APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1999, p. 2716–2722 Vol. 65, No. 6
0099-2240/99/$04.00+0

Increase in Alfalfa Nodulation, Nitrogen Fixation, and Plant Growth by Specific DNA Amplification in *Sinorhizobium meliloti*

MARCELA CASTILLO, MARGARITA FLORES, PATRICK MAVINGUI, ESPERANZA MARTÍNEZ-ROMERO, RAFAEL PALACIOS, AND GEORGINA HERNÁNDEZ*

Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Cuernavaca, Morelos, México

Received 14 December 1998/Accepted 11 March 1999

To improve symbiotic nitrogen fixation on alfalfa plants, *Sinorhizobium meliloti* strains containing different average copy numbers of a symbiotic DNA region were constructed by specific DNA amplification (SDA). A DNA fragment containing a regulatory gene (*nodD1*), the common nodulation genes (*nodABC*), and an operon essential for nitrogen fixation (*nifN*) from the *nod* regulon region of the symbiotic plasmid pSyma of *S. meliloti* was cloned into a plasmid unable to replicate in this organism. The plasmid then was integrated into the homologous DNA region of *S. meliloti* strains 41 and 1021, which resulted in a duplication of the symbiotic region. *Sinorhizobium* derivatives carrying further amplification were selected by growing the bacteria in increased concentrations of an antibiotic marker present in the integrated vector. Derivatives of strain 41 containing averages of 3 and 6 copies and a derivative of strain 1021 containing an average of 2.5 copies of the symbiotic region were obtained. In addition, the same region was introduced into both strains as a multicopy plasmid, yielding derivatives with an average of seven copies per cell. Nodulation, nitrogenase activity, plant nitrogen content, and plant growth were analyzed in alfalfa plants inoculated with the different strains. The copy number of the symbiotic region was critical in determining the plant phenotype. In the case of the strains with a moderate increase in copy number, symbiotic properties were improved significantly. The inoculation of alfalfa with these strains resulted in an enhancement of plant growth.

The construction of bacterial strains with an increased copy number of specific genes has been widely used for biotechnological applications. The most common procedure is to introduce the DNA of interest as a multicopy plasmid. In some cases gene amplification has been obtained through the natural mechanisms of homologous recombination, which usually occurs between reiterated sequences present in direct orientation (3).

DNA amplification is a common feature of the genome of prokaryotic organisms (3, 26). In some cases natural gene amplifications are associated with adaptive responses in different bacteria (26). Amplification may be induced in any part of the genome by cointegrating a plasmid carrying the region of interest into the homologous region of the genome. This results in a direct repeat of the region interposed by the vector used and constitutes an amplifiable or amplicon structure. Homologous recombination between the repeats leads to duplication and further amplification of the whole amplicon structure. This methology has been used to enhance the production of specific compounds (2, 13).

We have studied gene amplification phenomena in bacteria of the genus *Rhizobium* (27). These bacteria establish symbiosis with leguminous plants and elicit the formation of nitrogen-fixing nodules. By using random DNA amplification (RDA) in the symbiotic plasmid of *Rhizobium tropici* we have recently shown that DNA amplification may be used to obtain strains with enhanced competitiveness for nodulation (22).

Bacteria belonging to *Rhizobium* and related genera establish nitrogen-fixing symbiosis with legumes. The symbiosis is the result of the differentiation of both bacteria and plant cells mediated by an exchange of chemical signals between the partners. Initially, flavonoid compounds from the plant activate a regulatory circuit in the microsymbiont which results in the synthesis of signals known as nodulation (Nod) factors. These compounds are lipooligosaccharides that induce the initial steps of the differentiation of the plant cells (19).

The genome of *Sinorhizobium meliloti*, the microsymbiont of alfalfa, is comprised of a chromosome of 3.7 Mb and two megaplasmids of 1.4 and 1.7 Mb (10). Most of the nodulation (*nod*) and nitrogen fixation (*nif* and *fix*) genes are located in the 1.4-Mb replicon, which is known as the symbiotic plasmid (pSyma). An approximately 90-kb region of this replicon contains the different structural and regulatory genes that constitute the *nod* regulon. The *nod* regulon contains several operons that code for the enzymes that synthesize the Nod factors. Regulation is exerted by a complex circuit that includes four genes of the *nod* regulon: *nodD1*, *nodD2*, *nodD3*, and *SyrM* (17, 21, 24). Several *nif* and *fix* genes are located in the same region, interspersed with the elements that constitute the *nod* regulon.

With the aim of improving nodulation and nitrogen fixation in alfalfa plants, we have now induced amplification in a specific region of the symbiotic plasmid of *S. meliloti*. In contrast to the RDA strategy, we used specific DNA amplification (SDA) for this experimental approach.

We chose a particularly interesting DNA fragment from the *nod* regulon region and amplified it to different copy numbers. This fragment contains the regulatory gene *nodD1*; the nodulation genes *nodA*, *nodB*, and *nodC*, which encode the enzymes responsible for the synthesis of the core structure of Nod factors; and an operon which is essential for nitrogen fixation. To manipulate the copy number of this DNA fragment two strategies were used: (i) its cointegration into the homologous region of pSyma and amplification by the SDA procedure and (ii) its introduction on a multicopy plasmid. Derivatives of *S. meliloti* containing this fragment with an average copy number of 2.5 to 3 showed a significant increase in nodulation and nitrogen fixation and promoted alfalfa growth.

* Corresponding author. Mailing address: Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Ap. Postal 565-A, Cuernavaca, Morelos, México. Phone: (52) (73) 13-98-77. Fax: (52) (73) 11-67-10. E-mail: gina@cifn.unam.mx.

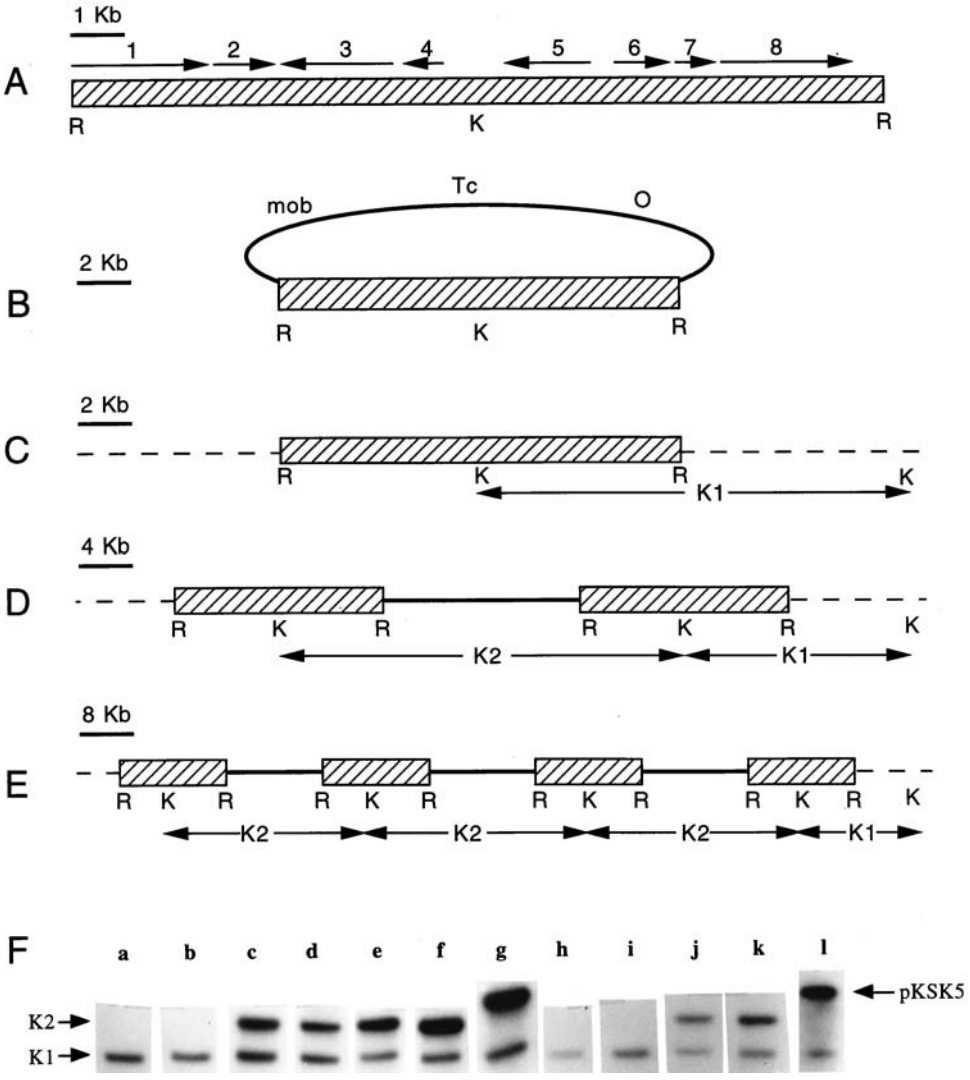

FIG. 1. Construction and characterization of *S. meliloti* derivatives with different copy numbers of a symbiotic DNA region. (A to E) Diagrams of relevant structures. Hatched areas indicate *Eco*RI fragment containing nodulation and nitrogen fixation gene sequences; the heavy solid line indicates the vector DNA sequences; the dashed line shows pSyma DNA sequences bordering the *Eco*RI fragment. Abbreviations: R, *Eco*RI sites; K, *Kpn*I sites; K1, wild-type *Kpn*I fragment; K2, recombinant *Kpn*I fragment; O, origin of replication; Tc, tetracycline resistance marker. Arrows indicate the positions and directions of transcription of the genes present in the *Eco*RI fragment: 1, *nolI*; 2, *nodN*; 3, *nifN*; 4, ORF110; 5, *nodD1*; 6, *nodA*; 7, *nodB*; 8, *nodC*. Panels: A, *Eco*RI fragment; B, pCH1; C, *S. meliloti* wild-type strain; D, *S. meliloti* containing a cointegration of pCH1 into the homologous region of pSyma; E, *S. meliloti* containing an amplification of the *Eco*RI fragment with a copy number of four. (F) Southern blots of *Kpn*I-digested total DNA hybridized with a *nod*A probe from (lanes): a and b, *S. meliloti* 41; c and d, CFNM101; e and f, CFNM102; g, CFNM104; h and i, *S. meliloti* 1021; j and k, CFNM103; l, CFNM105. In lanes a, c, e, g, h, j, and l the DNA was extracted from in vitro bacterial cultures. In lanes b, d, f, i, and k the DNA was extracted from nodule-recovered bacteria. The *Kpn*I fragments K1 and K2 and the pKSK5 plasmid are indicated.

MATERIALS AND METHODS

DNA manipulations. Total DNA was isolated from *Sinorhizobium* strains by using a DNA/RNA isolation kit (USB/Amersham, Cleveland, Ohio). Standard DNA manipulations such as restriction, agarose gel electrophoresis, and filter blot hybridization were performed as described previously (7). Probes were radiolabelled with ^{32}P by using a random priming DNA labelling kit (*redi*prime; Amersham).

Construction of *S. meliloti* strains carrying DNA amplifications in pSyma. The 8.5-kb *Eco*RI fragment from pKSK5 (16), kindly provided by E. Kondorosi, was purified and ligated into the *Eco*RI site of pSUP202 (30), generating plasmid pCH1 of 17 kb. This plasmid was introduced into *Escherichia coli* DH5α by transformation. From *E. coli*, pCH1 was mobilized to *S. meliloti* strains 41 (15) and 1021 (23) by triparental mating by using *E. coli* HB101(pRK2013) (6) as a helper. *Sinorhizobium* transconjugants were selected in minimal medium containing 5 μg of tetracycline (Tc) and 50 μg of spectinomycin per ml (for strain 41 derivatives) or 100 μg of spectinomycin per ml (for strain 1021 derivatives). These transconjugants contained a cointegration of pCH1 into the homologous region in pSyma. *Sinorhizobium* derivatives containing a higher level of amplification were selected by plating in different concentrations of Tc. Strains CFNM101 and CFNM103 were obtained from plates with 7.5 μg of Tc per ml, and strain CFNM102 was obtained from plates with 30 μg of Tc per ml. Strains CFNM104 and CFNM105 were obtained by mobilizing pKSK5 from *E. coli* DH5α into *S. meliloti* 41 or 1021, respectively, by triparental mating with *E. coli* HB101(pRK2013) (6) as a helper.

Determination of copy number. Southern blots of *Kpn*I-digested total DNA from *Sinorhizobium* strains were hybridized against an intragenic PCR product of the *nodA* gene. The copy number of the symbiotic fragment used in this study was quantified by densitometric integration of the corresponding hybridization signals on the autoradiograms. The intensity of the 10-kb *Kpn*I wild-type fragment was set at unity. The intensity of the recombinant *Kpn*I fragment from strains CFNM101, CFNM102, and CFNM103 (17 kb) and from strains CFNM104 and CFNM105 (25 kb) (see below) was divided by that of the 10-kb fragment to obtain the copy number of the corresponding recombinant fragment. The total copy number in each strain was the copy number of the recombinant fragment plus one.

Nod factor production. The lipooligosaccharides produced by *S. meliloti* wild-type and amplified strains were examined by reversed phase thin-layer chromatography (32) by using bacterial supernatants according to the procedures de-

scribed previously (18). Cultures were grown in the absence or in the presence of the inducer luteolin (1.2 μM). Cultures for Nod factor analysis were labelled with [^{14}C]glucosamine (0.5 μCi/ml, 52.6 μCi/mmol; Amersham), adjusting the same amounts of bacteria in each culture.

Plant growth conditions. Seeds from alfalfa (*Medicago sativa*) var. Iroquois were surfaced sterilized, germinated, and grown either in 12.5-cm-wide by 15.5-cm-high plastic growth pouches (Seed-Pack Growth Pouch; Vaughan's Seed Company, Downers Grove, Ill.) or in 20-cm-diameter by 16-cm-high plastic pots containing sterile vermiculite. Ten plants were grown in each growth pouch, and twenty plants were grown in each pot. Four days after germination, plants were inoculated with the *S. meliloti* strains. The bacterial inoculum was prepared from an overnight culture grown on rich medium plus the appropriate antibiotic, then washed, and adjusted to give an inoculum of ca. 10^9 cells per plant. Plants were incubated at 21°C and 80% humidity, with a 12-h photoperiod provided by cool white fluorescent lights at 110.4 μmol m^{-2} s^{-1}, and were watered with Jensen liquid medium (30) as necessary.

Nitrogenase activity and total nitrogen determinations. Alfalfa plants were harvested 60 days after inoculation and combined at random into samples of five plants each. Nitrogenase activities were determined in seven samples (35 plants) from each condition by the acetylene reduction method. The total nitrogen content of dry foliar samples was determined with an ANTEK 7000 (Antek Instruments, Inc., Houston, Tex.) nitrogen analyzer, as reported previously (5).

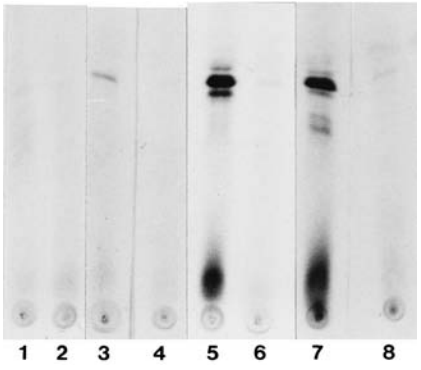

FIG. 2. Autoradiogram of a reversed-phase thin-layer chromatography of Nod factors labelled with [^{14}C]glucosamine. Lanes 1, 3, 5, and 7, luteolin-induced *S. meliloti*; lanes 2, 4, 6, and 8, noninduced *S. meliloti* wild-type strain and derivatives. Lanes: 1 and 2, *S. meliloti* 41; 3 and 4, CFNM101; 5 and 6, CFNM102; 7 and 8, CFNM104.

RESULTS

Construction of *S. meliloti* derivatives with different copy numbers of a symbiotic DNA region. An *Eco*RI fragment of 8.5 kb from the pSyma of *S. meliloti* 41 (Fig. 1A) contains several genes that participate in the establishment of symbiosis and in nitrogen fixation. These include the regulatory gene *nodD1*; the structural nodulation genes *nodA*, *nodB*, and *nodC* and their regulatory element *nod* box n1; and an operon essential for nitrogen fixation composed of gene *nifN* (previously called *fixF*) and an open reading frame (ORF) that codes for a protein of yet unassigned function (ORF110). In addition, the fragment contains genes *nolI* and *nodN*, which are part of an operon whose transcription is regulated by *nod* box n4, located 4 kb upstream of the fragment.

The fragment was purified from plasmid pKSK5 (16) and cloned into the *Eco*RI site of pSUP202 (30) to obtain plasmid pCH1 (Fig. 1B). This plasmid contains an origin of replication that is active in *E. coli* but not in *S. meliloti*, a mob site, and Tc and ampicillin resistance markers. pCH1 was mobilized from *E. coli* into *Sinorhizobium* by conjugation, and Tc-resistant transconjugants were selected. The transconjugants contained a cointegration of pCH1 into the homologous region of pSyma of *S. meliloti*. The resulting structure is a direct repeat of the 8.5-kb region separated by the cloning vector (Fig. 1D). Homologous recombination between the direct repeats may lead to further amplification. Amplified strains were selected by increasing the Tc concentration in the cell culture. These strains contain tandem repeats of the whole structure (Fig. 1E). Derivatives of *S. meliloti* 41 containing the *Eco*RI fragment in average copy numbers of 3 (CFNM101) and 6 (CFNM102) and a derivative of strain 1021 with an average copy number of 2.5 (CFNM103) were used for further experiments. In addition, pKSK5, which contains the same *Eco*RI fragment in a vector that replicates in *S. meliloti*, was introduced into both strains by conjugation from *E. coli*. This generated strains CFNM104 (from 41) and CFNM105 (from 1021) containing the fragment in an average copy number of 7.

To characterize the relevant structures of the amplified strains, total DNA was digested with restriction endonuclease *Kpn*I and subjected to gel electrophoresis. The *Eco*RI fragment contains one *Kpn*I site upstream of gene *nodD1*, while the vector of pCH1 does not contain *Kpn*I sites. Southern blots were hybridized by using an intragenic PCR product from *S. meliloti nodA* as a probe (Fig. 1F). The wild-type strains show one *Kpn*I fragment (K1) of 10 kb (Fig. 1F). As expected, the strains containing amplifications of the 8.5-kb *Eco*RI fragment show the same *Kpn*I fragment as the wild type and a new recombinant fragment of 17 kb (K2), whose relative intensity increases with the level of amplification (Fig. 1F). The strains that harbor the symbiotic region in a multicopy plasmid show the wild-type fragment and a fragment of 28.5 kb that corresponds to the linearized pKSK5. The copy number of the region was calculated from the relative intensities of both signals in each strain. Figure 1F shows the autoradiograms of the Southern blots from the wild-type strains and their derivatives.

Nod factor production. The patterns of Nod factor production were analyzed in the wild type and amplified derivatives of *S. meliloti*. The results obtained were in general agreement with what has been reported for *S. meliloti* (28). In the wild-type strains Nod factor production was faintly observed. With a moderate increase in copy number of the symbiotic region (strains CFNM101 and CFNM103), Nod factor production was clearly observed. The highest production of Nod factors was obtained with strain CFNM102, bearing the greatest number of copies of the amplified region, and strains CFNM103 and CFNM105, which contain the region in a multicopy plasmid. Data from *S. meliloti* 41 and its derivatives are shown in Fig. 2.

Symbiotic phenotype of alfalfa plants inoculated with the *S. meliloti* strains constructed. The ability of the different *S. meliloti* strains to nodulate alfalfa plants was determined. The nodulation kinetics of alfalfa plants inoculated in growth pouches are shown in Fig. 3. Strain CFNM101 shows a significant ($P \leq 0.03$) increase in nodulation, while strains CFNM102, CFNM104, and CFNM105 show a similar and significant ($P \leq 0.02$) decrease in nodulation compared to the respective wild-type strains (Fig. 3). At late postinoculation time points, strain CFNM103 shows a significant ($P \leq 0.01$) increase in nodulation with respect to the 1021 wild-type strain (Fig. 3B).

The symbiotic phenotype of alfalfa plants inoculated in pots with vermiculite in environmentally controlled growth chambers was determined. Plants were harvested after 60 days. Three independent experiments were performed, and similar results were obtained among them. Data from one such experiment are presented in Fig. 4. In agreement with the data from Fig. 3, the plants inoculated with CFNM101 or with CFNM103 show an increase in nodulation of 2.2- to 2.5-fold. These plants show a 2.6- to 2.8-fold significant increase ($P \leq 0.01$) in acetylene reduction activity per plant and a 2-fold significant increase ($P \leq 0.01$) in root and in foliar dry weight compared to the wild-type strain 41 or 1021, respectively. In addition, these

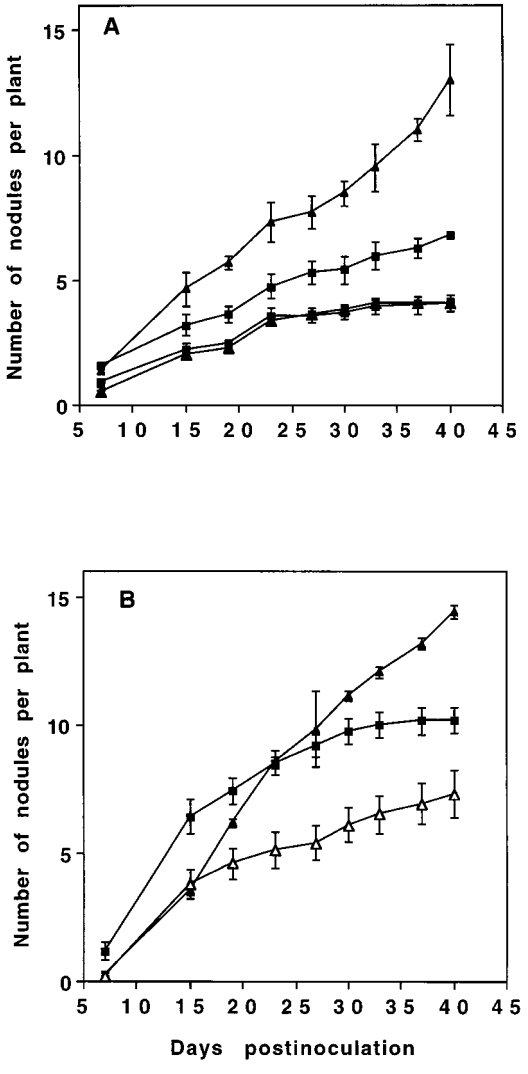

FIG. 3. Nodulation kinetics of alfalfa plants inoculated with different *S. meliloti* strains. Points are mean values ± the standard error from 100 plants inoculated with each strain. (A) ■, *S. meliloti* 41; ▲, CFNM101; △, CFNM102; □, CFNM104. (B) ■, *S. meliloti* 1021; ▲, CFNM103; △, CFNM105. In panel A, the open squares and triangles correspond to the two lowest lines; their values for each data point are almost identical, and therefore the symbols overlap, obscuring the fact that they should appear to be open, not solid.

plants have a significantly ($P \leq 0.01$) higher foliar nitrogen content per plant (2- to 2.2-fold). When alfalfa plants were inoculated with CFNM102, CFNM104, or CFNM105, they had significantly ($P \leq 0.01$) fewer nodules (33%), lower acetylene reduction activity (44%), decreased root and foliar dry weight (38 to 50%), and lower foliar nitrogen content (25 to 38%) compared to the respective wild-type strain. In fact, the values of growth (per dry weight basis) and of nitrogen content obtained for alfalfa plants inoculated with the latter strains containing higher copy numbers of the symbiotic region were similar to those obtained for control uninoculated plants watered without nitrogen (Fig. 4C).

Figure 5 shows representative alfalfa plants from the experiment described above. The improved growth and increased nodulation of alfalfa plants inoculated with strain CFNM101 is evident. In contrast, plants inoculated with strain CFNM102 or CFNM104 show poor growth, which is very similar to the growth of uninoculated plants watered without nitrogen, and they show decreased nodulation. The phenotypes of the alfalfa plants inoculated with the 1021 derivatives, CFNM103 or CFNM105 (not shown), were very similar to those inoculated with CFNM101 or CFNM104, respectively.

Southern blot analysis and densitometric quantification were performed with bacterial colonies recovered from surfaced sterilized alfalfa nodules in order to determine the stability of the amplified symbiotic region during symbiosis. The results shown in Fig. 1F indicate that each strain recovered from alfalfa nodules showed copy numbers similar to those of the strains used as an inoculum.

DISCUSSION

Together with previously reported results (22), our studies here show the potential of enhancing symbiotic properties by DNA amplification. At least under laboratory conditions, the manipulation of the *S. meliloti* genome by SDA may be a powerful tool for increasing nodulation, nitrogen fixation, and growth in alfalfa plants. When the DNA sequence and gene organization are unknown, RDA may be used. When the molecular basis and DNA organization of a particular function

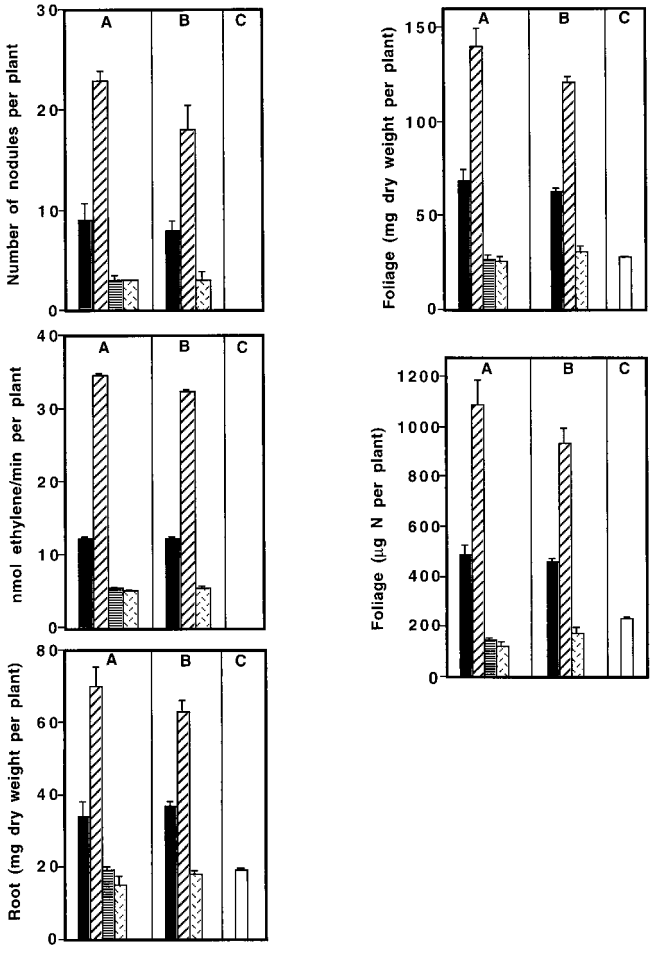

FIG. 4. Phenotype of alfalfa plants inoculated with different *S. meliloti* strains. The values of all the parameters are mean values ± the standard deviation from 35 plants; results were determined at 60 days postinoculation. Columns (A): ■, *S. meliloti* 41; ▨, CFNM101; ▤, CFNM102; ◪, CFNM104. Columns (B): ■, *S. meliloti* 1021; ▨, CFNM103; ◪, CFNM105. The open column in part C shows results for uninoculated control plants watered without nitrogen.

FIG. 5. Alfalfa plants inoculated with different *S. meliloti* strains. Representative plants from the experiment analyzed in Fig. 3 are shown. (A) An uninoculated control plant watered without nitrogen and plants inoculated with strains 41, CFNM101, CFNM102, and CFNM104 are shown from left to right. (B to E) Nodulated roots from plants inoculated with 41 (B), CFNM101 (C), CFNM102 (D), or CFNM104 (E). Bar, 1 cm.

are known, as is the case for symbiotic nitrogen fixation in *S. meliloti*, a more direct approach (SDA) may be a better choice.

The fact that most nodulation and nitrogen fixation genes are interspersed, yet clustered in a region of pSyma of *S. meliloti*, provides a good opportunity for increasing symbiotic nitrogen fixation by SDA. We chose a region containing three elements with key roles in symbiotic nitrogen fixation. The *nodD1* gene, activated by flavonoids, recognizes *nod* boxes upstream of the *nod* genes and positively regulates their expression (9, 11). The structural nodulation genes *nodABC* participate in the synthesis of the basic structure of the Nod factors. NodC catalyzes the polymerization of UDP-glucosamine (8), NodB is a de-N-acetylase (12) and NodA is a N-acyltransferase (25) which adds the acyl chain to the oligosaccharide core. *nodABC* mutants do not form nodules (20). The nitrogen fixation gene *nifN* is involved in nitrogenase FeMo cofactor biosynthesis, and *nifN* mutants do not fix nitrogen (1). Other components of this region are nodulation genes *nolI* and *nodN*. The transcription of these genes is regulated and starts in a region located 4 kb upstream of the *Eco*RI fragment. For this reason we assume that in the amplified state only one copy of these genes is transcribed.

The actual molecular mechanism of the observed symbiotic phenotypes is beyond the scope of this study. The increase in copy number of the regulatory gene *nodD1* and/or of the structural genes *nodABC* might result in an increase in nodulation.

The increase in nodulation may be in relation to a slight increase in Nod factor production observed in strains CFNM101 and CFNM103 in relation to the respective wild-type strains. Strains CFNM102, CFNM104, and CFNM105, with higher copy numbers, showed a greater amount of Nod factor production, but they showed reduced nodulation. The latter observation is consistent with the previous observation that strains bearing multicopy plasmids with *nod* genes have reduced nodulation (14). An increase in nodulation could lead to an increase in nitrogen fixation. In addition, the higher dosage of the *nifN* operon might have a direct effect on nitrogen fixation.

There are examples that show that the manipulation of structural or regulatory *nod* genes in rhizobia affects their symbiotic capacities. A *Rhizobium leguminosarum* strain carrying extra copies of structural nodulation genes (*nodABC*) in a multicopy plasmid was reported to have a reduced nodulation capacity on *Vicia* plants (14). The constitutive expression in different rhizobia of a chimeric *nodD1* gene, consisting of 75% of the *nodD1* gene of *S. meliloti* at the 5′ end and 27% of the *nodD* gene of *Rhizobium trifoli*, affected nodulation and nitrogen fixation when introduced into different rhizobia (31). Its expression in *R. trifoli* and *S. meliloti* resulted in an extension of the host range for nodulation to the tropical legumes *Macroptilium atropurpureum*, *Lablab purpureus*, and *Leucaena leucocephala* (31). Its expression in *R. trifoli* and *R. leguminosarum* resulted in a significant increase in nitrogen fixation during symbiosis with *Vicia sativa* and *Trifolium repens* (31).

When gene amplification is used for enhancing relatively simple functions such as the production of specific compounds, the increase in copy number is usually achieved by the introduction of multicopy plasmids. This approach has the disadvantage that the copy number depends on the replication characteristics of the vector used and is not easy to manipulate. When complex and tightly regulated functions are involved, the copy number may be critical. In these cases the approach based on amplification by recombination with the homologous DNA region is more convenient. This situation is clearly exemplified by the present study. When the symbiotic DNA region was introduced in a multicopy plasmid, the copy number was set to about seven; this actually resulted in a decrease in nodulation and nitrogen fixation. In contrast, induced DNA amplification in the homologous genomic region produced strains with different copy numbers. As shown here, a moderate increase in copy number (ca. three) produced an increase in nodulation, nitrogen fixation, and plant growth, while a higher copy number (ca. six) resulted in a decrease in these functions.

Efforts have been made to improve the symbiotic capability of *S. meliloti* in alfalfa. Genetically modified strains containing extra copies of *dct* (dicarboxylic transport) genes and a stronger *nifA* gene promoter show moderately increased nitrogen fixation abilities under some field conditions (4). In this case, the engineered strains caused a limited alfalfa yield increase over plants inoculated with the wild type, but the effects could also be attributed to the sites of insertion of the *dct* and *nif* genes (29). In this work we describe *S. meliloti* strains CFNM101 and CFNM103 which contain an average of 2.5 to 3 copies of the symbiotic region studied and show improved symbiotic abilities that result in an increase in nodulation, nitrogen fixation, and growth of alfalfa plants under environmentally controlled conditions. It will be interesting to determine whether the experimental approaches of RDA or SDA could be used for agricultural applications. In this regard, it is important to consider the two principal requirements for the development of improved strains for use as effective inoculants: a high symbiotic nitrogen fixation capacity and high competitive ability. Therefore, a good strategy may be to apply RDA and/or SDA to native *S. meliloti* strains in order to obtain improved engineered strains which are competitive and persistent in certain areas where alfalfa is cultivated.

ACKNOWLEDGMENTS

This work was supported in part by CONACyT (México) grant number L0013N.

We are grateful to Yolanda Pérez-Tejada, César Rodríguez, and Angeles Moreno for technical assistance; to Eva Kondorosi for providing a plasmid used; and to Michael Dunn for critically reading the manuscript.

REFERENCES

1. **Aguilar, O. M., H. Reiländer, W. Arnold, and A. Pühler.** 1987. *Rhizobium meliloti nifN* (*fixF*) gene is part of an operon regulated by a *nifA*-dependent promoter and codes for a polypeptide homologous to the *nifK* gene product. J. Bacteriol. **169:**5393–5400.
2. **Albertini, A. M., and A. Galizzi.** 1985. Amplification of a chromosomal region in *Bacillus subtilis*. J. Bacteriol. **162:**1203–1211.
3. **Anderson, R. P., and J. R. Roth.** 1977. Tandem genetic duplications in phage and bacteria. Annu. Rev. Microbiol. **31:**473–505.
4. **Bosworth, A. H., M. K. Williams, K. A. Albrecht, R. Kwiatkowski, J. Beynon, T. R. Hankinson, C. W. Ronson, F. Cannon, T. J. Wacek, and E. W. Triplett.** 1994. Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* with an extra copy of *dctABD* and/or modified *nifA* expression. Appl. Environ. Microbiol. **60:**3815–3832.
5. **Cevallos, M. A., S. Encarnación, A. Leija, Y. Mora, and J. Mora.** 1996. Genetic and physiological characterization of a *Rhizobium etli* mutant strain unable to synthesize poly-β-hydroxybutyrate. J. Bacteriol. **178:**1646–1654.
6. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
7. **Flores, M., V. González, S. Brom, E. Martínez, D. Piñero, D. Romero, G. Dávila, and R. Palacios.** 1987. Reiterated DNA sequences in *Rhizobium* and *Agrobacterium* spp. J. Bacteriol. **169:**5782–5788.
8. **Geremia, R. A., P. Mergaert, D. Geelen, M. Van Montagu, and M. Holsters.** 1994. The NodC protein of *Azorhizobium caulinodans* is an *N*-acetylglucosaminyltransferase. Proc. Natl. Acad. Sci. USA **91:**2669–2673.
9. **Göttfert, M., B. Horvath, E. Kondorosi, P. Putnoky, F. Rodriguez-Quiñones, and A. Kondorosi.** 1986. At least two *nodD* genes are necessary for efficient nodulation of alfalfa by *Rhizobium meliloti*. J. Mol. Biol. **191:**411–420.
10. **Honeycutt, R. J., M. McClelland, and B. W. S. Sobral.** 1993. Physical map of the genome of *Rhizobium meliloti* 1021. J. Bacteriol. **175:**6945–6952.
11. **Honma, M. A., and F. M. Ausubel.** 1987. *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. Proc. Natl. Acad. Sci. USA **84:**8558–8562.
12. **John, M., H. Röhrig, J. Schmidt, U. Wieneke, and J. Schell.** 1993. *Rhizobium NodB* protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. Proc. Natl. Acad. Sci. USA **90:**625–629.
13. **Kalio, P., A. Palva, and I. Palva.** 1987. Enhancement of α-amylase production by integrating and amplifying the α-amylase gene of *Bacillus amyloliquefaciens* in the genome of *Bacillus subtilis*. Appl. Microbiol. Biotechnol. **27:**64–72.
14. **Knight, C. D., L. Rossen, J. G. Robertson, B. Wells, and J. A. Downie.** 1986. Nodulation inhibition by *Rhizobium leguminosarum* multicopy *nodABC* genes and analysis of early stages of plant infection. J. Bacteriol. **166:**552–558.
15. **Kondorosi, A., G. B. Kiss, T. Forrai, E. Vincze, and Z. Banfalvi.** 1977. Circular linkage maps of *Rhizobium meliloti* chromosome. Nature **268:**525–527.
16. **Kondorosi, E., Z. Banfalvi, and A. Kondorosi.** 1984. Physical and genetic analysis of a symbiotic region of *R. meliloti*: identification of nodulation genes. Mol. Gen. Genet. **193:**445–452.
17. **Kondorosi, E., M. Buiré, M. Cren, N. Iyer, B. Hoffmann, and A. Kondorosi.** 1991. Involvement of the *syrM* and *nodD3* genes of *Rhizobium meliloti* in *nod* gene activation and in optimal nodulation of the plant host. Mol. Microbiol. **5:**3035–3048.
18. **Laeremans, T., I. Caluwaerts, C. Verreth, M. A. Rogel, J. Vanderleyden, and E. Martínez-Romero.** 1996. Isolation and characterization of the *Rhizobium tropici* Nod factor sulfation genes. Mol. Plant-Microbe Interact. **9:**492–500.
19. **Lerouge, P., P. Roche, C. Faucher, F. Maillet, G. Truchet, J. C. Promé, and J. Dénarié.** 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344:**781–784.
20. **Long, S. R., W. J. Buikema, and F. M. Ausubel.** 1982. Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod$^-$ mutants. Nature **298:**485–488.

21. **Maillet, F., F. Debellé, and J. Dénarié.** 1990. Role of the *nodD* and *syrM* genes in the activation of the regulatory gene *nodD3*, and the common and host-specific *nod* genes of *Rhizobium meliloti*. Mol. Microbiol. **4:**1975–1984.
22. **Mavingui, P., M. Flores, D. Romero, E. Martínez-Romero, and R. Palacios.** 1997. Generation of *Rhizobium* strains with improved symbiotic properties by random DNA amplification (RDA). Nat. Biotechnol. **15:**564–569.
23. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
24. **Mulligan, J. T., and S. R. Long.** 1989. A family of activator genes regulates expression of *Rhizobium meliloti* nodulation genes. Genetics **122:**7–18.
25. **Röhrig, H., J. Schmidt, U. Wieneke, E. Kondorosi, I. Barlier, J. Schell, and M. John.** 1994. Biosynthesis of lipooligosaccharide nodulation factors: *Rhizobium* NodA protein is involved in *N*-acylation of the chitooligosaccharide backbone. Proc. Natl. Acad. Sci. USA **91:**3122–3126.
26. **Romero, D., and R. Palacios.** 1997. Gene amplification and genomic plasticity in prokaryotes. Annu. Rev. Genet. **31:**91–111.
27. **Romero, D., G. Dávila, and R. Palacios.** 1998. The dynamic genome of *Rhizobium*, p. 153–161. *In* F. J. De Bruijn, R. Lupski, and G. M. Weinstock (ed.), Bacterial genomes. Chapman and Hall, New York, N.Y.
28. **Schultze, M., B. Quiclet-Sire, E. Kondorosi, H. Virelizier, J. N. Glushka, G. Endre, S. D. Géro, and A. Kondorosi.** 1992. *Rhizobium meliloti* produces a family of sulfated lipo-oligosaccharides exhibiting different degrees of plant host specificity. Proc. Natl. Acad. Sci. USA **89:**192–196.
29. **Scupham, A. J., A. H. Bosworth, W. R. Ellis, T. J. Wacek, K. A. Albrecht, and E. W. Triplett.** 1996. Inoculation with *Sinorhizobium meliloti* RMBPC-2 increases alfalfa yield compared with inoculation with a nonengineered wild-type strain. Appl. Environ. Microbiol. **62:**4260–4262.
30. **Simon, R., M. O'Connell, M. Labes, and A. Pühler.** 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. Methods Enzymol. **188:**640–659.
31. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, T. Tak, L. Goosen-de Roo, E. Pees, A. A. N. van Brussel, and B. J. J. Lugtenberg.** 1989. Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. J. Bacteriol. **171:**4045–4053.
32. **Spaink, H. P., A. Arts, G. Stacey, G. V. Bloemberg, B. J. J. Lugtenberg, and E. P. Kennedy.** 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Mol. Plant-Microbe Interact. **5:**72–80.
33. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. IBP Handbook, no. 15. Blackwell Scientific Publications, Ltd., Oxford, England.