three copies of *nodD* that are located within an 80-kb region of the Rm47a megaplasmid. We have designated the originally described *nodD* gene as *nodD1* and the other two copies as *nodD2* and *nodD3*. Southern blot and sequence analysis (of the *nodD2* gene; M.A.H., unpublished data) showed that the *R. meliloti* *nodD2* and *nodD3* genes are highly homologous to *nodD1*. The *nodD2* gene from Rm1021 corresponds to *nodD2* identified in *R. meliloti* strain 41 with respect to DNA sequence and genome location (ref. 29; M.A.H., unpublished data).

Additional functional *nodD* genes would explain the observation that a single *R. meliloti* *nodD1* mutant has no defective nodulation phenotype on alfalfa (17), whereas *R. trifolií* and *R. leguminosarum* *nodD* mutants fail to elicit any
nodules on their respective host plants (5, 7). As described in this paper, we tested this possibility by constructing single, double, and triple mutants in the different nodD genes and assayed their phenotypes on alfalfa and sweet clover. Our results showed that all three nodD genes are functional and that at least two nodD genes are required for optimal nodulation. The triple nodD mutant exhibited a nod− phenotype on both hosts, demonstrating that nodD is an essential nodulation gene in R. meliloti as it is in R. trifolii and R. leguminosarum. All single and double nodD mutants, with the exception of the nodD1-nodD3 mutant, elicited nodules on both host plants. The nodD1-nodD3 mutant exhibited a severe nodulation phenotype. Alfalfa plants inoculated with this mutant formed nodules at an extremely delayed rate relative to plants inoculated with wild type. No nodules appeared on sweet clover inoculated with the nodD1-nodD3 mutant. These data indicate that nodD2 is functional in alfalfa but not sweet clover nodulation.

Our data also suggest that the R. meliloti nodD genes may play different roles in the establishment of the symbiosis with two alternative hosts Medicago sativa (alfalfa) and Melilotus alba (sweet clover). Although the nodD1 and nodD3 mutants nodulated at the same rate on sweet clover, the nodD2 mutant nodulated at a faster rate than the nodD1 mutant on alfalfa. In addition, the nodD2 gene appears to have some minimal function in alfalfa nodulation but none in sweet clover nodulation. It appears that in alfalfa nodulation, the nodD1 gene is more important than the nodD3 gene; the nodD2 gene has a minimal role. In sweet clover nodulation, the nodD1 and the nodD3 genes appear to be equivalent, and the nodD2 gene has no function.

There are at least three possible explanations for the different phenotypes of the nodD mutants. (i) The nodD1, nodD2, and nodD3 products may be functionally interchangeable, but they may be expressed at different levels. (ii) They may differ in their ability to activate expression of the other nodulation genes. (iii) They interact with different root exudate factors (flavones) or with varying affinities for the same flavone. Correspondingly, one would expect that different host plants would produce different flavones and/or different amounts of the same flavones. A variety of combinations of these explanations is also possible.

Direct evidence that supports the production of host-specific root exudate factors has been obtained by Hermann Spink and co-workers (30). They compared the ability of nodD genes from different Rhizobium species to activate the nodA promoter of R. leguminosarum following addition of flavones or clover (host of R. trifolii) root exudate. Their results showed that the induction level of the nodA promoter was dependent on the origin of the nodD gene as well as the inducing substance. They concluded that, although the various nodD gene products were functionally interchangeable, they were not identical. It has been shown by Horvath and co-workers (10) that the nodD genes may encode determinants of host specificity by interacting with different plant factors. If R. meliloti NodD1 and NodD2 interact with different flavones, one would expect that sweet clover produces less or no “NodD2-specific” flavone compared to alfalfa.

Why does R. meliloti have three nodD genes? Under laboratory conditions at least two nodD genes are required for optimal nodulation of R. meliloti. It seems likely that the mutants that are delayed for nodulation in the test tube would be severely impaired in the field. R. meliloti forms an effective symbiosis on plants of at least three genera: Medicago, Melilotus, and Trigonella. The different nodD genes of R. meliloti may have evolved to optimize the interaction with specific flavones in the root.
Genetics: Honma and Ausubel

exudates of different host plants. Therefore, multiple nodD genes may define one level of host specificity in the interaction of *R. meliloti* with its legume hosts.

We are grateful to P. Boistard, J. Mulligan, S. Long, H. Spaink, R. Okker, and A. Kondorosi for strains, plasmids, and/or communication of results prior to publication. We thank K. Wilson for critical reading of the manuscript and R. Hyde for help in preparation. A. Peterson suggested the titration experiment. Finally, we are indebted to D. Marvel for noticing "those extra bands" on a Southern blot. This work was supported by a grant from Hoechst to Massachusetts General Hospital.