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Physical and Genetic Map of a *Rhizobium meliloti* Nodulation Gene Region and Nucleotide Sequence of *nodC*

THOMAS W. JACOBS, THOMAS T. EGELHOFF, AND SHARON R. LONG*
Department of Biological Sciences, Stanford University, Stanford, California 94305

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Infection of alfalfa by the soil bacterium *Rhizobium meliloti* proceeds by deformation of root hairs and bacterial invasion of host tissue by way of an infection thread. We studied an 8.7-kilobase (kb) segment of the *R. meliloti* megaplasmid, which contains genes required for infection. Site-directed Tn5 mutagenesis was used to examine this fragment for nodulation genes. A total of 81 *R. meliloti* strains with mapped Tn5 insertions in the 8.7-kb fragment were evaluated for nodulation phenotype on alfalfa plants; 39 of the insertions defined a 3.5-kb segment containing nodulation functions. Of these 39 mutants, 37 were completely nodulation deficient (Nod⁻), and 2 at the extreme *nif*-distal end were leaky Nod⁻. Complementation analysis was performed by inoculating plants with strains carrying a genomic Tn5 at one location and a plasmid-borne Tn5 at another location in the 3.5-kb nodulation segment. Mutations near the right border of the fragment behaved as two distinct complementation groups. The segment in which these mutations are located was analyzed by DNA sequencing. Several open reading frames were found in this region, but the one most likely to function is 1,206 bases long, reading from left to right (*nif* distal to proximal) and spanning both mutation groups. The genetic behavior of this segment may be due either to the gene product having two functional domains or to a recombinational hot spot between the apparent complementation groups.

Bacteria in the genus *Rhizobium* interact with the roots of leguminous plants to produce symbiotic nodules. *Rhizobium meliloti* infects and forms nodules on alfalfa (lucerne, *Medicago sativa*) and two related genera, *Melilotus* and *Trigonella*. Nodule development is initiated in the *R. meliloti*-alfalfa symbiosis by bacterium-plant interactions which result in the deformation of root hairs, elaboration of a characteristic invasion structure (the infection thread), and stimulation of cell divisions in the host (12, 18). The biochemical mechanisms underlying this process have been difficult to elucidate, because the events occur transiently in a very small portion of the root cells (3). By inducing mutations in *R. meliloti* and subsequently testing the symbiotic phenotype, we have begun to identify genes controlling the role of the bacterium in the infection process (23, 28). Identification of the bacterial genes and gene products involved in nodulation will provide a valuable tool for elucidating the cellular mechanisms by which nodule formation proceeds.

Bacterial genes required for symbiotic function have been identified through mutation in many species and often have been shown to be located on large indigenous plasmids of various *Rhizobium* species (reviewed in references 5, 13, 22). In *R. trifolii*, *R. leguminosarum*, and *R. meliloti*, at least some nodulation (*nod*) genes are located on such plasmids, linked to the nitrogen fixation (*nif*) loci (2, 15, 20, 23, 33, 37). Tn5 mutagenesis was used to identify a nodulation gene region in *R. meliloti* 1021 (24). This region was then cloned and localized to an 8.7-kilobase (kb) *Eco*RI fragment located some 20 kb 3′ to the *nifHDK* genes (23). In this paper, we present a transposon Tn5 map of the 8.7-kb fragment and a fine-structure analysis of the *nif*-proximal portion of this nodulation gene segment. Although mutations extending over a 0.9-kb portion of the fragment behave as two apparent complementation groups, DNA sequencing indicates that one long open reading frame (*nodC*) is the likely functional gene. The sequences for *nodC* in *R. meliloti* 41 and *R. leguminosarum* were concurrently determined by Torok et al. (33a) and Rossen et al. (34a), respectively, and show a high degree of conservation with *R. meliloti* 1021 *nodC*.

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* Corresponding author.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* HB101 and LE392 have been described previously (25); strain W3350 is F^- *galK2 galT1*. *R. meliloti* 1021 is a streptomycin-resistant (Str^r) derivative of SU47 (28). Nodulation-defective (Nod⁻) derivatives of *R. meliloti* 1021 are shown in Fig. 2. Plasmids pBR325, pRK290, and pLAFR1 have been described previously (7, 14, 17). Vector pWB5a, in which the πvx polylinker (25) is inserted into the *Eco*RI site of pRK290, was constructed and generously donated by W. E. Buikema (Harvard University). Descriptions and sources for bacteriophages MP1 through MP4, N3, 7a, and ϕ11 and for ϕM5 are found in references 26 and 16, respectively.

Bacterial media. Bacteria were maintained on L broth (LB) (31) or TY (4) rich medium and M9-sucrose (29) minimal medium. Bacteria to be infected with phage lambda were grown in YM medium (25). Selective media were supplemented with ampicillin (50 mg/liter), chloramphenicol (50 mg/liter), gentamicin (100 mg/liter), neomycin (50 mg/liter), streptomycin (500 mg/liter), or tetracycline (10 mg/liter), alone or in combination. We added antibiotics to agar-containing media immediately before pouring plates.

DNA manipulations. Plasmid DNA was purified from 5-ml overnight cultures by the alkaline lysis method (6). Large-scale preparations of amplifiable plasmids were carried out by the cleared-lysate procedure (19). Restriction enzyme digests and ligations were performed according to enzyme supplier recommendations (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Bio-Rad Laboratories, Richmond, Calif.). *E. coli* was rendered competent and trans-

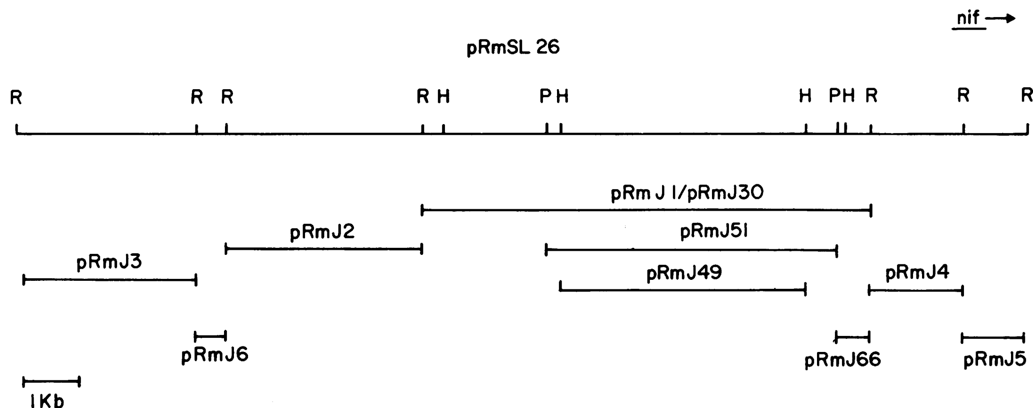

FIG. 1. Subclones derived from pRmSL26. pRmJ1 through pRmJ6 are *Eco*RI fragments cloned into the *Eco*RI site of pBR325. pRmJ30, pRmJ49, pRmJ51, and pRmJ66 were cloned into pRK290 or a derivative, pWB5a, as described in the text. Abbreviations for restriction sites: R, *Eco*RI; H, *Hin*dIII; P, *Pst*I. *Pst*I and *Hin*dIII sites are shown for pRmJ1 only.

formed with plasmid DNA by standard procedures (25). DNA molecules were routinely separated and visualized on 0.7 to 1.5% agarose minigels. When precise fragment sizes were to be determined, gels that were 24 by 13 by 0.5 cm were used. DNA restriction fragments were isolated from agarose minigels for subcloning by electroelution (25) or by a low-melting-point agarose method (11).

Total *Rhizobium* DNA was purified by a modification of standard procedures. The strain used was grown overnight to saturation in 5 ml of selective LB at 30°C, washed once in 10 mM $MgSO_4$, pelleted, frozen at −20°C, and suspended in 5 ml of TES (10 mM Tris [pH 8], 100 mM disodium EDTA, 150 mM NaCl). Lysozyme was added to a concentration of 0.2 mg/ml, and the cells were incubated at 37°C for 1 h. The solution was brought to 3% sodium dodecyl sulfate and incubated for 1 h at 65°C. Buffer-saturated phenol (6 ml) was added, mixed vigorously, and separated from the aqueous phase by centrifugation. Residual phenol was removed by chloroform extractions. DNA was precipitated by the addition of 0.6 volume of isopropanol and suspended in 1 ml of TE (10 mM Tris [pH 8], 1 mM disodium EDTA). The yield was approximately 1 mg.

Subclones of pRmSL26. The individual *Eco*RI fragments of clone pRmSL26 were subcloned from CsCl gradient-purified pRmSL26 DNA. The DNA was digested with *Eco*RI and ligated with pBR325 and then transformed into *E. coli* LE392, selecting for ampicillin resistance (Ap^r) and screening for insertional inactivation of chloramphenicol resistance (Cm^r). Transformation of *E. coli* with 0.7 μg of ligated DNA yielded 6.6×10^5 Ap^r tetracycline resistance (Tc^r) transformants, 15% of which were Cm^s. Clones containing the 8.7-, 3.8-, 3.5-, 1.8-, 1.25-, and 0.6-kb *Eco*RI subfragments of pRmSL26 were identified by rapid plasmid screening of the Ap^r Tc^r Cm^s transformants. These subclones were named pRmJ1 through pRmJ6, respectively. The 8.7-kb *Eco*RI insert of pRmJ1 was also subcloned into pLAFR1 (pRmJ30). Other fragments internal to the 8.7-kb *Eco*RI fragment were cloned into appropriate sites in pWB5a: the 5.5-kb *Pst*I fragment was cloned as pRmJ51; the 4.6-kb *Hin*dIII fragment, as pRmJ49; and the 0.7-kb *Pst*I-*Eco*RI fragment, as pRmJ66 (Fig. 1).

Filter hybridizations. Total bacterial DNA was purified as described above. DNA was digested with restriction enzymes, separated by electrophoresis, transferred to nitrocellulose filters, and hybridized with nick-translated ^{32}P-labeled DNA probes according to published procedures (25). Filters were routinely hybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and washed five times in 2× SSC and 0.5% sodium dodecyl sulfate at 65°C after hybridization.

Transposon Tn*5* mutagenesis. Cloned *R. meliloti* DNA was subjected to Tn*5* mutagenesis with phage NK467 by a modification of the technique of Ruvkun and Ausubel (34). *E. coli* W3350 was transformed with the plasmid to be mutagenized and grown to a density of 4.5×10^8 cells per ml in YM medium. Phage NK467 was added at a multiplicity of infection of 1.0 and the solution was brought to 10 mM $MgSO_4$ and incubated at 30°C for 30 min without shaking. LB (4 volumes) was then added and the culture was shaken at 30°C for 3 to 4 h. The culture was then diluted 10-fold with LB and grown to saturation under neomycin (30 mg/liter) selection. *E. coli* was transformed with plasmids prepared from the saturated culture, and plasmids were analyzed from colonies growing under neomycin-tetracycline selection. Mutations generated by this procedure were mapped and marker exchanged (homogenotized) into the *R. meliloti* genome by the methods described below.

Bacterial genetic techniques. Conjugations of pRK290- or pBR325-based recombinant plasmids were carried out triparentally as previously described with the helper plasmid pRK2013 (14). Conjugation of pPH1JI into *R. meliloti* for homogenotization experiments was performed biparentally (29, 35). Cells to be conjugated were grown in TY medium, mixed together on LB plates, and incubated at 30°C overnight. Cells were scraped from the plates, washed once in 10 mM $MgSO_4$, and plated on selective media. *E. coli* was counterselected with streptomycin (500 mg/liter) or with M9-sucrose minimal medium. Merodiploid *R. meliloti* strains for complementation tests were constructed by conjugating Tn*5*-containing clones (in pRK290, Tc^r) into *R. meliloti* 1021 carrying genomic Tn*5* mutations (streptomycin resistance-neomycin resistance [Sm^r Nm^r]). Nm^r Sm^r Tc^r transconjugants were picked, purified, and tested for symbiotic phenotype.

DNA sequence analysis. DNA sequencing was carried out by both the chemical cleavage (27) and dideoxy termination (1, 36) methods. For the chemical cleavage method, restriction fragments were isolated from pRmJ1 or its derivatives after end labeling with ^{32}P-labeled nucleotides by either T4 polynucleotide kinase or DNA polymerase I (Klenow fragment). *Sal*I-*Sst*I or *Sph*I-*Sst*I genomic fragments and *Hpa*I-*Hin*dIII or *Hpa*I-*Eco*RI Tn*5*-genome junction fragments were

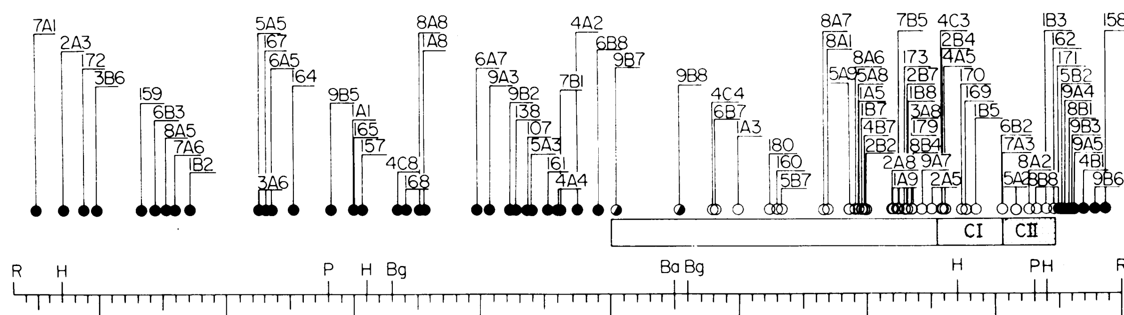

FIG. 2. Map of Tn*5* inserts generated in the 7.8-kb nodulation gene segment contained in pRmJ1 and pRmJ30. Position of insert is shown by a vertical line; the number of the mutation is listed horizontally at the top of the line. Symbols: ●, Nod^+; ○, Nod^-; ◑, $Nod^{+/-}$ (leaky). The divisions on the scale equal 100 bp. Restriction site abbreviations are defined in the legend to Fig. 1 and as follows: Ba, *Bam*HI; Bg, *Bgl*II.

subcloned into phage vectors M13mp11 (30) or M13mp18 (32) for dideoxy termination sequencing with Amersham's 17-base-pair (bp) universal primer and ^{35}S-labeled nucleotides. In some cases, Tn*5*-genome junction restriction fragments were eluted from agarose gels and sequenced directly (41) with a synthetic primer (3′-GTTCATCGCAGGACTTG-5′) complementary to a segment 15 to 32 bases from the end of Tn*5* (T. T. Egelhoff, R. Fisher, T. W. Jacobs, J. Mulligan, and S. R. Long, DNA, in press). Unless otherwise specified above, methods and reagents for dideoxy termination sequencing were obtained from Amersham Corp. (Arlington Heights, Ill.). The sequences of both strands were determined for the entire segment, and all indicated restriction sites were crossed at least once. DNA sequence data were analyzed by the SEQ20 computer program of A. Delaney (University of British Columbia, Vancouver) and by the RE, AA, and OM programs of Richard Pattis (Stanford University) on a Texas Instruments Professional Computer. Hydropathic analysis of open reading frames (ORFs) was carried out with the SUDS program of T. W. Jacobs based on published parameters and algorithms (8, 21).

Symbiotic assays. *R. meliloti* strains carrying site-directed Tn*5* mutations were screened for their nodulation phenotypes on alfalfa seedlings (*M. sativa* cv. AS-13R; Ferry-Morse Co., Mountain View, Calif.). Seeds were sterilized and planted on agar slopes as described previously (28). Bacterial strains were grown to saturation in TY medium, washed once, suspended in 10 mM $MgSO_4$, and added to each tube containing 3-day-old seedlings at a density of 10^9 bacteria per tube. Each strain was tested on at least five plant tubes. Seedlings were observed for nodule formation after 3 to 4 weeks of growth at 28°C under 2,200-lx fluorescent light intensity on a 16-h day, 8-h night cycle. Fåhraeus microscope assays for root hair reaction were carried out as described previously (40).

RESULTS AND DISCUSSION

Mutagenesis of nodulation region. The six *Eco*RI subfragments of *R. meliloti* 1021 DNA from the cosmid clone pRmSL26 were subcloned into pBR325. The narrow (pBR325) and broad (pLAFR1) host-range subclones of the 8.7-kb *Eco*RI nodulation gene fragment were designated pRmJ1 and pRmJ30, respectively. These and other subclones are shown in Fig. 1. The optimal method for site-directed mutagenesis of the 8.7-kb *Eco*RI fragment was as follows. Tn*5* insertions in pRmJ30 were generated and mapped in *E. coli*, and mutated plasmids were conjugally transferred into *R. meliloti* and homogenotized by a method exploiting plasmid incompatibility (34). An alternative approach was used to generate a few of the mutants discussed below. Tn*5* insertions were generated in pRmJ1, conjugally transferred into *R. meliloti*, and homogenotized by plasmid host-range restriction (9). This method was generally inefficient because of background transposition of Tn*5* before homogenotization (data not shown).

A total of 81 Tn*5* insertions were mapped and homogenotized into the 8.7-kb *Eco*RI fragment. Of these mutations, 39 resulted in an altered nodulation phenotype. Transposon Tn*5* insertions throughout the left (*nif*-distal) portion of the 8.7-kb fragment had no effect on nodulation (Fig. 2). In contrast, insertions in a region extending between 0.5 and 4.0 kb from the right (*nif*-proximal) *Eco*RI site produced a Nod^- phenotype. Similar results have been reported in *R. meliloti* 41 (20).

All 37 Tn*5* insertions in the segment between 0.5 and 3.3 kb from the right *Eco*RI site produced a complete loss of nodulation (between insertions 6B7 and 162, inclusive). In the segment immediately *nif* distal to these, near the central *Bgl*II and *Bam*HI sites, two nonnodulating Tn*5* mutants were identified (9B7 and 9B8; Fig. 2) which show a variable Nod^- phenotype: in some trials, no nodules formed on any plants; in others over 50% of the plants developed nodules. This apparently incomplete penetrance may reflect variability in the genotype of the host plants, because alfalfa is an outcrossing species. Alternatively, the gene product(s) of this locus (loci) may be involved in the efficiency of nodule formation or may regulate other *nod* genes. A further possibility is that the Tn*5* insertions in this region may precisely excise at high frequencies, leading to reversion of the phenotype. However, *R. meliloti* 1021, unlike other *Rhizobium* strains, showed no precise excision-reversion of 12 Tn*5*-induced auxotrophic mutants (28; S. R. Long, unpublished data). Rare revertants could stimulate nodule formation while permitting Tn*5*-bearing Nod^- strains to coinvade (24), precluding a definitive test of reversion.

Light microscopic examination showed that none of these *nod* region mutants elicited the characteristic root hair curling response on alfalfa. However, some branching and swelling of root hairs was found on plants inoculated with Nod^- strains whose lesions were in region CII (Fig. 2 and below). This reaction took place only slightly above background level.

Because interactions between the bacterial and plant cell surfaces may be involved in infection, we examined the nodulation mutants for alterations in their surface. No alterations in colony morphology or in growth rate were apparent. To test for one particular class of surface change, six mutants (9B7, 4C4, 5B7, 8B4, and 8A2) were tested as

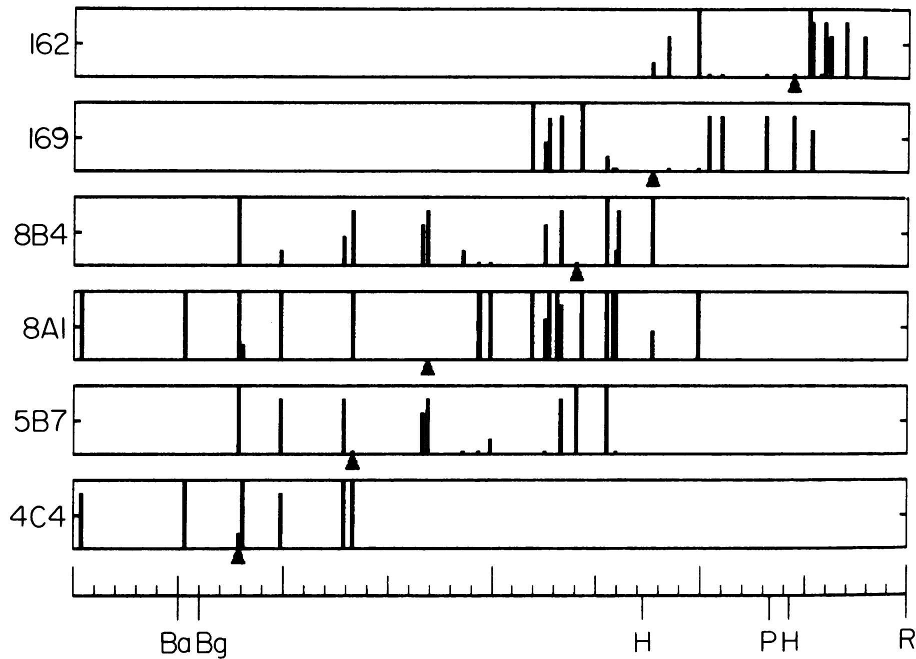

FIG. 3. Complementation analysis of Nod⁻ mutants. Each histogram (horizontal panel) represents a series of experiments in which the recipient strain was the genomic Tn*5* Nod⁻ mutant named at left whose mutation was mapped as indicated (▲) with respect to the restriction map shown at bottom. Positions of vertical bars in each histogram correspond to map locations of pRmJ30::Tn*5* derivatives individually conjugated into the recipient indicated at left. Height of bar represents proportion of inoculated plants which was nodulated by that host-plasmid combination. Full vertical scale equals 100% for each histogram. Shortest bars shown represent 0% nodulation. Abbreviations are as defined in the legends to Fig. 1 and 2.

hosts for eight *R. meliloti* bacteriophages: MP1, MP2, MP3, MP4, 7a, N3, and ϕ11 (26) and ϕM5 (16). All of these except ϕ11 infect the wild-type *R. meliloti* parent strain 1021. Spot tests with lysates of these phages on bacterial lawns showed that all of the mutants were unchanged in their sensitivity to infection by the seven phages which infect strain 1021, and none had become a host for ϕ11.

Genetic analysis of *nod* genes in the 8.7-kb fragment. We performed complementation analysis to examine the organization of the *nod* genes in the 8.7-kb fragment. Subclones of wild-type DNA and Tn*5*-mutated pRmJ30 derivatives were introduced into particular Nod⁻ mutant strains carrying a mapped genomic Tn*5* insertion. Each merodiploid strain was inoculated into five tubes containing one or two plants each. Nodulation phenotype was scored at 3 to 4 weeks after inoculation.

Mutations mapping at the *nif*-proximal (right) end of the 8.7-kb fragment appeared to fall into two groups. The rightmost group, CII, was defined by six mutants (7A3, 5A2, 8A2, 1B3, and 162). The mutants originally used to identify and clone this nodulation segment, strains 1027 and 1126 (23, 24), by both physical and genetic criteria are included in this group. Mutant 162 is restored to Nod⁺ by clones with Tn*5* insertions to the left of 7A3 and to the right of 162 but not by clones with Tn*5* insertions within CII or by pRmJ51, indicating a gene which spans the segment shown in Fig. 3.

Immediately *nif* distal to CII is group CI, defined by six mutants (2A5, 4C3, 2B4, 169, 1B5, and 6B2). Mutant 169 is restored to Nod⁺ by pRmJ30 derivatives containing insertions to the right of 6B2 and to the left of 8B4; some insertions located at the left, such as 9A7 and 2A5, show inconsistent Nod⁺ restoration of mutant 169. Group 1 mutants are not restored to Nod⁺ by pRmJ49 or pRmJ66. Apparent complementation between mutants in groups 1 and 2 might indicate either two genuine complementation groups or the presence of a small defined region in which elevated levels of recombination occur.

Similar pair tests of mutations mapping approximately 1.5 to 3.2 kb from the right end of the 8.7-kb fragment yielded inconsistent results. Restoration of nodulation occurred in most combinations of Tn*5* mutant pairs (Fig. 3). Recombination between the genomic and plasmid-borne genes can regenerate wild-type copies of the gene, yielding false-positive results in these *trans*-complementation tests. Nodulation would be a strong selection for such a recombinant; wild-type cells which are outnumbered by Nod⁻ mutants 10^4-fold still form nodules (23), and such nodules may

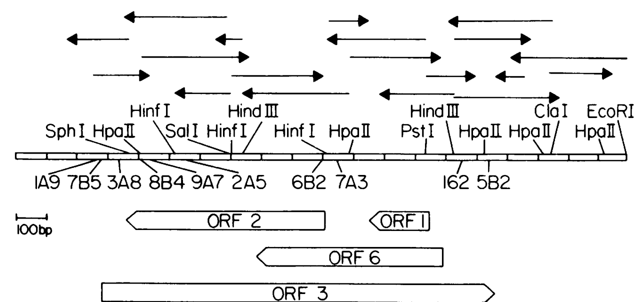

FIG. 4. Strategy used and results obtained in sequencing CI-CII region. Upper arrows represent separate sequence determinations combined to produce the result shown in Fig. 5. Restriction sites are shown above the scale, and sequenced Tn*5* mutations are shown below. Arrows at bottom represent ORFs in the sequence.

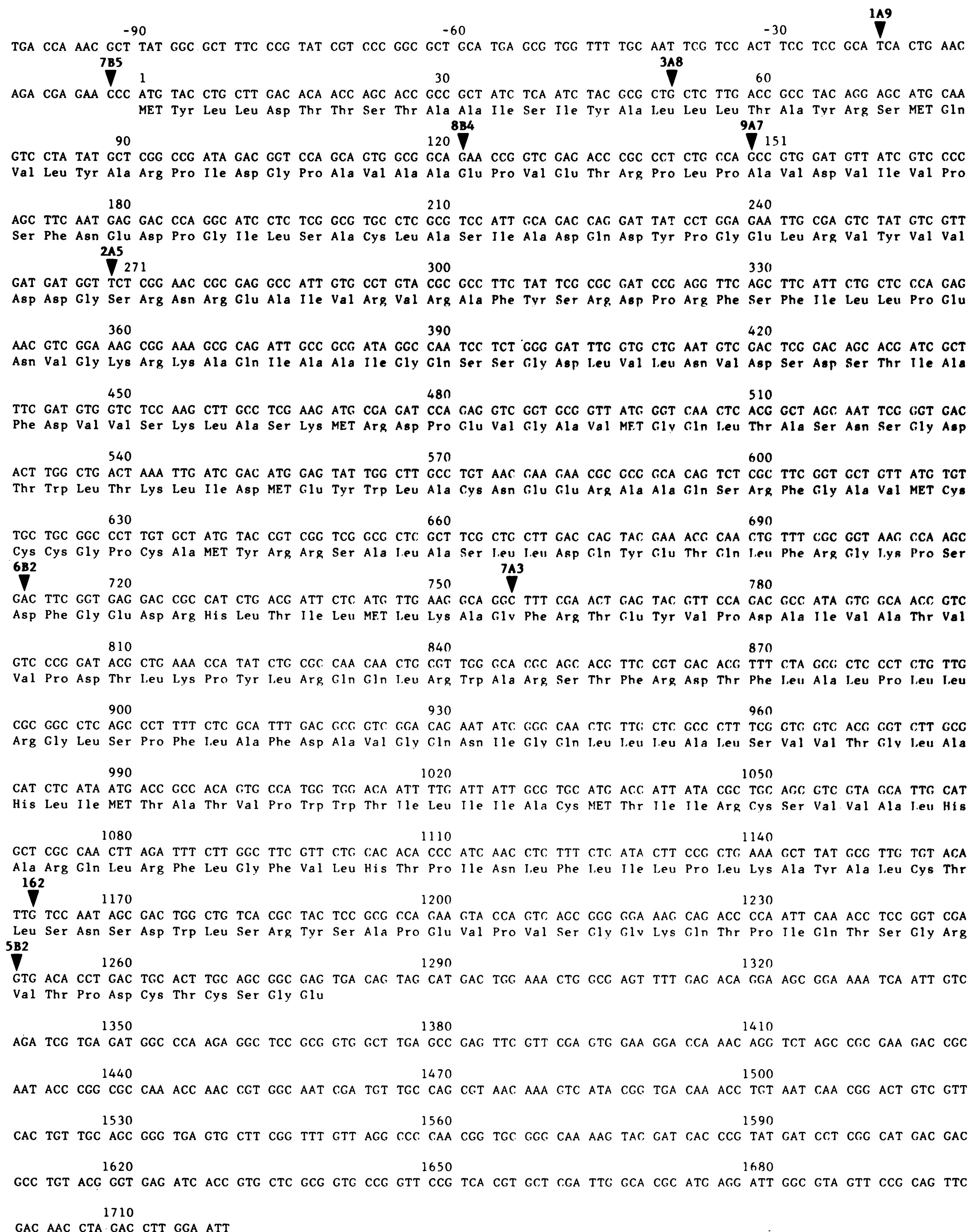

FIG. 5. Nucleotide sequence of CI-CII segment with translation of ORF 3. Positions of Tn5 insertions are shown at left end of the 9-bp genomic repeat generated by Tn5 transposition.

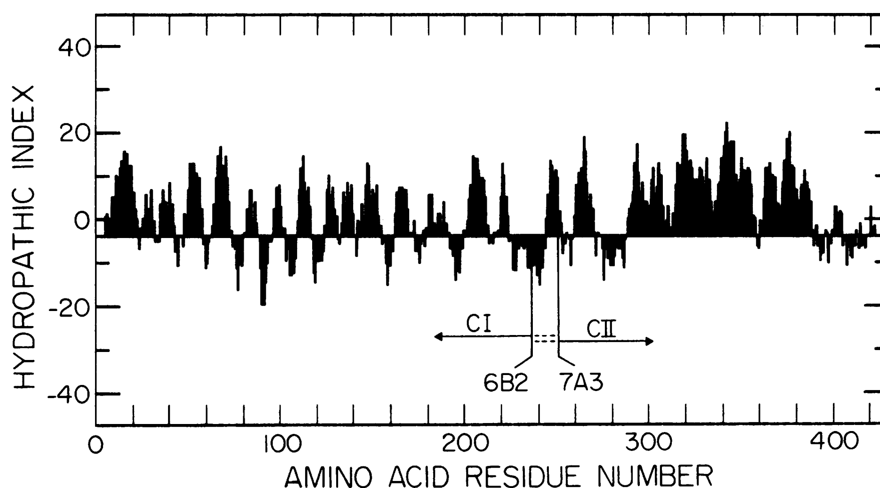

FIG. 6. Hydropathy analysis of ORF 3 translation product. Algorithm and parameters were taken from Kyte and Doolittle (21), using span = 7. Positive hydropathic indices are hydrophobic, negative values are hydrophilic. The protein is 426 amino acids long with a predicted molecular size of 46,715 daltons. The calculated Grease Index (21) is + 0.22.

contain mixtures of the Nod$^-$ and wild-type bacteria. In the case of complementation tests with pairs of mutants with mutations toward the middle of the 8.7-kb fragment, the lengthy homologous sequences flanking the transposons on both sides may permit elevated levels of recombination. Thus, our results indicate that for *nod* genes, unlike *Rhizobium nif* loci (35), paired mutant combinations in a wild-type (i.e., recombination-proficient) background may not generally suffice for complementation tests. Definitive complementation tests should be performed in a recombination-deficient background. Bacteria should be recovered from nodules formed by complementing mutant pairs and their genotypes analyzed to confirm that they have maintained the merodiploid state.

DNA sequence of the CI-CII region. We determined the DNA sequence of the CI-CII region to understand better its apparent genetic organization. The DNA sequencing strategy is described above and shown in Fig. 4. The sequence has three ORFs which read from right to left (ORFs 1, 2, and 6) of 65, 160, and 270 amino acids, respectively. ORFs 2 and 1 appeared to correspond to groups CI and CII, respectively. There is also a longer ORF (ORF 3, 426 amino acids) proceeding from left to right (Fig. 4). RNA studies of this region have shown little or no transcription in either direction (T. Egelhoff, unpublished observations), so we were unable to choose the functional ORF by correlation with biochemical data. However, we suspected that a DNA-sequence determination of the insertion positions of the Tn*5* mutants used in the above complementation tests would eliminate some of the possible ORFs.

Tn*5*-genome junction sequence analysis (Fig. 5) revealed that the mutants which define the CI-CII boundary (6B2 and 7A3, respectively) are 46 bp apart. This boundary does not correlate with the boundary between ORFs 1 and 2. Neither does ORF 6 correlate with a boundary of either complementation group. However, ORF 3 spans the segment including all of the mutants used to define both groups, with the exception of the Nod$^+$ strain 5B2, in which the transposon is located 30 bp upstream from the stop codon of the ORF. This insertion results in an ORF 3-Tn*5* fusion in which the carboxy terminus of ORF 3 (-Val-Thr-Pro-Asp-Lys-Thr-Cys-Ser-Gly-Glu-Stop) is replaced by the sequence -Leu-Thr-Leu-Ile-His-Lys-Stop, in which one amino acid is unchanged and the local charge character of the peptide might be sufficiently conserved as to retain function.

On the basis of the lengths of the ORFs and the relative positions of the Tn*5* insertions, ORF 3 is the most likely candidate for a functioning gene in this region. By hybridization analysis (20) and sequencing (33a, 34a), this ORF appears to be highly conserved in *R. meliloti* 41 (99% amino acid homology) and *R. leguminosarum* 248 (71% amino acid homology). By consensus and comparison, it has been designated *nodC*. It should be noted that reading frames which proceed in the opposite direction exist in the strain 41 and *R. leguminosarum* sequences as well, and the functionality of *nodC* remains to be proven. There are multiple methionine codons in the N-terminal part of ORF 3, and the translation start site will need to be determined by analysis of the in vivo gene product. Translation initiation at the first methionine (Fig. 5, base 1) would yield an amino terminus with signal peptide character (42), although the presence of a negatively charged amino acid differs from the typical procaryotic signal sequences studied so far (38). A consensus sequence for *Rhizobium* protein leader peptides is not yet available.

If the observed biological effects are in fact the result of mutations in the one putative gene (*nodC*), then the behavior of mutants as two groups remains unexplained. If the restoration to a Nod$^+$ phenotype is occurring by marker rescue rather than by complementation, then recombination would necessarily be occurring at a significantly higher rate within the 46 bp between 6B2 and 7A3 than in the 400- to 500-bp segments defined by either of the groups. Thus, this intervening segment might contain a recombinational hot spot. An examination of the sequence shows no Chi-site (GCTGGTGG) (39) or its complement; however, it is possible that other sequences display high recombinational activity in *Rhizobium* species.

Alternatively, the N-terminal and C-terminal domains of the *nodC* protein might reconstitute an active molecule if produced separately. Tn*5* is known to produce some nonpolar mutations in *R. meliloti* (10), and there are multiple methionines in ORF 3 at which translational reinitiation might occur. Therefore, it is possible that the genomic and plasmidborne components of our merodiploid constructs each produce partial gene products. Such intracistronic

complementation would imply that *nodC* should display two functional domains. Indeed, the amino acid sequence of *nodC* reveals two domains of contrasting hydropathy (8, 21), the C-terminal half being more hydrophobic than the N-terminal half (Fig. 6). That two sections of the *nodC* locus may behave independently is consistent with the finding that CI and CII mutants show differing apparent complementation of *R. trifolii* Nod⁻ mutants (R. Fisher, J. Tu, and S. R. Long, submitted for publication).

To distinguish between the possibilities of recombination and of intragenic complementation and to relate the function boundaries for this nodulation gene to the sequence, it will be necessary to carry out complementation tests in a recombination-deficient background. Fine-structure genetic analysis should provide an entry to studying the function of individual *Rhizobium* genes in plant infection.

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