Presence of Megaplasmids in *Rhizobium tropici* and Further Evidence of Differences between the Two *R. tropici* Subtypes

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Using a modified Eckhardt method, we visualized replicons larger than 1,000 kb in *Rhizobium tropici* strains belonging to both subgroup A and subgroup B. The megaplasmid of *R. tropici* CFN299 was characterized. This megaplasmid is different from a cointegrate of various plasmids and from the chromosome. Hybridization of Eckhardt blots of 15 *R. tropici* strains with fragments derived from the megaplasmids of the type strains of subgroups A and B revealed that the megaplasmids are subgroup specific.

*Rhizobium* spp. form nitrogen-fixing nodules on the roots of leguminous plants. In *Rhizobium* strains plasmids constitute an important part of the genome (11, 18, 28). Megaplasmids larger than 1,000 kb have been found in *Rhizobium meliloti* (32), *Rhizobium fredii* (20), and *Rhizobium galegae* strains (24). In *Rhizobium* strains, genes essential for nodule formation and nitrogen fixation are located on plasmids called symbiotic plasmids (pSym) (12, 25), and these genes have been shown to be located on megaplasmids in *R. meliloti* (11, 32).

*Rhizobium tropici* strains nodulate *Phaseolus vulgaris* L. beans and other tropical legumes, including *Leucaena* sp. trees. Two subgroups, corresponding to type A and type B strains, have been described for this species on the basis of differences in rRNA genes and differences in several other phenotypic characteristics, including resistance to metals and antibiotics and the ability to metabolize certain carbon sources (19). We analyzed 15 previously described (19) *R. tropici* strains, including members of both subgroups, for their plasmids by using a modification (37) of the Eckhardt procedure (4) in which horizontal agarose gels are used (Fig. 1A). A replicon larger than 1,000 kb that has not been observed previously was found in all of the *R. tropici* strains, as well as in *R. fredii* USDA 191 (13) and *R. meliloti* RCR 2011, but not in *Rhizobium loti* NZP2037 (3), *Rhizobium leguminosarum* bv. viciae VF39 (29), *R. leguminosarum* bv. trifolioli USDA 2152, or *Rhizobium etli* CFN422 (30) (Fig. 2). The nature of the replicon was determined in type A strain CFN299. To verify that this replicon was not a cointegrate of smaller plasmids, the homology between the megaplasmid and smaller plasmids was examined. Each of the smaller plasmids used (185, 225, and 410 kb) was transferred to an *Agrobacterium tumefaciens* plasmidless strain (16; this study), purified from it, and used as a probe for the CFN299 plasmid. We observed no homology with any of the plasmids (Fig. 3, lanes 1 to 4). In order to distinguish between a chromosomal and a megaplasmid, an Eckhardt blot was hybridized to a 300-bp fragment of the *R. etli* 16S rRNA genes (34). No hybridization was detected (Fig. 3, lane 5), indicating that the replicon was different from the chromosome.

When *R. tropici* CFN299 was cross-mated with *Escherichia coli* S17 pSup 5011 (35), the CFN299 mutants (which were resistant to 60 mg of kanamycin per liter) were analyzed to locate Tn5. Hybridization between pSup 5011 and blotted plasmid patterns revealed that the frequencies of Tn5 insertion for each replicon were 52% for the chromosome, 30% for the megaplasmid, 10% for the pSym, and 8% for plasmids a and b. These values are consistent with the estimated molecular weights of the chromosome and the plasmids (17) and clearly show that insertions occurred independently in each replicon.

Eckhardt blots of CFN299 were hybridized to the following different genes related to symbiosis: *nifHDK* (from pCQ15 [30]), *ips* (from pCOS309 [2]), *exoF* (from pEX80 [5]), *dctABD* (from pRTI505 [7]), and the citrate synthase gene (from pMP7 [27]). Using high-stringency hybridization conditions, we found that only the *exoF* gene hybridized to the megaplasmid. In *R. meliloti* *exoF* is also located on a megaplasmid, as are other *exo* genes (15). Although exopoly saccharide-deficient mutants of *R. tropici* have been described (21), the localization of the exopoly saccharide genes has not been reported previously. Our results suggest that genes for exopoly saccharide production could be harbored in the *R. tropici* megaplasmid. The *nifHDK* and citrate synthase genes hybridized to pSym, as reported previously (Fig. 3).

To study the structural conservation of these megaplasmids in *R. tropici* strains, fragments of the megaplasmids from strains CFN299 (type A) and CIAT899 (type B) were used as probes. To do this, each megaplasmid was labeled with Tn5-mob and transferred to plasmid-free *A. tumefaciens* strains by using RP4-4 as a helper. The latter was transferred from VF39 SM/PRtVF39c::Tn5-mob/RP4-4 (10). With this procedure, only deleted megaplasmids that were 100 to 450 kb long were obtained in *A. tumefaciens* transconjugants. These organisms were grown in Luria broth (33) supplemented with rifampin (200 mg/liter) and neomycin (60 mg/liter). Two agrobacteria, each harboring 450-kb fragments from either CFN299 or CIAT 899 (9), were used as sources of the probes for hybridization to Eckhardt blots (Fig. 1B and C). The CFN299 megaplasmid was structurally conserved in strains BR10043, BR846, BR842, BR836, BR835, and BR833, which belong to the same subgroup (Fig. 1B, lanes 1 to 7). Likewise, the CIAT 899 megaplasmid was structurally conserved in strains BR847, BR852, BR857, BR858, and BR859, which belong to the same subgroup (Fig. 1C, lanes 8 to 13). No homology between megaplasmids in members of the two subgroups was found. Therefore, the megaplasmids are subgroup specific.

*R. tropici* is native to South America but has also been isolated from nodules of *P. vulgaris* from acid soils in Kenya (18). Very recently, type A *R. tropici* strains have also been isolated from nodules of *P. vulgaris* plants grown in sandy soils in southwestern France (1). In this ecological study, nodule
isolates were characterized for their plasmid contents, and in some cases a high-molecular-weight band (like the one described in this paper) was also observed in these strains.

Megaplasmids have sometimes been considered minichromosomes in members of the genus Rhizobium because they harbor genes that may be important for bacterial survival (6). Furthermore, it has not been possible to remove them from bacteria in the laboratory. Megaplasmids have been found to be more difficult to transfer from one bacterium to another than smaller plasmids are (as we observed). In taxonomic and systematic studies it has been recommended that bacterial species should be established on the basis of chromosomal characteristics. The contribution of megaplasmids to the definition of genetically significant groups should not be denied, in view of the large amount of DNA contained in megaplasmids and in view of the relative stability of megaplasmids in bacterial lineages.

DNA-DNA hybridization experiments revealed that the level of homology was relatively low (36%) when type A and type B R. tropici strains were compared in two independent studies (14, 17). Other workers have pointed out additional differences between the two types (23). Type B strains produce chlorosis in bean plants under certain growth conditions, while type A strains do not (26). A PCR analysis of repetitive se-
quences clearly distinguished R. tropici type A strains from type B strains (36). Likewise, we found that while both types of strains harbor megaplasmids that are structurally conserved in strains belonging to the same subgroup, there is no overall homology between the megaplasmids in members of the two subgroups. The megaplasmids are therefore subgroup specific. The subgroup specificity of the megaplasmids represents a new finding for this species. This additional difference between the two subgroups could support the proposal that in the future taxonomists should define these two groups as subspecies or even different species, as originally proposed by Martinez et al. (17) and more recently by van Berkum et al. (36).

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REFERENCES