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Genes Essential for Nod Factor Production and Nodulation Are Located on a Symbiotic Amplicon (AMP*Rtr*CFN299pc60) in *Rhizobium tropici*

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Amplifiable DNA regions (amplicons) have been identified in the genome of *Rhizobium etli*. Here we report the isolation and molecular characterization of a symbiotic amplicon of *Rhizobium tropici*. To search for symbiotic amplicons, a cartridge containing a kanamycin resistance marker that responds to gene dosage and conditional origins of replication and transfer was inserted in the nodulation region of the symbiotic plasmid (pSym) of *R. tropici* CFN299. Derivatives harboring amplifications were selected by increasing the concentration of kanamycin in the cell culture. The amplified DNA region was mobilized into *Escherichia coli* and then into *Agrobacterium tumefaciens*. The 60-kb symbiotic amplicon, which we termed AMP*Rtr*CFN299pc60, contains several nodulation and nitrogen fixation genes and is flanked by a novel insertion sequence IS*Rtr1*. Amplification of AMP*Rtr*CFN299pc60 through homologous recombination between IS*Rtr1* repeats increased the amount of Nod factors. Strikingly, the conjugal transfer of the amplicon into a plasmidless *A. tumefaciens* strain confers on the transconjugant the ability to produce *R. tropici* Nod factors and to nodulate *Phaseolus vulgaris*, indicating that *R. tropici* genes essential for the nodulation process are confined to an ampliable DNA region of the pSym.

Bacteria belonging to *Rhizobium* and related genera are able to establish a symbiosis with leguminous plants which culminates in the formation of nitrogen-fixing nodules. Nodule formation is the result of a cellular differentiation process mediated by molecular signal exchange which involves the expression of specific genes in both partners. In response to plant flavonoid compounds, the bacteria produce a family of lipo-chito-oligosaccharide molecules, called nodulation (Nod) factors, which in turn elicit nodule development on roots or stems of the legumes (5, 30). *Rhizobium* nodulation (*nod*, *nol*, and *noe*) genes involved in the production of Nod factors are located primarily on large plasmids, called symbiotic plasmids or pSyms (3, 28).

In *Rhizobium*, both plasmid and chromosome replicons contain a large amount of reiterated DNA sequences (9). These repeated DNA sequences include complete operons, specific genes, regulatory sequences, and insertion sequence (IS) elements (27). The recently reported sequence of the pSym of *Rhizobium* sp. strain NGR234 revealed that most of the repeated sequences correspond to IS-type elements (11). We have shown that repeated sequences participate in recombination events leading to genomic rearrangements. We have used the term "amplicon" to denote a DNA region, bordered by direct repeated sequences, that has the potential to be amplified. The first *Rhizobium* amplicon was identified in *Rhizobium etli* CFN42 and consists of a 120-kb DNA region bordered by *nifHDK* repeats (25). Subsequently, other amplicons have been identified either on the chromosome or on the pSym or other plasmids of *R. etli* (10, 26). Amplification may also be induced potentially in any region of the genome by genetic manipulations that generate amplicon-type structures. We have recently applied a random DNA amplification strategy to the pSym of *Rhizobium tropici* to generate strains with improved symbiotic properties (20).

Natural DNA amplification, generated by repeated sequences present in the genome, occurs at high frequency under laboratory conditions (10, 25, 26). Little, however, is known about its consequences in the *Rhizobium*-legume symbiosis. In this study, we demonstrate the presence of natural DNA amplification in the symbiotic region of the pSym of *R. tropici* CFN299. A symbiotic amplicon, which we call AMP*Rtr*CFN299pc60, was isolated and characterized. Since mobilization of this amplicon into *Agrobacterium tumefaciens* enables the transconjugant to produce *R. tropici* Nod factors and to form nodules on *Phaseolus vulgaris*, we conclude that the genes essential for nodulation are confined to an amplicon structure.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown on Luria-Bertani medium (1% peptone, 0.5% yeast extract, 1% NaCl) supplemented with the following antibiotics at the indicated concentrations (micrograms per milliliter): ampicillin, 100; chloramphenicol, 15; gentamicin, 25; kanamycin, 50; nalidixic acid, 20; and tetracycline, 10. *Rhizobium* and *Agrobacterium* strains were cultivated at 30°C on PY medium (0.5% peptone, 0.3% yeast extract, 10 mM $CaCl_2$) with the following antibiotics, at the indicated concentrations (micrograms per milliliter), when required: chloramphenicol, 30; kanamycin, 30 to 300; nalidixic acid, 20; neomycin, 60; and rifampin, 100. Triparental matings, using *E. coli* 1830/pJB3JI as a helper, were performed as described previously (20).

DNA manipulations. Standard techniques such as isolation of genomic DNA, plasmid purification, DNA cloning and restriction, agarose gel electrophoresis, DNA labelling, and filter blot hybridization were performed as described previously (9, 20). Plasmid profiles were obtained by using a modified Eckhardt technique (6) as described by Hynes and McGregor (14). A genomic library of *R.*

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
R. tropici		
CFN299	Wild-type strain isolated from bean	19
CFNX302	*nodP*::*GDYN3* Km^r	This study
CFNX303	Amplified derivative, Km^r	This study
CFNX304	Amplified derivative, Km^r	This study
A. tumefaciens		
GMI9023	Plasmidless, Rif^r	29
CFNA303	GMI9023, pEYM5/AMP*Rtr*CFN299pc60	This study
CFNA304	GMI9023, pEYM5/AMP*Rtr*CFN299pc60	This study
CFNA305	GMI9023, pSym::Tn*5* of CFN299	This study
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 *endA1 recA1 hsd*R17 (r_K^- m_K^+) *thi-1 supE44* λ^- *gyrA96*	Gibco BRL
HB101	F^- *mcrB mrr hsdS20* (r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 glK2 xyl-5 rspL20* (Sm^r) *supE44* λ^-	Gibco BRL
S17-1	Tp^r294 *recA*, chromosomally integrated RP4 derivative	34
CFNC303	DH5α, pEYM5/AMP*Rtr*CFN299pc60	This study
CFNC304	DH5α, pEYM5/AMP*Rtr*CFN299pc60	This study
Plasmids		
pUC19	Sequencing vector, Ap^r	21
pIC20-R	Cloning vector, Ap^r	18
pJQ200sk	*B. subtilis sacB* containing suicide vector, Gm^r	23
pSUP205	Cosmid vector, Tc^r	34
pJB3JI	*R68*::*45* tra^+ IncP, Tc^r Ap^r Km^s	4
pPAM37	pJQ200sk containing *nodHPQ* of *R. tropici* CFN299 with *nodP*::*GDYN3*	This study

tropici CFN299 was made by cloning partially *Eco*RI-digested total DNA into the cosmid vector pSUP205. DNA amplification was quantified by densitometric integration of the hybridization signals from autoradiographs, using the Eagle Eye II system (Stratagene, La Jolla, Calif.).

Isolation of *R. tropici* strains carrying an amplification in the pSym. For this purpose, a 2.2-kb *Pst*I-*Sal*I DNA fragment containing the *nodHPQ* genes of *R. tropici* strain CFN299 cloned into pJQ200sk was used. A cartridge constituted by a 6.5-kb *Bgl*II-*Pst*I DNA fragment from plasmid pTn*5*luxB (15a) was ligated into pIC20R and cut out by *Bgl*II-*Bam*HI digestion, giving rise to GDYN3. GDYN3 contains a gene encoding kanamycin resistance (*Km*) from Tn*903* as well as conditional origins for conjugal transfer (*oriT*) and replication (*oriV*) of the broad-host-range plasmid RK2 (8). GDYN3 (*Km-oriVT*) was then inserted into the unique *Bam*HI site of the *nodP* gene. The resulting recombinant plasmid, designated pPAM37, was transformed into *E. coli* S17-1 and from there transferred into *R. tropici* wild-type strain CFN299 by biparental mating. Double recombinants of *R. tropici* carrying GDYN3 in the pSym were selected on PY medium containing nalidixic acid 5% sucrose, and 30 μg of kanamycin per ml. The site of GDYN3 insertion was ascertained by Southern hybridization (see Results). One of these *R. tropici* exconjugants was named CFNX302. To isolate *R. tropici* derivatives carrying amplifications, strain CFNX302 was grown at 30°C in PY broth medium to log phase. Serial dilutions were prepared in a solution containing 10 mM $MgSO_4$ and 0.01% (vol/vol) Tween 40. Appropriate dilutions were plated on PY agar plates supplemented with 50, 100, 150, 200, 250, and 300 μg of kanamycin per ml. After incubation at 30°C for 4 days, small and large colonies were obtained. Analysis of plasmid profiles showed that only the larger colonies (which appeared at a frequency of 10^{-4}) exhibited amplification in the pSym (see Results). Two of the clones carrying amplifications were isolated on 150 μg of kanamycin per ml and designated CFNX303 and CFNX304.

Mobilization of *Rhizobium* symbiotic amplicons into *E. coli* and *A. tumefaciens*. *E. coli* DH5α and *A. tumefaciens* GMI9023 containing plasmid pEYM5 were used as recipients. Plasmid pEYM5, which carries a helper function (*trfA*) of replication at *oriV* (35a), was introduced into the strains by biparental mating with S17-1/pEYM5 as a donor. *Rhizobium* amplicons were first mobilized into *E. coli* DH5α/pEYM5 by using *Rhizobium* amplified derivatives CFNX303 and CFNX304 as donors in triparental matings. *E. coli* exconjugants containing *Rhizobium* amplicons as stable plasmids were selected on LB agar plates supplemented with nalidix acid, chloramphenicol, and kanamycin. The plasmids isolated from 60 *E. coli* exconjugants, 30 clones per mating, had identical restriction patterns. Two of these *E. coli* exconjugants, CFNC303 and CFNC304, were used as donors to transfer the *Rhizobium* amplicon into *A. tumefaciens* GMI9023/pEYM5 by triparental mating. *A. tumefaciens* exconjugants bearing *Rhizobium* amplicons were selected on PY agar plates containing chloramphenicol, neomycin, and rifampin. Two of these *A. tumefaciens* exconjugants were designated CFNA303 and CFNA304.

Cloning and sequencing of endpoints of the amplicon. The smaller 2.5-kb *Eco*RI fragment that borders the amplicon was purified from agarose gels by using a Nucleotrap kit (Macherey-Nagel, Düren, Germany) and cloned into pUC19. The nucleotide sequence of the cloned fragment was determined with a ALF DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). Sequencing reactions were performed with alkaline-denaturated double-stranded DNA as a template. Oligonucleotides were designed to complete the nucleotide sequence determination of both DNA strands. Sequences were analyzed using the GCG (version 8.0.1-UNIX 1994; University of Wisconsin, Madison) and PCGENE (Intelligenetics, Mountain View, Calif.) software packages. Sequence comparisons were done by using the BLAST program (1).

TLC and HPLC analysis of Nod factors. Nod metabolites for thin-lay chromatography (TLC) and high-pressure liquid chromatography (HPLC) analysis were obtained as described previously (16). Briefly, 1 ml (TLC) or 10 ml (HPLC) of liquid minimal medium (22) was inoculated with fresh overnight cell cultures to an optical density at 600 nm of 0.1. For *A. tumefaciens*, 0.1% (wt/vol) Casamino Acids was added to the medium (pH 7.0). When required, apigenin (1.2 μmol per liter) was added as a *nod* gene inducer. Nod metabolites were radiolabeled with 2 μCi of [^{35}S]sulfate or 0.1 μCi of D-[U-^{14}C]glucosamine. After 12 h of growth, *Rhizobium* or *Agrobacterium* cell supernatants were passed through a C_{18} Sep-Pak cartridge (Waters Millipore, Milford, Mass.), and hydrophilic molecules were washed out with 10 ml of autoclaved deionized water. Nod metabolites were then eluted with 3 ml of methanol and dried under N_2 at 35°C. For TLC, Nod metabolite fractions were loaded onto reverse-phase TLC plates (RP-18 F_{254s}; Merck, Darmstadt, Germany). Migration was performed with methanol-ammonia (5.5 N; 9:1, vol/vol) as the mobile phase. Radiolabeled compounds were revealed by autoradiography on Hyperfilm-ßmax (Amersham). For HPLC, samples were applied to a reverse-phase C_{18} cartridge (LiChropher 100 RP-18, 5 μm; Merck), and Nod metabolites were eluted with the gradient described previously (22). The eluate was monitored at 220 nm, and analysis was performed by comparison with elution times of purified sulfated (10 min) and nonsulfated (14 min) Nod factors.

Plant nodulation assays. Plant nodulation assays were performed as described previously (20). Seeds of *P. vulgaris* cultivars Negro Jamapa, N-8-116, and BAT-477 were surface disinfected and germinated on water agar plates. Germinated seedlings were transferred to 250-ml Erlenmeyer flasks containing Fahraeus (7) medium with vermiculite; 5×10^5 *Rhizobium* or *Agrobacterium* cells were used to inoculate plant roots. The identity of nodule isolates was ascertained by antibiotic resistance and confirmed when necessary by Southern blot hybridization analysis of plasmid profiles.

Light and electron microscopy. Microscopy of nodules was performed as described previously (36). Nodules were harvested from bean seedlings 21 days after inoculation and fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer. Segments were washed in buffer, postfixed for 2 h at 4°C in 1% osmium

tetroxide, dehydrated in ethanol, and embedded in Epon resin or in ore. Light microscopy was performed with thick sections (about 1 μm) stained with 0.05% toluidine blue. Scanning microscopy was performed at 15 kV with a JEOL 5410 LB microscope. For electron microscopy, ultrathin sections (about 80 nm) were stained in aqueous 2% uranyl acetate and observed at 60 kV with a JEOL 1200 EX2 electron microscope.

Nucleotide sequence accession number. The nucleotide sequence of IS*Rtr1* is under GenBank accession no. AF041379.

RESULTS

Experimental strategy to search for *Rhizobium* symbiotic amplicons. The genetic element GDYN3 was constructed (see Materials and Methods), and a recombinant plasmid (pPAM37) containing the *nodHPQ* genes interrupted in *nodP* by the GDYN3 element was introduced into *R. tropici* by conjugation from *E. coli*. Double recombinants of *R. tropici* bearing the *nodP*::*GDYN3* allele in the homologous region of the pSym were selected (Fig. 1A). The site of the insertion of GDYN3 was ascertained by hybridization of Southern blots of total DNA digested with *Eco*RI from recombinant strains (not shown). Recombination between direct repeats flanking the region containing GDYN3 leads to a tandem duplication of a whole amplicon structure. The tandemly duplicated region may serve as a substrate for recombination, leading to further amplification or deletion (Fig. 1B). *R. tropici* derivatives carrying amplifications were selected by increasing the kanamycin concentration in the culture medium. Amplification was ascertained by the analysis of plasmid profiles (see below).

When deletion events occur in amplified regions, closed circular structures containing the whole amplified region in either a monomeric or multimeric form are generated. The presence of *oriT* and *oriV* on the excised amplicons provides the possibility for their isolation. To do this, *R. tropici* amplified derivatives were used as donors using *E. coli* containing plasmid pEYM5 as the recipient (Fig. 1C). This IncQ plasmid has a broad host range and harbors the *trfA* gene, which encodes a transactivator of *oriV*. Exconjugants of *E. coli* were selected, and all harbored a plasmid of approximately 66 kb containing *Rhizobium* sequences (see below). Amplicons were then mobilized from *E. coli* exconjugants into *A. tumefaciens* GMI9023 carrying plasmid pEYM5 (Fig. 1D). *A. tumefaciens* exconjugants bearing *Rhizobium* amplicons were selected. The presence of *Rhizobium* sequences in the *A. tumefaciens* exconjugants was confirmed (see below).

All steps of the experimental strategy (gene replacement to introduce the GDYN3 element, amplification in *Rhizobium*, in vivo cloning of the amplicon sequence in *E. coli*, and its transfer to *A. tumefaciens*) were monitored by the analysis of plasmid profiles of the different strains involved (Fig. 2). Wild-type *R. tropici* (CFN299) harbors four plasmids (pa, pb, pc, and pd). Plasmid pc of 490 kb is the pSym. As expected, the pSym pattern of amplified derivatives shows bands of larger size. Hybridization of plasmid profiles with a *nodHPQ* probe confirmed the presence of monomers, dimers, and trimers of closed circular DNA containing amplicon sequences in amplified derivatives. *E. coli* (CFNC303 and CFNC304) and *A. tumefaciens* (CFNA303 and CFNA304) transconjugants contained a plasmid of approximately 66 kb that hybridized with the *R. tropici nodHPQ* probe.

Characterization of the *R. tropici* symbiotic amplicon. The amplicon identified was named AMP*Rtr*CFN299pc60 (see Discussion). *nodHPQ* genes and the amplicon DNA sequence, represented by plasmid pCFNC303 (isolated from *E. coli* CFNC303), were used to hybridize against colonies from a genomic library of total *R. tropici* DNA made in pSUP205. Several overlapping cosmids were isolated. The relative positions of five overlapping cosmids covering the whole amplicon are shown in Fig. 3a. Localization of the amplicon sequences in the cosmid contig was performed by hybridization of AMP*Rtr*CFN299pc60 against the ordered cosmids digested with *Eco*RI (not shown). Comparative hybridization analysis of total DNA digested with *Eco*RI of different strains (the wild-type strain, the derivative containing GDYN3, the amplified strains, as well as *E. coli* and *A. tumefaciens* bearing amplicons)

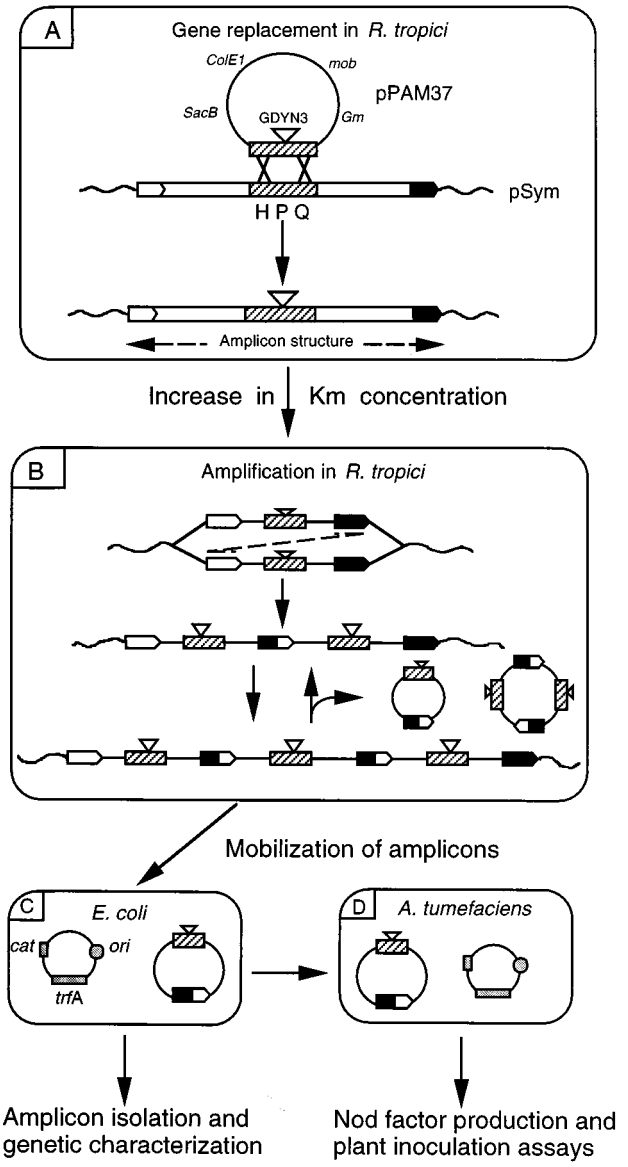

FIG. 1. Experimental approach to identify *Rhizobium* symbiotic amplicons. (A) Plasmid pPAM37, containing the subcloned *nodHPQ* genes with the GDYN3 insertion in *nodP*, was introduced via conjugation into the wild-type *R. tropici* strain CFN299. When a double-crossover event occurred between the two copies of *nodHPQ*, *Rhizobium* exconjugants bearing the *nodP*::*GDYN3* allele in the pSym were selected by resistance to kanamycin and sucrose. To select for amplification, the *Rhizobium* exconjugant was grown in higher kanamycin concentrations. (B) Recombination between the direct repeats leads to duplication and further amplification (see text). Closed circular structures containing amplicon DNA sequences are released during the amplification process (see text). These structures were mobilized into *E. coli* (C) and then into *A. tumefaciens* (D) harboring plasmid pEYM5 with a transactivator *trfA* gene of *oriV*. *Gm*, gentamicin resistance gene; ▭, ▬, direct repeats; ∼, *Rhizobium* sequences outside the amplicon; *cat*, chloramphenicol resistance gene.

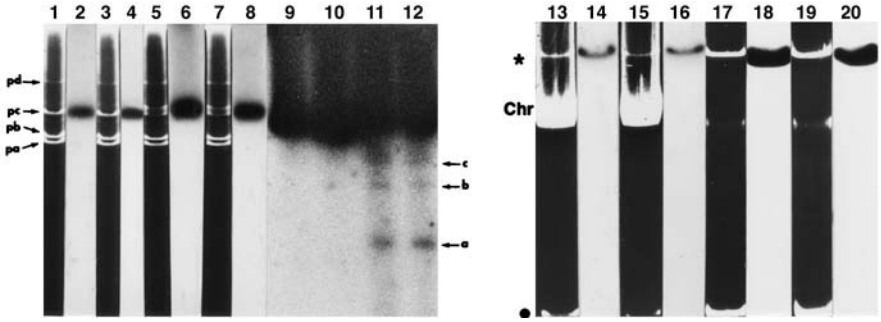

FIG. 2. Plasmid profiles of *R. tropici*, *A. tumefaciens*, and *E. coli* strains, stained with ethidium bromide (odd-numbered lanes) or hybridized against a *nodHPQ* probe (even-numbered lanes). Strains used were *R. tropici* CFN299 (wild type) (lanes 1 and 2), *R. tropici* CFNX302 containing the GDYN3 element interrupting the *nodP* gene (lanes 3 and 4), *R. tropici* amplified derivative CFNX303 (lanes 5 and 6), and *R. tropici* amplified derivative CFNX304 (lanes 7 and 8) (lanes 9, 10, 11, and 12 are overexposures of the corresponding autoradiographs of lanes 2, 4, 6, and 8), *A. tumefaciens* CFNA303 bearing AMP*Rtr*CFN299pc60 (lanes 13 and 14), *A. tumefaciens* CFNA304 bearing AMP*Rtr*CFN299pc60 (lanes 15 and 16), *E. coli* CFNC303 bearing AMP*Rtr*CFN299pc60 (lanes 17 and 18), and *E. coli* CFNC304 bearing AMP*Rtr*CFN299pc60 (lanes 19 and 20). pa (185 kb), pb (220 kb), pc (490 kb), and pd (>1,000 kb) are plasmids. a, b, and c, closed circular DNA; ∗, AMP*Rtr*CFN299pc60; Chr, chromosome DNA; ●, plasmid pEYM5.

against the DNA from AMP*Rtr*CFN299pc60 (not shown) allowed the construction of the physical map of the amplicon (Fig. 3). In the wild-type strain, seven regions were defined (Fig. 3b). Regions A and Z contain the borders of the amplicon. The inner regions were named B, C, D, E, and F. Regions A, C, D, E, and Z correspond to single *Eco*RI fragments. Regions B and F contain two and three *Eco*RI sites, respectively, that were not mapped between them. As expected, in

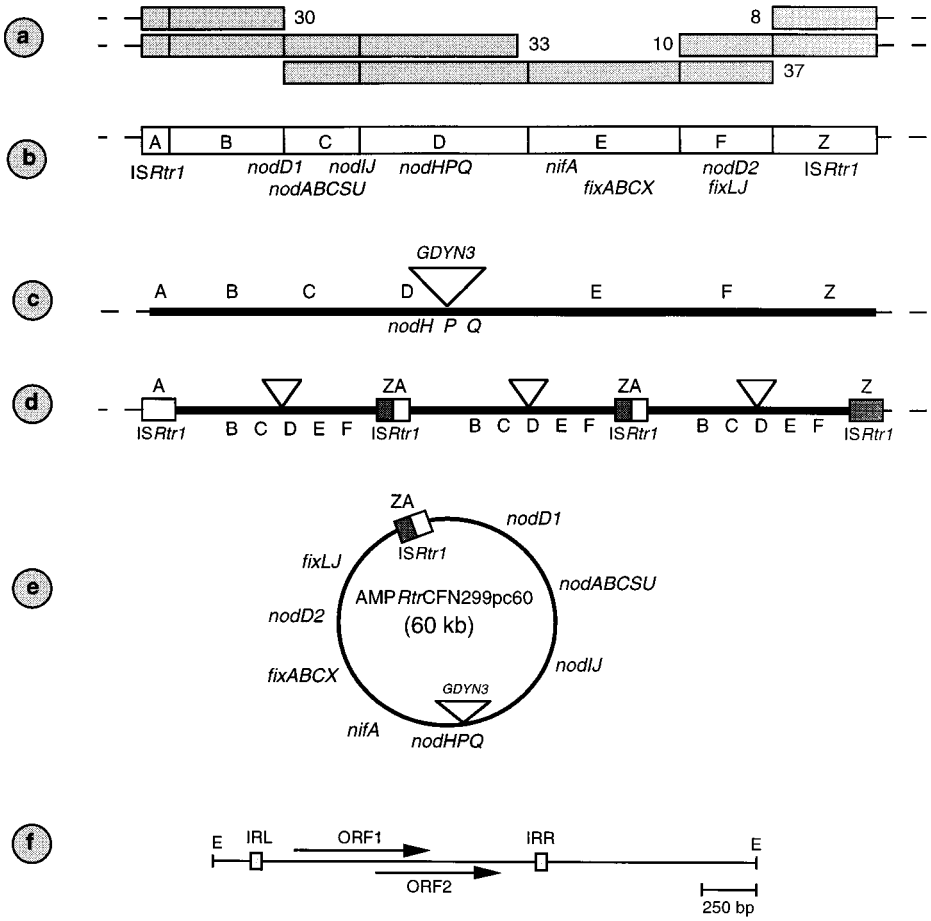

FIG. 3. Characterization of AMP*Rtr*CFN299pc60. (a) Overlapping cosmids (indicated by numbers) containing amplicon sequences. (b) Map of amplicon region in wild-type CFN299 showing the locations of different genes. (c) Schematic representation of strain CFNX302 showing the position of the GDYN3 insertion. (d) Schematic representation of the amplified state. (e) Schematic representation of the AMP*Rtr*CFN299pc60 sequence present as a stable plasmid in *E. coli* or *A. tumefaciens*. Rectangles in panels a and b show the different regions (indicated by letters) mentioned in Results; triangles in panels c to e indicate the GDYN3 element; ZA in panels d and e indicates the region generated by recombination between the amplicon borders represented by the insertion sequence IS*Rtr1* (f). *nif* and *fix*, nitrogen fixation genes; *nod*, nodulation genes; E, *Eco*RI; IRL and IRR, left and right 28-bp inverted repeats; ORF, open reading frame.

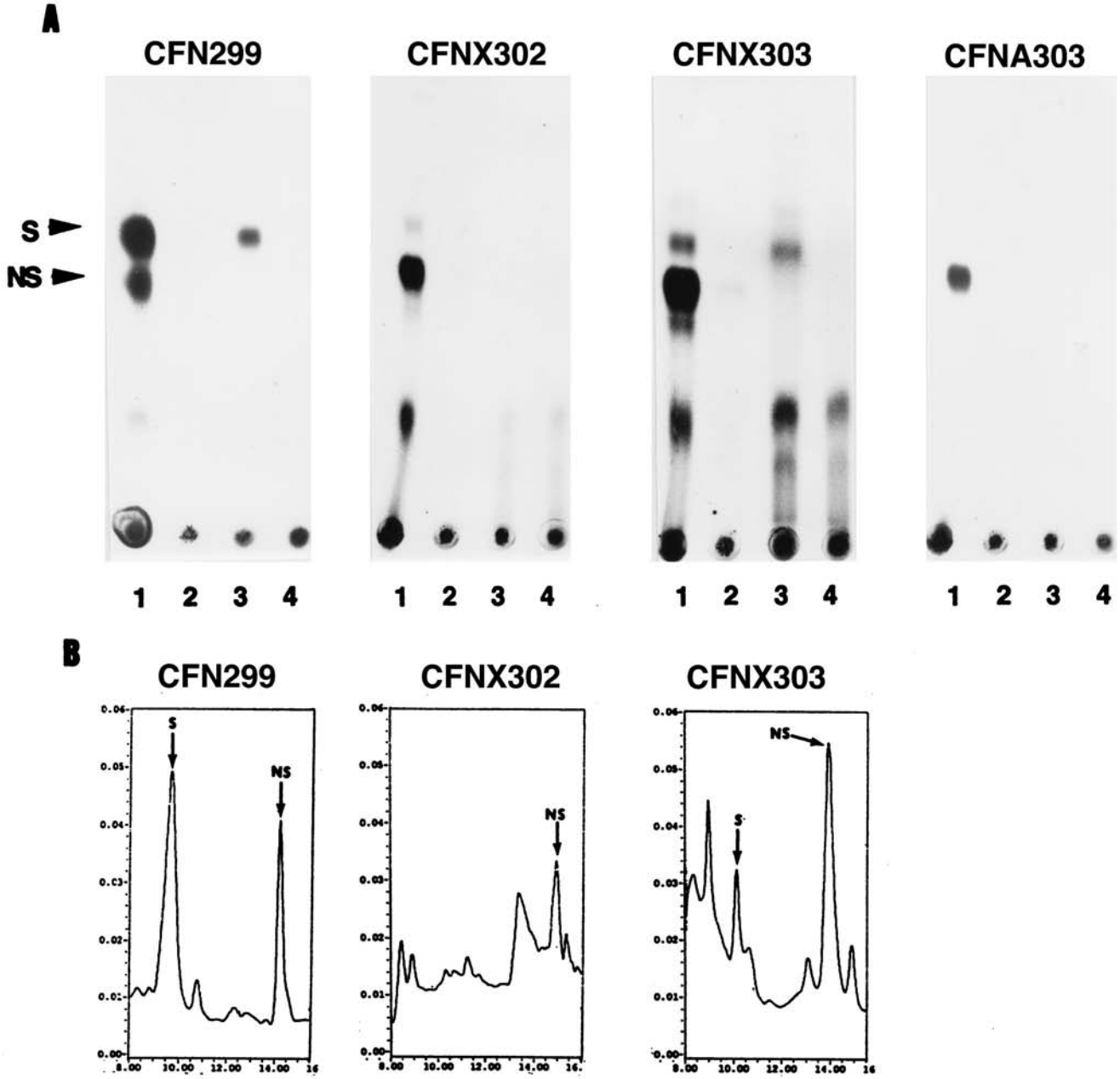

FIG. 4. Nod factors produced by *R. tropici* and *A. tumefaciens* strains. (A) Reverse-phase TLC of ^{14}C-labeled (lanes 1 [induced] and 2 [noninduced]) and ^{35}S-labeled (lanes 3 [induced] and 4 [noninduced]) samples. (B) HPLC profiles. *y* axis, absorption units; *x* axis, retention time in minutes (only molecules eluting between min 8 and 16 are shown). S and NS, sulfated and nonsulfated Nod factors. CFN299, *R. tropici* wild type; CFNX302, *R. tropici* containing the GDYN3 element interrupting the *nodP* gene; CFNX303, *R. tropici* amplified derivative; CFNA303, *A. tumefaciens* bearing AMP*Rtr*CFN299pc60.

the *R. tropici* strain containing the GDYN3 element (CFNX302), region D was larger (Fig. 3c). The *R. tropici* amplified strains contained an additional *Eco*RI fragment that corresponds to the joint fragment (region ZA) of amplification (Fig. 3d). All of the *Eco*RI fragments corresponding to regions B, C, D, E, F, and ZA were amplified, while fragments A and Z remained in single copies in the amplified strains. The average level of amplification in strains CFNX303 and CFNX304 was four to five copies of the whole amplicon structure. The amplicon in *E. coli* and *A. tumefaciens* does not contain regions A and Z but contains the joint fragment ZA (Fig. 3e). As expected, fragment ZA hybridizes against the A and Z borders.

Hybridization of various homologous and heterologous probes against DNA from ordered cosmids, *R. tropici* wild-type and amplified derivatives, and AMP*Rtr*CFN299pc60 (not shown) led to the identification of several symbiotic genes within the amplicon (Fig. 3b and e). Other gene sequences which were detected in the *R. tropici* genome but do not form part of the amplicon include *nodD3*, *nodD4*, *nodL*, and *nifHDK* (not shown).

Determination of AMP*Rtr*CFN299pc60 endpoints. To investigate the nature of the repeated sequences that border AMP*Rtr*CFN299pc60, we cloned and determined the DNA sequence of region A. Sequence analysis revealed the presence

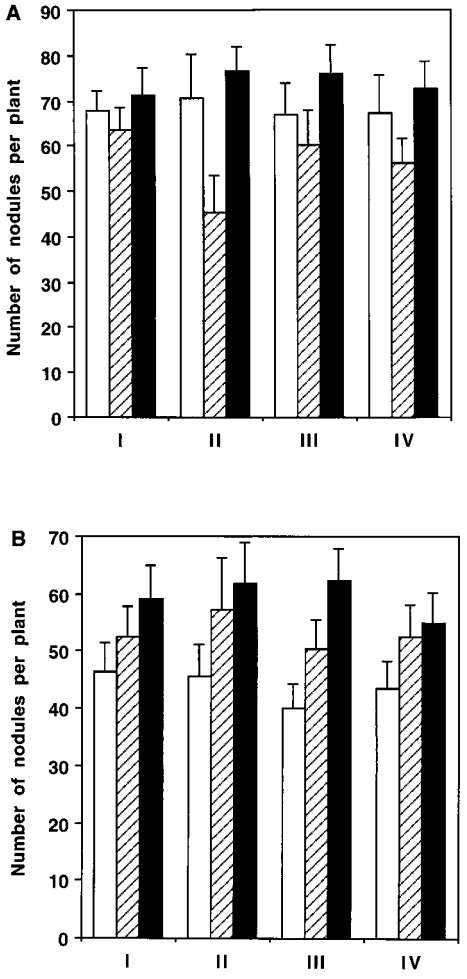

FIG. 5. Nodulation assays with different *R. tropici* strains and bean cultivars. The average number ± 95% confidence interval of nodules per plant 18 days after inoculation is presented. For each point, 8 to 10 plants were used. (A) Cultivar Negro Jamapa; (B) cultivar N-8-116. *R. tropici* strains used were wild-type CFN299 (open bars), CFNX302 containing the GDYN3 element (hatched bars), and CFNX303 amplified derivative (black bars). I to IV indicate four different experiments.

of a novel IS, designated IS*Rtr1*, of 1,364 bp terminated by two 28-bp imperfect inverted repeats (Fig. 3f). IS*Rtr1* contains two open reading frames, ORF1 and ORF2, of 651 and 516 nucleotides, respectively. The derived amino acid sequences of these two ORFs shows ≤51% similarity with transposases. The GC content of IS*Rtr1* is 60.8%, which matches values reported for the *Rhizobium* genome (56 to 62%), suggesting an endogenous origin. However, BLAST analysis using the GenBank and EMBL databases indicates that IS*Rtr1* is more closely related to IS elements from gram-positive bacteria such as IS*1245* of *Mycobacterium avium* (12) or IS*1164* of *Rhodococcus rhodochrous* J1 (15).

A PCR product internal to the IS*Rtr1* sequence was used as a probe to hybridize against Southern blots of *Eco*RI-digested DNA from the *R. tropici* wild-type and amplified strains, the isolated AMP*Rtr*CFN299pc60, and an *A. tumefaciens* transconjugant containing the pSym of CFN299 (not shown). As there is no *Eco*RI site within the IS*Rtr1* DNA sequence, we conclude that IS*Rtr1* or closely related elements are present in at least six copies in the genome. Five are located in the pSym which include the two borders of the described amplicon, one more internal and two external to the amplicon. In addition, the probe revealed the previously identified joint fragment ZA in amplified strains.

Production of Nod factors by *Rhizobium* amplified derivatives and *Agrobacterium* bearing AMP*Rtr*CFN299pc60. Our results confirm the finding (16, 22) that the wild-type CFN299 produces both sulfated and nonsulfated Nod factors whereas the NodP mutant fails to produce sulfated Nod factors (Fig. 4). Surprisingly, the amplified derivative CFNX303 exhibited a TLC pattern different from that of the parental CFNX302 strain. It consists of (i) the clear presence of sulfated Nod factors, albeit in quantities lower than in the wild-type strain, (ii) an evident overproduction of nonsulfated Nod factors, and (iii) the presence of new products in comparison to both wild-type CFN299 and parental CFNX302 strains. HPLC profiles corroborate the TLC results (Fig. 4B). Interestingly, *A. tumefaciens* CFNA303 bearing AMP*Rtr*CFN299pc60 was able to excrete nonsulfated Nod factors (Fig. 4A), suggesting that AMP*Rtr*CFN299pc60 contains sufficient genetic information for Nod factor production.

Nodulation phenotypes. In comparison to the wild-type CFN299, the strain with a mutation in *nodP* (CFNX302) formed fewer nodules on cultivar Negro Jamapa and more nodules on cultivar N-8-116 (Fig. 5), in corroboration of previous results (16). Interestingly, in cultivar Negro Jamapa, the amplified strain CFNX303 produced more nodules than the parental strain CFNX302 (Fig. 5A). However, for only two (II and IV) of four experiments performed were differences significant ($P < 0.05$). In cultivar N-8-116 (Fig. 5B), the amplified strain CFNX303 induced significantly more nodules than the wild-type strain in the four experiments performed ($P < 0.05$). This strain showed a tendency to form more nodules than the parental strain CNFX302, although this increase in nodulation was significant only in experiment III ($P < 0.05$).

The nodulation capacity of the *A. tumefaciens* strain harboring AMP*Rtr*CFN299pc60 (CFNA303) was tested on three different bean cultivars: Negro Jamapa, N-8-116, and BAT-477. In all bean plants tested, strain CFNA303 induced nodules. Typically, *A. tumefaciens* CFNA303 produced 15 to 20 nodules per plant (not shown), compared to 40 to 70 for the wild-type *R. tropici* CFN299. Nodules elicited by *A. tumefaciens* CFNA303 were white and smaller than those induced by *Rhizobium* strains. Light, scanning, and transmission electron micrographs of nodules formed by *A. tumefaciens* CFNA303 showed infected plant cells, albeit with fewer bacteria per infected cell than nodules induced by *R. tropici* wild-type strain (Fig. 6). Furthermore, both contained poly-β-hydroxybutyrate granules (Fig. 6C).

DISCUSSION

In this study, the procedure for identification and mobilization of *Rhizobium* amplicons was improved. The use of conditional origins of replication (*oriV* of RK2) and conjugal transfer (*oriT* of RK2) allowed both the introduction of the GDYN3 element into the *Rhizobium* genome (in the absence of the transactivator TrfA of *oriV* of RK2) as well its mobilization into different strains, including *A. tumefaciens* (in the presence of the *trfA* gene).

We propose the term "symbiotic amplicons" to refer to amplifiable DNA units of the genome carrying genes essential for symbiosis and suggest the following nomenclature: "AMP" followed by the abbreviated genus (capital letter), species (lowercase letter), and name of the strain, followed by the replicon in which the amplicon is located ("p" for plasmid, followed by the corresponding letter, or "ch" for chromosome) and the size

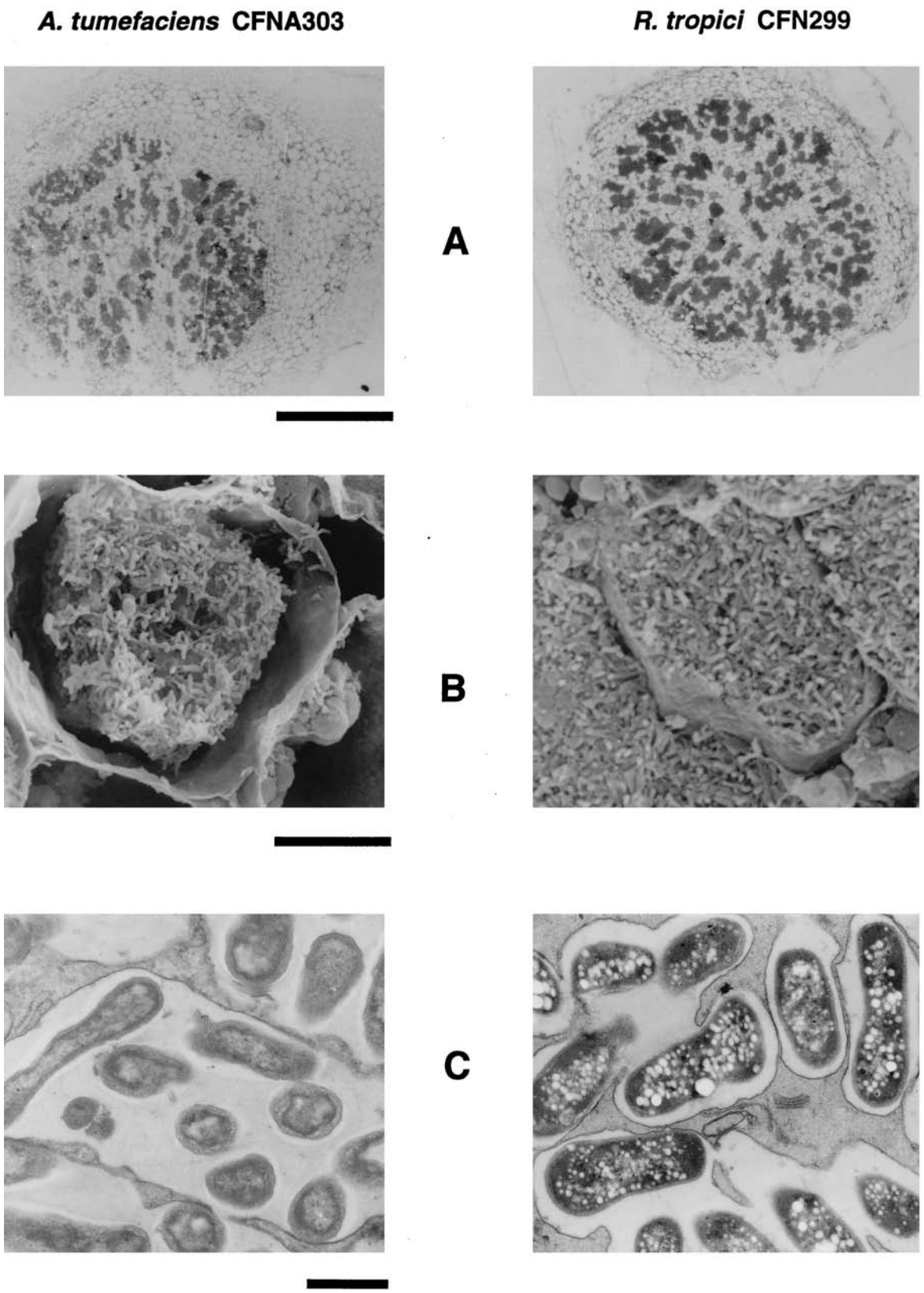

FIG. 6. Light (magnification, ×40; bar = 200 μm) (A), scanning (×2,000; bar = 10 μm (B), and transmission (×7,500; bar = 1 μm) (C) electron micrographs of bean nodules containing *A. tumefaciens* CFNA303 harboring AMP*Rtr*CFN299pc60 and wild-type CFN299 showing infected cells. In panel C, poly-β-hydroxybutyrate granules are present inside the bacteria.

of the amplicon in kilobases. Accordingly, the *R. tropici* symbiotic amplicon is termed AMP*Rtr*CFN299pc60. The previously reported symbiotic amplicon of *R. etli* (25) should be termed AMP*Re*CFN42pd120.

AMP*Rtr*CFN299pc60 consists of a 60-kb stretch of DNA flanked by IS*Rtr1* repeats. IS*Rtr1* is a novel IS element closely related to IS*1245* and IS*1164* from gram-positive bacteria (12, 15). Symbiotic genes including *nodD1*, *nodD2*, *nodABCSUIJ*, *nodHPQ*, *nifA*, *fixLJ*, and *fixABCX* are part of this amplicon. Amplification of AMP*Rtr*CFN299pc60 is probably generated through homologous recombination between IS*Rtr1* repeats. This model of rearrangement is supported by the presence of the joint fragment in the *R. tropici* amplified strains and in the amplicon DNA isolated from *E. coli*. Similar recombination events have been proposed to explain amplification of plasmid and chromosomal DNA regions in different bacteria (2, 13, 35), including pSym regions of *R. etli* (10, 25).

As the presence of reiterated sequences is a common feature of the *Rhizobium* genome, we believe that amplicon structures and DNA amplification events may occur in the different *Rhizobium* species. Actually, the DNA sequence of the symbiotic plasmid of *Rhizobium* sp. strain NGR234 (11) suggests the existence of several symbiotic amplicons.

The effect of the quality and quantity of Nod factors in regard to nodulation capacity in *Rhizobium* is complex (24). As previously reported, in *R. tropici* a mutation in *nodP* impairs the production of sulfated Nod factors without altering that of the nonsulfated forms, thus decreasing the total amount of Nod factors produced. This, in turn, decreases nodulation in some bean cultivars while improving it in others (reference 16 and Fig. 5). Our results show that in amplified strains, Nod factor production clearly increases. Moreover, some sulfated Nod factors are produced. Amplified strains formed more nodules than both wild-type and parental mutant strains. This increment was, however, significant in only some experiments (Fig. 5).

The presence of sulfated Nod factors in amplified strains was unexpected since mutation in *nodP* impairs the synthesis of PAPS (3′-phosphoadenosine 5′-phosphosulfate), the activated form of sulfate required for sulfation of the Nod factor core (31, 32). A plausible explanation is that the overproduction of nonsulfated Nod factors may utilize PAPS produced by an alternative pathway such as that described for *R. meliloti* (33). Actually, in *R. tropici* CFN299 a chromosomal DNA sequence showing homology with a locus of *E. coli* involved in the synthesis of PAPS was recently found (17).

Demonstration that the symbiotic amplicon AMP*Rtr*CFN 299pc60 contains functional genes essential for Nod factor production and nodulation processes was achieved after its transfer into *A. tumefaciens*. Transconjugants of *A. tumefaciens* were able to produce nonsulfated Nod factors like those found in the *R. tropici* strain containing the GDYN3 element in *nodP*. Moreover, *A. tumefaciens* bearing AMP*Rtr*CFN299pc60 induced nodules on beans containing intracellular bacteria. Obviously, these nodules did not fix nitrogen since AMP*Rtr*CFN 299pc60 does not contain the structural *nifHDK* genes.

The fact that our experimental procedure makes the transfer of amplicons into different bacteria possible will facilitate analysis of Nod factor production and its effects on nodulation in both different plants and different bacterial genomic backgrounds. Whether amplicons can also function as transferable elements under natural conditions and increase the flow of plasmid-borne genetic information among bacteria remains to be explored.

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