Generation of *Rhizobium* strains with improved symbiotic properties by random DNA amplification (RDA)

Patrick Mavingui, Margarita Flores, David Romero, Esperanza Martínez-Romero, and *Rafael Palacios

Department of Molecular Genetics, Nitrogen Fixation Research Center, National University of Mexico, P.O. Box 565-A, Cuernavaca, Morelos, Mexico.
**Corresponding author: (e-mail: palacios@cifn.unam.mx).*

Received 26 November 1996; accepted 15 April 1997

To select for bacterial strains with enhanced phenotypes, random fragments of a whole genome, or a defined region of the genome, are cloned in a nonreplicating vector. The resulting plasmids are integrated by recombination into the homologous DNA region of the original strain. Integration gives rise to a nontandem direct duplication of the corresponding DNA region separated by the vector moiety of the plasmid. Recombination between the direct repeats leads to tandem duplication and further amplification of the entire integrated DNA, including the vector. Bacteria harboring the amplified DNA are selected by increasing the dosage of an antibiotic corresponding to a resistance marker of the integrated vector. Pooled strains carrying amplifications are then challenged with a selective pressure for the desired phenotype. After repeated selection cycles, the most fit strains are isolated. We used this process, which we called random DNA amplification, to select *Rhizobium* strains with increased competitiveness for nodule formation. Derivatives containing randomly amplified DNA regions of the symbiotic plasmid of *Rhizobium tropici* CFN299 strain were generated. Pools of amplified strains were inoculated onto various tropical legumes. After several cycles of selection through plants, amplified derivatives showing an increased competitiveness for nodule formation with the leguminous plant *Macroptilium atropurpureum* were obtained.

Keywords: combinatorial chemistry, DNA amplification, bacterial selection, nodulation, symbiotic competitiveness

There is abundant evidence that DNA amplification occurs naturally in the genome of prokaryotic organisms[1,2]. In some cases gene amplifications have been associated with adaptive responses such as growth on poor or limiting carbon sources[3,4], utilization of exotic carbon compounds[5], increased virulence[6,7], and increased antibiotic resistance in different bacteria[8].

Natural DNA amplification usually occurs by homologous recombination between reiterated DNA sequences present in direct orientation. The location of such sequences determines potentially amplifiable regions known as AUDs (amplifiable units of DNA) or amplicons[9,10]. An amplicon consists of a DNA segment bordered by two repeated DNA sequences present in direct orientation. Homologous recombination between these repeated sequences results in either a tandem duplication or a deletion of the amplicon sequence. Recombination between the tandemly duplicated regions may lead to either further amplification or reversion to the original genomic structure. Amplification of a defined region of the genome is then a dynamic state whose copy number may vary, and the nonamplified state may be restored without disrupting the architecture of the genome[9,10].

Amplification may potentially be induced in any region of the genome by using genetic manipulations that generate duplications. Some workers have exploited induced DNA amplification to enhance the production of particular compounds[11,12]. In these cases duplications were generated by cointegrating plasmids carrying the gene of interest into the homologous region of the genome.

We describe a strategy, called random DNA amplification (RDA), for the improvement of bacterial strains through the selection of desired properties without the need for identifying the specific genes involved in the process. To exemplify this strategy we applied RDA in *Rhizobium* and used competitiveness for nodule formation as the selectable property.

Bacteria of the genus *Rhizobium* interact with the roots of leguminous plants leading to the formation of nitrogen-fixing nodules. Nodule formation is the result of a cell differentiation process mediated by chemical signals, the production of which involves a large number of genes from both symbionts[13–15]. The symbiotic association has a certain degree of specificity because different *Rhizobium* species nodulate particular groups of legumes[16]. Strains within a given *Rhizobium* species possess different abilities to establish symbiosis. Under conditions where different strains coexist, a particularly important characteristic is the capacity to compete for nodule invasion.

The *Rhizobium* genome is a complex structure composed of the chromosome and several large plasmids. The plasmids range from 150 to 1700 kb, and the complete set of plasmids of a particular strain may represent 25% to 50% of the total genome[17]. Genes involved in the plant nodulation process have been found mostly on a large plasmid, the so called symbiotic plasmid (pSym)[18,19]. We have found that the *Rhizobium* genome—chromosome and

plasmids—contains a large amount of reiterated sequences[20], which undergo rearrangements at high frequency[21], particularly gene amplification[9,22,23]. These characteristics make *Rhizobium* an ideal organism for using the RDA strategy.

We applied RDA to the pSym (410 kb) of *Rhizobium tropici*, a broad-host-range species that nodulates several tropical legumes at different efficiencies[24]. This strategy allowed the generation of strains with improved competitive abilities for nodule formation in the legume *Macroptilium atropurpureum*.

Results

Rationale of the RDA strategy. This strategy is based on the generation of amplicon-type structures and may be applied to the whole or in a defined region of the genome. These structures are generated by the cointegration of recombinant plasmids containing randomly cloned DNA sequences into the homologous region of the genome. To obtain cointegration, the cloning vector must be incapable of replication in the recipient strain. The amplicon-type structures produced consist of nontandem direct duplications of the corresponding DNA segments separated by the length of the integrated vector. Recombination between the direct repeated DNA segments leads to tandem duplication and further amplification of the amplicon-type structure. Amplified derivatives are selected by using specific markers from the original cloning vector that respond to gene dosage. Pools of strains carrying amplifications are challenged with an appropriate selective condition. If DNA amplification of certain DNA regions gives some advantage under the particular conditions used, then the most fit strains may be selected. The amplified regions of the selected strains can be recovered owing to the dynamic nature of DNA amplification events, where homologous recombination among the tandem repeats may lead not only to further amplification but to the excision of closed circular structures as well. The excised structures are equivalent to monomers or multimers of the original recombinant plasmids because they contain the vector and the corresponding cloned DNA regions. These structures can be transferred by transformation or conjugation and recovered as stable plasmids in strains that allow their replication[9].

Application of RDA to obtain *Rhizobium* strains with improved competitiveness for nodule formation. For this purpose we used *R. tropici*, a broad-host-range species that nodulates different tropical legumes[24]. The strain used, *R. tropici* CFN299, harbors a pSym of 410 kb (ref. 25). To show the potential of RDA, we utilized leguminous plants that are poorly nodulated by this *Rhizobium* species[26]. The selectable property used was the competitiveness of strains for nodule formation.

The strategy developed is illustrated in Figure 1. A cosmid library of pSym fragments (30 to 40 kb) of *R. tropici* CFN299 was constructed in *Escherichia coli*. The cloning vector, pSUP205, contains an origin of replication for *E. coli* and a tetracycline-resistance marker (Fig. 1A). This vector can be transferred to, but does not replicate in, *Rhizobium*. One hundred *E. coli* clones containing recombinant cosmids from the pSym library were individually conjugated into *R. tropici* CFN299. *Rhizobium* exconjugants were selected by tetracycline-resistance. Because the cosmid could not replicate in *Rhizobium*, selection picked those cells in which the entire cosmid had integrated into the homologous region of the resident pSym (Fig. 1B). This cointegration resulted in the formation of an amplicon-type structure. Recombination between the direct repeated regions led to tandem duplication and further amplification of the region, including both the corresponding pSym sequence and the integrated vector (Fig. 1C). Amplification is accompanied by elevated resistance to tetracycline due to the increase in copy number of the

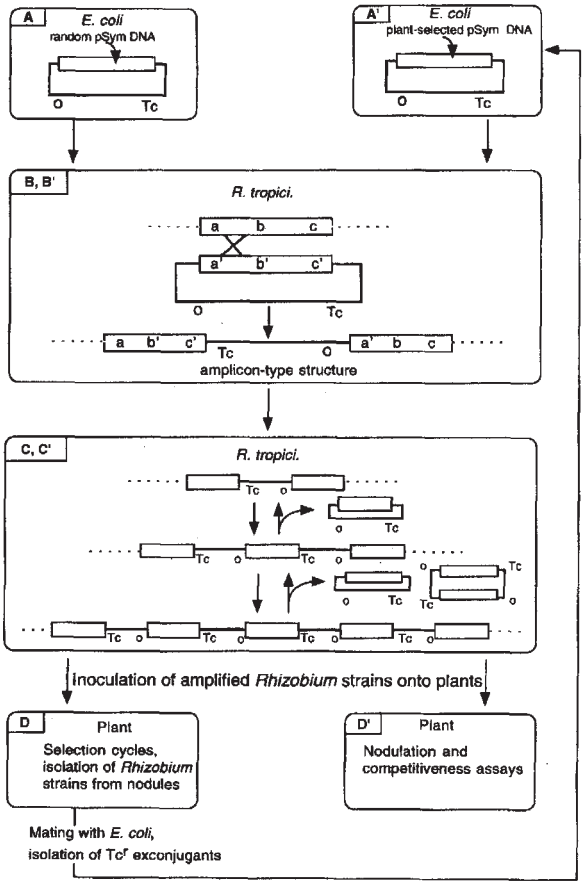

Figure 1. Diagram illustrating the RDA experimental design. (A) Cosmid library of random DNA fragments of the pSym of *R. tropici* CFN299 strain constructed in the cloning vector pSUP205. □, *Rhizobium* DNA sequences; ——, vector sequences; O, origin of replication; Tc, tetracycline resistance marker. Individual *E. coli* clones were conjugated with the original *R. tropici* strain and tetracycline resistant (Tc') *Rhizobium* exconjugants were isolated. This resulted in the cointegration of recombinant plasmids into the homologous region of the pSym. (B) Cointegration event. A region in the pSym (abc) was recombined with a homologous region (a'b'c') carried by the introduced plasmid. This resulted in the generation of an amplicon-type structure within the pSym (see text). -----, *Rhizobium* pSym sequences outside the amplicon. Amplified *Rhizobium* derivatives were then selected by increasing the tetracycline concentration in the culture medium. (C) The generation and dynamics of amplification (see text). Amplified *Rhizobium* strains were used as inoculum for leguminous plants. (D) After several plant selection cycles *Rhizobium* strains were isolated from nodules (see text). *Rhizobium* nodule isolates were conjugated with *Escherichia coli*, and tetracycline-resistant *E. coli* exconjugants were isolated. These exconjugants harbored plasmids containing the original cloning vector and the plant-selected *Rhizobium* DNA sequences (A'). These plasmids were used to generate cointegrations in the pSym homologous region (B') and amplifications (C') as described above. These amplified *Rhizobium* strains were compared with the wild-type strains in regard to nodulation kinetics and competitiveness (D').

tetracycline-resistance gene of the vector. Accordingly, *Rhizobium* derivatives containing amplified regions were selected by increasing the tetracycline concentration in the culture medium. Each selected strain was analyzed, and the presence of amplification was confirmed (see below).

DNA amplification is a dynamic state in which the copy number of the amplified sequence can increase or decrease as a result of homologous recombination between the tandem repeats. This results in the excision of closed circular structures consisting of

monomers or multimers of the amplified DNA sequence (Fig. 1C). Some of these may reinsert, while others will be lost by dilution as the cells divide. However, these structures can be recovered as stable plasmids in *E. coli*[9] (see below).

In order to select *Rhizobium* derivatives with enhanced competitiveness for nodule formation, amplified strains were inoculated onto different tropical legumes (Fig. 1D). Ten pools each containing a mixture of 10 amplified strains were used to inoculate the following leguminous plants: *Cajanus cajan* (L.), *Canavalia ensiformis* (L.), *Glycine max* (L.), *M. atropurpureum* (Urb.) and *Vigna umbellata* (Thumb). Two pools (C and D) showed earlier nodulation than the wild-type strain with *M. atropurpureum*. No clear differences were observed with other leguminous plants. Both pools and the control wild-type strain were used for further inoculation cycles in *M. atropurpureum*. After the third cycle, *R. tropici* derivatives were isolated from the nodules and conjugated with *E. coli*. Forty-eight exconjugants of *E. coli* exhibiting tetracycline resistance were isolated and analyzed for the presence of plasmids. All of them harbored plasmids containing *Rhizobium* sequences. Moreover, such plasmids showed an identical restriction pattern (see below). Plasmids from four *E. coli* strains, CFNC301(a-d), containing the *Rhizobium* sequences selected by *M. atropurpureum* were conjugated back into *R. tropici* CFN299 (Fig. 1A'). *Rhizobium* exconjugants harboring cointegrations were obtained (Fig. 1B') and amplified (Fig. 1C') as described above, generating four amplified derivatives named CFNX301(a-d). Finally, the new amplified *Rhizobium* derivatives were compared with the wild-type strain for kinetics of nodule formation and competitiveness (Fig. 1D').

Analysis of amplified *R. tropici* strains selected by the plant. The 100 original cosmids used for the cointegration into the pSym showed heterogeneity as observed by their restriction patterns. Only in a few cases were identical cosmid patterns detected. The restriction patterns of the cosmid sets that originated pools C (lanes 1 to 10) and D (lane 11 to 20) of amplified *Rhizobium* derivatives are shown (Fig. 2A). After plant selection cycles of pools C and D and conjugation of nodule-isolated strains with *E. coli*, the plasmids recovered from *E. coli* exconjugants were analyzed. All the plasmids containing *Rhizobium* sequences derived from pools C and D showed the same restriction pattern (Fig. 2A, lane 21). This common restriction pattern was present among the cosmids that gave rise to pools C (lane 3) and D (lane 17) of amplified *Rhizobium* derivatives.

To ascertain that the *Rhizobium* derivatives contained amplifications in the pSym, we analyzed their plasmid profiles hybridized against the integrating vector pSUP205. The 100 initial *Rhizobium* derivatives and CFNX301(a-d) strains had amplified regions. These were observed by the increase in the size of the pSym and by the presence of monomers and multimers of closed circular structures, as illustrated for strains CFNX301a and b (Fig. 2B). Furthermore, we performed a Southern blot analysis of the genomic DNA of CFNX301(a-d) derivatives hybridized against the plasmid recovered from *E. coli* CFNC301a strain. The four amplified derivatives exhibited a similar pattern. All the fragments revealed more intense hybridization signals compared with those of the wild-type strain. The results obtained with strains CFNX301a and b are presented (Fig. 2C). On average there were four copies of the amplified region in each strain.

Kinetics of nodule formation and competitiveness of amplified *Rhizobium* derivatives. The kinetics of nodule formation were analyzed by inoculating *M. atropurpureum* plants with the wild-type CFN299, the plant-recycled wild-type strain, and with the four amplified derivatives CFNX301(a-d). No differences were found between the original and the plant-recycled wild-type

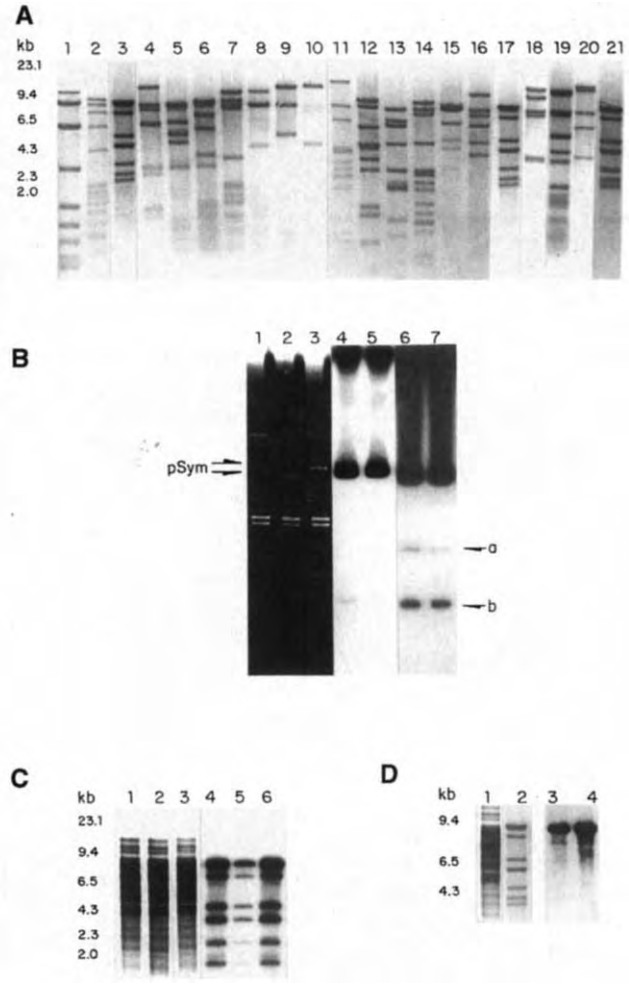

Figure 2. Characterization of recombinant plasmids and *R. tropici* strains. (A) EcoRI fragment patterns stained with ethidium bromide of the cosmid sets of the pSym library used to generate the amplified *Rhizobium* derivatives corresponding to pools C (lanes 1–10) and D (lanes 11–20) and of the plasmid present in *E. coli* strain CFNC301a (lane 21). The DNA molecular size markers are indicated on the left-hand side. (B) Plasmid profiles of *R. tropici* strains stained with ethidium bromide (lanes 1–3) and hybridized against the vector pSUP205 (lanes 4–7); lanes 6 and 7 are an overexposure of the corresponding autoradiographs presented in lanes 4 and 5. Wild-type *R. tropici* CFN299, lane 2; amplified *R. tropici* strain CFNX301a, lanes 1, 4, and 6; amplified *R. tropici* strain CFNX301b, lanes 3, 5, and 7. pSym: symbiotic plasmid; a, b: closed circular DNA. (C) EcoRI fragment patterns of total genomic DNA stained with ethidium bromide (lanes 1–3) and hybridized against the plasmid recovered from *E. coli* strain CFNC301a (lanes 4–6). Wild-type *R. tropici* strain CFN299, lanes 2 and 5; *R. tropici* strain CFNX301a, lanes 1 and 4; *R. tropici* strain CFNX301b, lanes 3 and 6. (D) EcoRI fragment patterns of total genomic and plasmid DNA stained with ethidium bromide (lanes 1 and 2) and hybridized against a *R. tropici nod*D3 probe (lanes 3 and 4). Wild-type *R. tropici* strain CFN299, lanes 1 and 3; plasmid pCFNC301a, lanes 2 and 4.

strains. Four plants per each amplified derivative were compared with 16 plants inoculated with the wild-type strain. Data from the 16 plants of each group were combined and are presented (Fig. 3). Amplified derivatives induced nodules earlier than the wild-type strain, judged either by the number of nodules per plant (Fig. 3A) or by the percentage of nodulated plants (Fig. 3B). By using the nonparametric Kolmogorov-Smirnov two-sample test[27], the differences between the two groups (amplified derivatives and wild-type strain) were found to be statistically signifi-

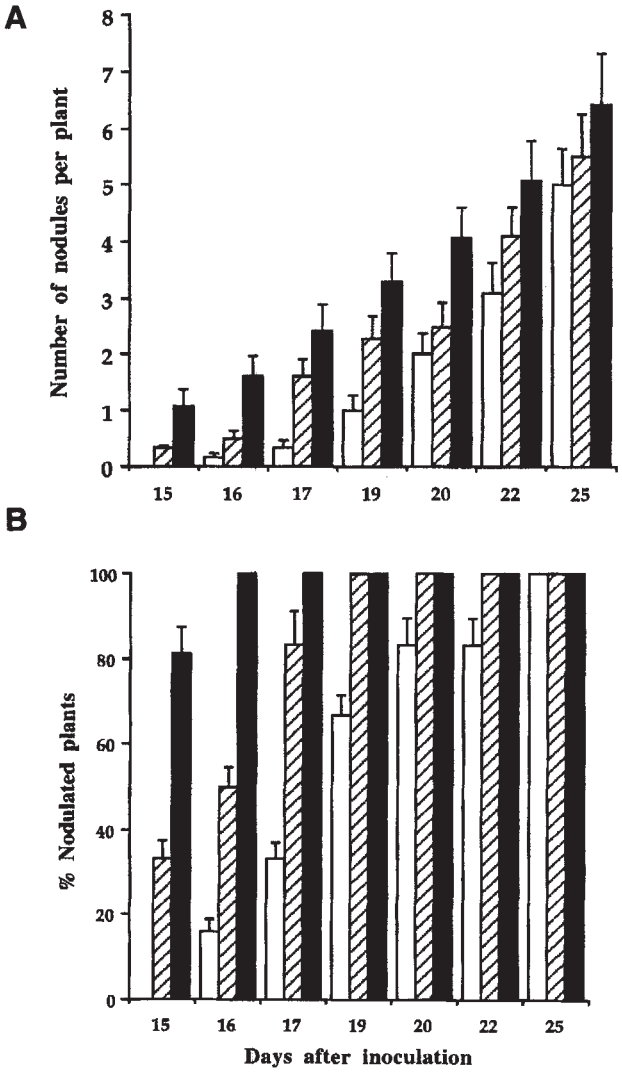

Figure 3. Kinetics of nodule formation on *M. atropurpureum* elicited by *R. tropici* wild-type CFN299 and derivatives carrying amplifications. CFN299 (open bars); CFNX301a (black bars); and CFNX301a/CFN299 in 1:10 ratio of inoculum (hatched bars). The values represent the median ±95% confidence interval of 16 replicates of inoculation with individual strains (see text) and of six replicates for coinoculation. The experiment was repeated 4 times; representative results are shown.

cant ($p<0.0001$) during the first days of nodulation. Significance continues ($p<0.01$) over the course of nodulation kinetics measurements, except for the percentage of nodulated plants, which became similar for both groups on day 25 after inoculation because all the plants were nodulated. When the data were analyzed separately for each of the four amplified strains, all of them showed significantly earlier nodulation than the wild-type strain (data not shown).

Earlier nodulation has been reported to be correlated with competitiveness[28]. We explored the ability of the amplified derivative CFNX301a to compete with the wild-type CFN299 for nodule formation during coinoculation at different ratios. At a 1:1 ratio of inoculation, kinetics of *M. atropurpureum* nodulation (not shown) was similar to that obtained with amplified derivatives inoculated independently. When a ratio of 1:10 (amplified vs wild-type) of inoculum was applied to plants, nodules also appeared earlier, as seen with amplified strains inoculated alone in comparison to the wild-type strain (Fig. 3). When the wild-type strain outnumbered

Table 1. Competitiveness of amplified derivatives relative to wild-type.

Amplified strain	Inoculum ratio*	Nodule occupancy† % Amplified	% wild
CFNX301a	1:1	100[a]	0[b]
	1:10	42[a]	58[b]
	1:100	16[a]	84[b]

*Ratio of amplified strain to wild-type in the inoculum. †Percentage of 4 nodules per plant occupied by the tested strains. Values are means from 6 plants. a,b: significally different for each inoculum ratio ($p<0.05$) as determined by χ^2 test.

amplified derivative by 100-fold, nodulation kinetics ressembled those of CFN299 alone but with a slightly earlier and increased nodulation (not shown). These results indicate that the presence of the amplified strain in the inoculum improves plant nodulation. The improvement of nodule formation by the amplified strain was correlated with its competitive ability as defined by nodule occupancy (Table 1). Indeed, at a 1:1 ratio of inoculation, the amplified derivative occupied all of the nodules formed on *M. atropurpureum*. When a tenfold excess of the wild-type strain was applied, the amplified derivative competed well by occupying 42% of nodules. Even with an inoculum containing a 100-fold excess of the wild-type strain, a significant proportion of nodules (16%) contained the amplified strain.

Search for symbiotic gene sequences in plant-selected *Rhizobium* DNA. The *Rhizobium* DNA recovered in *E. coli* plasmids pCFN301a-d is a region of 30 kb. This region was hybridized under high stringency conditions against various probes of *R. tropici* genes involved in the plant nodulation process. These included *nod* genes A,B,C,I,J,H,P,Q,S,U and the five different copies of *nod*D. As expected, all the probes showed positive hybridization signals with the wild-type *R. tropici* strain CFN299 (data not shown). The 30 kb pSym region selected by the plant showed hybridization only with the probe from gene *nod*D3 (Fig. 2D). The EcoRI DNA fragment revealed in plasmid pCFNC301a was identical in size (8 kb) to that observed in the wild-type strain.

Discussion

We describe a novel strategy, RDA, useful for the generation of bacterial strains with improved selectable properties. The potential success of the RDA approach relies on imposing a powerful selective pressure to the amplified derivatives generated in the procedure. If amplification of a certain region of the genome confers an advantage under the particular conditions used, then the most fit strains may be selected.

In this study we used competitiveness for nodule formation as a selective pressure for amplified *Rhizobium* derivatives. Competitiveness for nodule formation is a complex phenomenon and is considered a limiting step for field legume inoculation. Little is known regarding the molecular basis of competitiveness, and many different mutations affect this process[29]. In some cases genetic manipulation has resulted in an increase in competitiveness[30–32]. For these reasons we considered competitiveness for nodule formation a particularly interesting biological property to be addressed by the RDA strategy. The results show that the application of RDA to the pSym of *R. tropici* resulted in the generation of strains with improved competitive ability for nodulation of the leguminous plant *M. atropurpureum*. In fact, *M. atropurpureum* selected only one type of amplified derivative from a large collection of strains carrying amplifications of different regions within the pSym of *R. tropici* CFN299. Strains CFNX301(a-d) constructed by directed amplification using the cosmid that confered to *R. tropici* an

advantage for *M. atropurpureum* clearly showed earlier nodulation and higher competitiveness for nodule formation compared with the wild-type strain.

It is important to point out that in the experiments presented here we used only one cycle of inoculation as the initial selective pressure. Because pools of 10 amplified strains were used, only highly competitive strains responsible for earlier nodulation when present in approximately 10% of the inoculated population could be selected. Strains with less pronounced effects might be obtained by either increasing the number of plant nodulation cycles for the initial selection step or decreasing the number of strains in each pool.

Selection of specific amplified strains depends strictly on the particular conditions used. Because the main purpose of this study was to show the potential of RDA, laboratory conditions were employed. If RDA is used with the aim of selecting strains with potential utility in agriculture, defined greenhouse or field conditions should be employed in the selection cycles.

In addition to its potential biotechnological value, RDA can be used as a first step in the analysis of sequences involved in complex biological functions. In fact, the amplified DNA regions selected by RDA can be recovered as stable plasmids in *E. coli*. This allows their molecular characterization. In the present study the amplified DNA region from the *Rhizobium* derivatives selected by several inoculation cycles in the leguminous plant *M. atropurpureum* was transferred into *E. coli* by conjugation. The *Rhizobium* DNA recovered from *E. coli* is a region of 30 kb from the pSym of *R. tropici* strain CFN299. A preliminary characterization of this region was made by hybridization against various probes of *Rhizobium* genes involved in the plant nodulation process. In the *Rhizobium* species studied so far the majority of *nod* genes are clustered in a particular region of the pSym. Such genes are organized into coordinately regulated operons forming a functional unit known as the *nod* regulon. The major function of the *nod* regulon is the production of chemical signals (Nod factors) that induce the plant responses that culminate in nodule formation. The central regulatory element of the *nod* regulon is the transcriptional activator NodD, encoded by the *nod*D gene. In some *Rhizobium* species several copies of *nod*D that encode similar but not identical NodD proteins have been found. The different NodD proteins may be involved in the determination of specificity for plant nodulation[15]. In the case of *R. tropici*, five copies of *nod*D genes (*nod*D1 to *nod*D5) were found in the pSym of CIAT899 strain[33]. Different genes involved in the production of Nod factors have also been identified in this *R. tropici* species. These include nod genes A,B,C,I,J,H,P,Q,S, and U (refs. 34 to 37). The 30-kb pSym region recovered in this study showed hybridization only with the gene *nod*D3. To correlate the symbiotic phenotype with the amplification of specific DNA sequences, a more comprehensive characterization of the 30-kb pSym region is necessary.

RDA results in the generation of cell populations that contain amplifications of different DNA sequences. Because these amplifications are in a dynamic state, each particular region amplified is present at a different copy number in individual cells of the population. An alternative strategy to increase the copy number of DNA sequences, which has been used extensively, involves introducing DNA fragments on multicopy plasmids into the strains to be selected[38]. In this case the plasmid copy number is determined by the particular origin of replication. Although it appears more laborious, the RDA approach might present an advantage because bacterial cells with different copy numbers of the corresponding regions may be selected from the cell population.

We propose that, if an appropriate selective pressure is defined for a particular biological function, RDA can be used as a general strategy to generate bacterial strains with improved properties for either fundamental research or biotechnological purposes.

Experimental approach

DNA manipulations. Plasmid DNA was isolated from *E. coli* using a Wizard plus minipreps kit (Promega, Madison, WI). Plasmid profiles of *Rhizobium* were obtained by a modified Eckhardt technique[39], as described by Geniaux et al.[40] Genomic DNA was isolated from *Rhizobium* using a DNA/RNA isolation kit (USB, Amersham, Cleveland, OH). Standard DNA manipulations such as restriction, agarose gel electrophoresis, and filter blot hybridization were performed as described by Flores et al.[20] Probes were radiolabelled with ^{32}P by a random priming DNA labelling kit (*redi*prime, Amersham). DNA amplification was quantified by densitometric integration of the hybridization signals from autoradiographs using the Eagle Eye II system (Stratagene, La Jolla, CA).

Construction of the pSym cosmid library. The pSym of *R. tropici* CFN299, 410 kb, was isolated from an *Agrobacterium tumefaciens* transconjugant devoid of endogenous plasmids[25] following the procedure used by Hirsch et al.[41] DNA was partially digested with EcoRI to obtain fragments of 30 to 40 kb, and then ligated into the cosmid vector pSUP205, which contains an origin of replication from *E. coli*. pSUP205 can be transferred to, but does not replicate in, *Rhizobium*[42]. After in vitro packaging and infection of *E. coli* HB101, the mixture was plated on LB (1% peptone, 0.5% yeast extract, 1% NaCl) agar plates containing 10 μg/ml tetracycline for the selection of *E. coli* clones containing recombinant cosmids.

Construction of *R. tropici* strains carrying cointegration in the pSym, and further amplification. For this purpose, 100 *E. coli* clones containing recombinant cosmids were conjugated individually with *R. tropici* CFN299 strain in a triparental mating using *E. coli* HB101/pRK2013 as a helper[43]. The *R. tropici* exconjugants with cointegrated cosmids in the homologous regions of pSym were selected on PY (0.5% peptone, 0.3% yeast extract, 10 mM $CaCl_2$) agar plates containing 20 μg/ml nalidixic acid and 5 μg/ml tetracycline. To select *Rhizobium* derivatives carrying amplifications, *Rhizobium* exconjugants were cultivated overnight in liquid PY medium supplemented with 10 μg/ml tetracycline, and appropriate dilutions were plated onto PY agar plates containing 10 μg/ml tetracycline. *Rhizobium* colonies carrying amplifications were verified by Southern blot analysis of their plasmid profiles hybridized with the vector pSUP205 as a probe.

Plant inoculation and selection cycles. For plant inoculation assays, the following tropical legumes were used: *Cajanus cajan* (L.) Millsp., *Canavalia ensiformis* (L.) DC, *Glycine max* (L.) Merr. Peking, *Macroptilium atropurpureum* (Urb.) and *Vigna umbellata* (Thumb.) ohwi & ohashi. Seeds were surface-disinfected by immersing them for 10 min in concentrated sulfuric acid followed by thorough rinsing with sterile water. Seeds were germinated on water agar plates, and seedlings were inoculated by dipping the roots in bacterial suspensions of inocula. The seedlings were transferred to 250-ml Erlenmeyer flasks containing Fahraeus[44] agar without added nitrogen and were grown in a growth chamber under a 16-h photoperiod. Day and night temperatures were 29°C and 18°C, respectively, with a relative humidity of 80%.

For plant selection of *R. tropici* strains with enhanced symbiotic properties, roots were inoculated with 10 pools (A–J) of *Rhizobium* amplified strains each containing 10 different derivatives. Three plants of each of the legumes were inoculated with each pool containing a total of 10^5 cells. Plants inoculated with the wild-type CFN299 were used as a control. Plants were checked daily for nodule appearence. Earlier nodulation was used as a criterion to select plants for further inoculation cycles. In the case of *M. atropurpureum*, plants inoculated with two pools (C and D) clearly showed earlier nodulation than those inoculated with the wild-type strain. Accordingly, nodules from *M. atropurpureum* plants inoculated with either pools C or D were used as inoculum for further nodulation cycles. Four nodules from each of the three plants originally inoculated with each pool were combined, surface-disinfected 1 min in 70% ethanol and 5 min in sodium hypochlorite, crushed, and used as inoculum for six plants. For the third plant selection cycle, four nodules from each of the plants were combined, crushed, and used as inoculum for six new plants. Two nodules from each of these six new plants (a total of 24 nodules) were individually crushed, resuspended in 10 mM $MgSO_4$, and plated on PY medium containing 20 μg/ml nalidixic acid with or without 5 μg/ml tetracycline. Two clones from each nodule were used for in vivo cloning of *Rhizobium* sequences in *E. coli* (see below). The plasmids isolated from the 48 *E. coli* exconjugants had an identical restriction pattern (see Results). Four of these *E. coli* clones—

CFNC301(a-d)—were used to construct new *R. tropici* strains by the cointegration and amplification of the selected sequences (see above). *R. tropici* amplified strains were designated CFNX301(a-d) and used to study nodulation kinetics and competitiveness.

Comparative nodulation kinetics and competitiveness assays. Comparative nodulation kinetics assays were performed by inoculating plants with 10^5 cells per seedling. Four plants were inoculated with each of the four amplified derivatives CFNX301(a-d), and 16 plants were inoculated with the wild-type strain. Plants were checked daily for nodule appearance. The number of nodules formed and the percentage of nodulated plants were followed up until 25 days after inoculation. The identity of the inoculated strains was ascertained by plating nodule suspensions on PY medium and then testing resistance of individual colonies with the appropriate antibiotics. The number of *Rhizobium* cells per nodule was similar for all of inoculated strains, about 10^6 per nodule.

For nodulation competitiveness tests, inocula were prepared by mixing the amplified strain CFNX301a with the wild-type strain CFN299 in different ratios: 1:1, 1:10, and 1:100, which contained 10^5 cells of the amplified strain and 10^5, 10^6, and 10^7 cells of wild-type strain, respectively. Six plants were inoculated per inoculum combination. The nodulation kinetics were followed up as mentioned above. On day 25 after inoculation, four nodules per plant (representing 24 nodules per inoculum ratio) were individually crushed, and appropriate dilutions were plated on PY agar containing 20 μg/ml nalidixic acid with or without 5 μg/ml tetracycline. The proportion of nodules occupied by each strain was evaluated by the ratio of clones growing on each medium and confirmed by hybridization of plasmid profiles of 60 clones obtained from each medium (with or without tetracycline) using the cloning vector pSUP205 as a probe.

In vivo cloning of amplified *Rhizobium* DNA into *E. coli*. *Rhizobium*-amplified derivatives isolated from nodules after the third inoculation cycle in *M. atropurpureum* were used as donors in a triparental mating using *E. coli* DH5α as a recipient and *E. coli* HB101/pRK2013 as helper. *E. coli* exconjugants containing *Rhizobium* sequences as stable plasmids were selected on LB agar plates supplemented with 20 μg/ml nalidixic acid and 10 μg/ml tetracycline.

Acknowledgments

We are very grateful to S. Pueppke for providing legume seeds. We thank Angeles Moreno and Jose A. Gama for technical assistance, and Michael Dunn and Michael Hynes for helpful comments on the manuscript. This work was supported in part by grants from the Rockefeller Foundation (USA) and CONACYT (Mexico).

1. Anderson, R.P. and Roth, J.R. 1977. Tandem genetic duplications in phage and bacteria. *Annu. Rev. Microbiol.* **31:**473–505.
2. Petes, T.D. and Hill, C.W. 1988. Recombination between repeated genes in microorganisms. *Annu. Rev. Microbiol.* **22:**147–168.
3. Neuberger, M.S. and Hartley, B.S. 1981. Structure of an experimentally evolved gene duplication encoding ribitol dehydrogenase in a mutant of *Klebsiella aerogenes*. *J. Gen. Microbiol.* **122:**181–191.
4. Sonti, R.V. and Roth, J.R. 1989. Role of gene duplications in the adaptation of *Salmonella typhimurium* to growth on limiting carbon sources. *Genetics* **123:**19–28.
5. Ghosal, D., You, I.-S., Chatterjee, D.K., and Chakrabarty, A.M. 1985. Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4. *Proc. Natl. Acad. Sci. USA* **82:**1638–1642.
6. Mekalanos, J.J. 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. *Cell* **35:**253–263.
7. Corn, P.G., Anders, J., Takala, A.K., Käyhty, H., and Hoiseth, S.K. 1993. Genes involved in *Haemophilus influenzae* type b capsule expression are frequently amplified. *J. Infect. Dis.* **167:**356–364.
8. Peterson, B.C. and Rownd, R.H. 1985. Drug resistance gene amplification of plasmid NR1 derivatives with various amounts of resistance determinant DNA. *J. Bacteriol.* **161:**1042–1048.
9. Flores, M., Brom, S., Stepkowski, T., Girard, M.L., Dávila, G., Romero, D., et al. 1993. Gene amplification in *Rhizobium*: Identification and in vivo cloning of discrete amplifiable DNA regions (amplicons) from *Rhizobium leguminosarum* biovar *phaseoli*. *Proc. Natl. Acad. Sci. USA* **90:**4932–4936.
10. Roth, J.R., Benson, N., Galitski, T., Haack, K., Lawrence, J.G., and Miesel, L. 1996. Rearrangements of the bacterial chromosome: formation and applications, pp. 2256–2276 in: Escherichia coli *and* Salmonella typhimurium*: cellular and molecular biology*, 2nd. ed. Neidhardt, F.C., Curtiss III, R., Ingrham, J.L., Lin, E.C.C., Low Jr., K., Magasanik, B., et al. (eds.) American Society for Microbiology Press, Washington, DC.
11. Albertini, A.M. and Galizzi, A. 1985. Amplification of a chromosomal region in *Bacillus subtilis*. *J. Bacteriol.* **162:**1203–1211.
12. Kallio, P., Palva, A., and Palva, I. 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:**64–71.
13. Fisher, R.F. and Long, S.R. 1992. *Rhizobium*-plant signal exchange. *Nature* **357:**655–660.
14. Dénarié, J., Debellé, F., and Rosenberg, C. 1992. Signaling and host range variation in nodulation. *Annu. Rev. Microbiol.* **46:**497–531.
15. Schultze, M., Kondorosi, E., Ratet, P., Buiré, M., and Kondorosi, A. 1994. Cell and molecular biology of *Rhizobium*-plant interactions. *Int. Rev. Cytol.* **156:**1–74.
16. Martínez-Romero, E. and Caballero-Mellado, J. 1996. *Rhizobium* phylogenies and bacterial genetic diversity. *Crit. Rev. Plant Sci.* **15:**113–140.
17. Honeycutt, R.J., McClelland, M., and Sobral, B.W.S. 1993. Physical map of the genome of *Rhizobium meliloti* 1021. *J. Bacteriol.* **175:**6945–6952.
18. Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I., and Kondorosi, A. 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. *Mol. Gen. Genet.* **184:**318–325.
19. Rosenberg, C., Boistard, P., Dénarié, J., and Casse-Delbart, F. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. *Mol. Gen. Genet.* **184:**326–333.
20. Flores, M., González, V., Brom, S., Martínez, E., Piñero, D., Romero, D., et al. 1987. Reiterated DNA sequences in *Rhizobium* and *Agrobacterium*. *J. Bacteriol.* **169:**5782–5788.
21. Flores, M., González, V., Pardo, M.A., Leija, A., Martínez, E., Romero, D., et al. 1988. Genomic instability in *Rhizobium phaseoli*. *J. Bacteriol.* **170:**1191–1196.
22. Romero, D., Brom, S., Martínez-Salazar, J., Girard, M.L., Palacios, R., and Dávila, G. 1991. Amplification and deletion of a *nod-nif* region in the symbiotic plasmid of *Rhizobium phaseoli*. *J. Bacteriol.* **173:**2435–2441.
23. Romero, D., Martínez-Salazar, J., Girard, L., Brom, S., Dávila, G., Palacios, R., et al. 1995. Discrete amplifiable regions (amplicons) in the symbiotic plasmid of *Rhizobium etli* CFN42. *J. Bacteriol.* **177:**973–980.
24. Martínez-Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P., and Pardo, M.A. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int. J. Syst. Bacteriol.* **41:**392–394.
25. Martínez, E., Palacios, R., and Sánchez, F. 1987. Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. *J. Bacteriol.* **169:**2828–2834.
26. Hernández-Lucas, I., Segovia, L., Martínez-Romero, E., and Pueppke, S.G. 1995. Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. *Appl. Environ. Microbiol.* **61:**2775–2779.
27. Hollander, M. and Wolfe, D.A. 1973. *Nonparametric statistical methods.* John Wiley & Sons, Inc., New York.
28. Dowling, D.N. and Broughton, W.J. 1986. Competition for nodulation of legumes. *Annu. Rev. Microbiol.* **40:**131–157.
29. Triplett, E.W. 1990. The molecular genetics of nodulation competitiveness in *Rhizobium* and *Bradyrhizobium*. *Mol. Plant-Microbe Interact.* **3:**199–206.
30. Bromfield, E.S.P., Lewis, D.M., and Barran, L.R. 1985. Cryptic plasmid and rifampicin resistance in *Rhizobium meliloti* influencing nodulation competitiveness. *J. Bacteriol.* **164:**410–413.
31. Sanjuan, J. and Olivares, J. 1991. Multicopy plasmids carrying the *Klebsiella pneumoniae nifA* gene enhance *Rhizobium meliloti* nodulation competitiveness on alfalfa. *Mol. Plant-Microbe Interact.* **4:**365–369.
32. Beattie, G.A. and Handelsman, J. 1993. Evaluation of a strategy for identifying nodulation competitiveness genes in *Rhizobium leguminosarum* biovar *phaseoli*. *J. Gen. Microbiol.* **139:**529–538.
33. van Rhijn, P.J.S., Feys, B., Verreth, C., and Vanderleyden, J. 1993. Multiple copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816. *J. Bacteriol.* **175:**438–447.
34. Vargas, C., Martínez, L.J., Megías, M., and Quinto, C. 1990. Identification and cloning of nodulation genes and host specificity determinants of the broad host range *Rhizobium leguminosarum* bv. *phaseoli* strain CIAT899. *Mol. Microbiol.* **4:**1899–1910.
35. Waelkens, F., Voets, T., Vlassak, K., Vanderleyden, J., and van Rhijn, P. 1995. The *nodS* gene of *Rhizobium tropici* strain CIAT899 is necessary for nodulation on *Phaseolus vulgaris* and on *Leucaena leucocephala*. *Mol. Plant-Microbe Interact.* **8:**147–154.
36. Folch-Mallol, J.L., Marroquí, S., Sousa, C., Manyani, H., López-Lara, I.M., van der Drift, K.M.G.M., et al. 1996. Characterization of *Rhizobium tropici* CIAT899 nodulation factors: the role of *nodH* and *nodPQ* genes in their sulfation. *Mol. Plant-Microbe Interact.* **9:**151–163.
37. Laeremans, T., Caluwaerts, I., Verreth, C., Rogel, M.A., Vanderleyden, J., and Martínez-Romero, E. 1996. Isolation and characterization of the *Rhizobium tropici* Nod factor sulfation genes. *Mol. Plant-Microbe Interact.* **9:**492–500.
38. Simon, R. and Priefer, U.B. 1990. Vector technology of relevance to nitrogen fixation research, pp. 13–49 in *Molecular biology of symbiotic nitrogen fixation*. Gresshoff, P.M.P (ed). CRC Press, Boca Raton, FL.
39. Eckhardt, T. 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. *Plasmid* **1:**584–588.
40. Géniaux. E., Flores, M., Palacios, R., and Martínez, E. 1995. Presence of megaplasmids in *Rhizobium tropici* and further evidence of differences between the two *R. tropici* subtypes. *Int. J. Syst. Bacteriol.* **45:**417–426.
41. Hirsch, P.R., Van Montagu, M., Johnston, A.W.B., Brewin, N.J., and Schell, J. 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **120:**403–412.
42. Simon, R., O'Connell, M., Labes, M., and Pühler, A. 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other Gram-negative bacteria. *Methods Enzymol.* **188:**640–659.
43. Figurski, D.H. and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependant on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **76:**1648–1652.
44. Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a single glass technique. *J. Gen. Microbiol.* **16:**374–381.