Effects of *Medicago truncatula* Genetic Diversity, Rhizobial Competition, and Strain Effectiveness on the Diversity of a Natural *Sinorhizobium* Species Community

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We investigated the genetic diversity and symbiotic efficiency of 223 *Sinorhizobium* sp. isolates sampled from a single Mediterranean soil and trapped with four *Medicago truncatula* lines. DNA molecular polymorphism was estimated by capillary electrophoresis–single-stranded conformation polymorphism and restriction fragment length polymorphism on five loci (*IGS*<sub>NOD</sub>, *typA*, *virB11*, *avhB11*, and the 16S rRNA gene). More than 90% of the rhizobia isolated belonged to the *Sinorhizobium medicae* species (others belonged to *Sinorhizobium meliloti*), with different proportions of the two species among the four *M. truncatula* lines. The *S. meliloti* population was more diverse than that of *S. medicae*, and significant genetic differentiation among bacterial populations was detected. Single inoculations performed in tubes with each bacterial genotype and each plant line showed significant bacterium-plant-line interactions for nodulation and N<sub>2</sub> fixation levels. Competition experiments within each species highlighted either strong or weak competition among genotypes within *S. medicae* and *S. meliloti*, respectively. Interspecies competition experiments showed *S. meliloti* to be more competitive than *S. medicae* for nodulation. Although not highly divergent at a nucleotide level, isolates collected from this single soil sample displayed wide polymorphism for both nodulation and N<sub>2</sub> fixation. Each *M. truncatula* line might influence *Sinorhizobium* soil population diversity differently via its symbiotic preferences. Our data suggested that the two species did not evolve similarly, with *S. medicae* showing polymorphism and variable selective pressures and *S. meliloti* showing traces of a recent demographic expansion. Strain effectiveness might have played a role in the species and genotype proportions, but in conjunction with strain adaptation to environmental factors.

The rhizobium-legume nitrogen-fixing association is a good model for studying symbiosis and coevolution between organisms. In this mutualistic association, bacteria form nodules on plant roots (more rarely on the stem), where atmospheric nitrogen is reduced to ammonium available for the plant. Among the various plant-bacterium couples studied so far, the *Medicago truncatula* association with *Sinorhizobium meliloti* is particularly interesting (8) and has often been studied as a model system for genetic description of the molecular pathways involved in the establishment of the symbiosis (16). *M. truncatula* has the ability to form an efficient symbiosis with two bacterial species, *S. meliloti* (9) and its sister species, *Sinorhizobium medicae* (29).

The genetic diversity of these two bacterial species, especially *S. meliloti*, has usually been described as quite important (2, 7, 11, 40). This bacterial diversity, assessed by phenotypic or genotypic analyses, has been reported to be influenced by several factors, including geographical location (32), soil factors (14), and plant species (19, 37). Furthermore, at an interspecies level, several studies on *S. meliloti* and *S. medicae* natural populations, trapped from various soils (using several *Medicago* species), reported either the balanced coexistence of the two bacterial species (3, 23) or the dominance of one of them (3, 31, 33, 43).

At a plant genotype level, host nodulation specificity could also influence the bacterial diversity in soil. Significant variations were detected among rhizobial populations associated with different plant lines of *Vicia faba* (38), *Pisum sativum* (10), or *Medicago sativa* (26). Symbiotic specificity studies among *M. truncatula* lines were based on single-strain inoculations. Some of them showed qualitative symbiotic polymorphism for nodulation (Nod<sup>+</sup>/Nod<sup>−</sup>) and nitrogen fixation (Fix<sup>+</sup>/Fix<sup>−</sup>) among a wide collection of *M. truncatula* fixed lines inoculated with diverse *S. meliloti* strains (35, 39). Other studies demonstrated that different plant-rhizobium combinations displayed different quantitative levels of effectiveness, described as the input that a bacterial nitrogen-fixing symbiosis makes to plant nitrogen metabolism, with *S. meliloti* (27) and *S. medicae* species (17).
However, as a host plant usually interacts with several symbionts, these studies do not reflect possible quantitative variations of symbiotic effectiveness within a bacterial population encompassing diverse genotypes. Studying plant symbiotic specificity when put in contact with a single soil would reflect what occurs in a natural ecosystem compared to artificial single inoculations.

Nodulation competition among *S. meliloti* and *S. medicae* genotypes, at both intra- and interspecies levels, is another biotic factor which could also influence the genetic structure of bacterial populations recovered from soil. Strain competitiveness (defined as the ability to dominate nodulation) experiments were mostly performed to compare inoculated strains versus native bacteria, in order to enhance the agricultural yield by selecting the best strains for nodulation and nitrogen fixation efficiency (21, 42).

In the *M. truncatula*-Sinorhizobium symbiotic system, it is still unclear (3) if the plant cultivar and competition among bacterial genotypes acting at both inter- and intraspecies levels are true selective forces driving the partner choice in nitrogen fixation association. Furthermore, a study of the nitrogen fixation polymorphism of these bacteria within a single geographical site has never been performed. With the aim of improving our understanding of the bacterial population structure and diversity maintenance in this symbiotic association, we carried out a study whose objectives were (i) to explore the genetic diversity of symbiotic bacteria recovered from a single soil and trapped with four different *M. truncatula* lines, (ii) to test for significant symbiotic divergence among *Sinorhizobium* populations sampled with four different *M. truncatula* lines, (iii) to perform competition assays within and between the two bacterial species *S. meliloti* and *S. medicae* to evaluate strain competitiveness, and (iv) to compare the fixation efficiencies of single-strain inoculants and multistrain mixtures.

**MATERIALS AND METHODS**

**Bacterial trapping from soil samples.** Soil samples were collected in an old abandoned vineyard fallow parcel at Saint Bauzille de la Sylve, France (43°33'5/H11032, 3°33'E). The soil was characterized as loamed limestone with a pH of 8.3. Soil samples were taken at five points (at a 2-m distance from each other) in the plot (depth, 5 to 25 cm) and were homogenized in the laboratory in a single bulk batch.

Aliquots of soil were put into contact with four *M. truncatula* fixed lines, originally sampled from different countries: (i) DZA315-16 (Algeria), (ii) F83-005 (France), (iii) PRT180-21 (Portugal), and (iv) the reference line, *M. truncatula* Jemalong A17. These lines were chosen based on the data of Ronfort et al. (30), which showed large genetic distances among them using microsatellite DNA. After seed sterilization, five individuals per line were cultivated for 2 months on a soil aliquot, and nodules were collected from plant roots and stored at −80°C in 20% glycerol till they were used. From 9 to 14 nodules per plant were surface sterilized, and isolation of a single bacterial clone per nodule was performed.

**DNA extraction and marker choice for genetic-diversity analyses.** Bacteria were grown in 20 ml yeast extract-mannitol liquid medium for at least 3 days at 27°C under agitation (41), and total genomic DNA was isolated (3).

The genome of *S. meliloti* Sm1021 comprises one chromosome (3.65 Mb) and two megaplasmids (pSymA and pSymB), whereas that of *S. medicae* strain WSM419 comprises in addition one small plasmid. We amplified five genomic regions evenly distributed along the genome and implicated in different bacterial functions. Markers are located either within genes or adjacent to them within an intergenic spacer (IGS). We used two markers involved in symbiosis: *IGS\_nod* (on pSymA), framed by nodulation genes involved in symbiosis (*nodE* and *nodG*), and *typA* (on the chromosome), which is essential for symbiosis on *M. truncatula* Jemalong A17 (20). We also used two paralogous genes, *virB11* and *avhB11*, implicated in effector translocation and conjugation, respectively, in *A. tumefaciens* tumefaciens. In this study, we named loci *virB11* and *avhB11* as described in *A. tumefaciens*. In the *S. meliloti* reference strain (Sm2011), a single gene, named *virB11*, has been found (on pSymA) with high similarity to *virB11* of *A. tumefaciens*. On the other hand, the *S. medicae* reference strain (WSM419) also contains a single locus, also named *virB11*, but with high similarity to *virB11* of *A. tumefaciens*. Furthermore, we studied the 16S rRNA gene, which allows species discrimination between *S. meliloti* and *S. medicae*.

**PCR–CE-SSCP analyses.** Allelic discrimination was performed by capillary electrophoresis—single-stranded conformation polymorphism (CE-SSCP) (36). For the PCRs used for the SSCP, the primers of each set were labeled with two different fluorescent dyes, FAM (5-carboxyfluorescein) and HEX (5-carboxy-4',5',2',4',3',7-tetrahydro-2'H-chromen-1-one) (Table 1), and we used PCR mixes and cycling conditions without a touchdown program, as described previously (3).

Precipitation of the PCR products was performed by adding 1/10 sodium acetate volume (sodium acetate-EDTA buffer [1.5 M sodium acetate, pH 8.4, and 250 mM EDTA]) and 4 volumes of 95% ethanol and centrifuging the mixture at 4,000 rpm and 10°C for 35 min. The pellet was washed in 100 µl 70% ethanol and then centrifuged again for 15 min. The DNA was resuspended in 50 µl sterile Tris-HCl (10 mM).

**CE-SSCP analyses of PCR products** were performed on a MegaBACE 1000 DNA Analysis System (Amersham Biosciences) equipped with 96 coated capillaries using 70-min runs at 40°C and 8 kV. The raw data were exported in RSD format, different runs were aligned, and peaks were detected with MegaBACE Genetic Profiler V1.5 software (Amersham).

**Nucleotide sequencing and PCR-RFLP analyses of the 16S rRNA gene.** We sequenced each different CE-SSCP allele obtained. For similar alleles, we randomly selected and sequenced from 1 to 26 individuals each. PCR amplifications were performed as described above using unlabeled primers, except for *virB11* and *avhB11* (Table 1). Bands were extracted from 1% agarose gels and purified using the Qiaquick PCR purification kit (Qiagen). Sequencing reactions were

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**TABLE 1. Primers used in the study**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primers</th>
<th>Sequence (5’–3’</th>
<th>Tm a (°C)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IGS_nod</em></td>
<td>nod283R_HEX</td>
<td>GCGATCCATTACGCCGTGAT</td>
<td>53</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>nod717F_FAM</td>
<td>CATTTCTGGCATATCAAGC</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td><em>typA</em></td>
<td>typ1706R_HEX</td>
<td>GGGCTGTAGTCGTTGAAACA</td>
<td>59</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td>typ1707F_FAM</td>
<td>CACCAAGATAGGACGACGAC</td>
<td>56</td>
<td>212</td>
</tr>
<tr>
<td><em>virB11</em></td>
<td>virB11F_383</td>
<td>AGCCGGCTACAGATCTGTG</td>
<td>53 b</td>
<td>492</td>
</tr>
<tr>
<td></td>
<td>virB11R_860</td>
<td>CRTC GGCAATCTCGGCCGAG</td>
<td>53 b</td>
<td>483</td>
</tr>
<tr>
<td><em>avhB11</em></td>
<td>avhB11F_383</td>
<td>GAGGACGGATCAGCGGCA</td>
<td>57</td>
<td>1,500</td>
</tr>
<tr>
<td></td>
<td>avhB11R_860</td>
<td>CGAGGTTAGATCTGGGTCAG</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>FGPS 1509</td>
<td>AAGGAGGAAGTCAGCGGCA</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>gene</td>
<td>FGPS 6</td>
<td>GAGAGGTTAGATCTGGGTCAG</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

*a* Tm, melting temperature.

*b* A touchdown PCR program was used (20 cycles of annealing at 63°C [decreasing by 0.5°C at each cycle] followed by 25 cycles of annealing at 53°C).
IGSNOD positive controls received 5 mM nitrogen \([\text{Ca(NO}_3\text{)}_2]\). The plants were grown for inoculum 2 days later. Negative control plants received only sterile water, and ml of liquid sterile nutritive solution diluted four times. After sterilization and

- Divergence among bacterial populations isolated from each plant line was low for each locus, especially for \(S.\ meliloti\) and \(S.\ medicae\). The genetic differentiation among bacterial populations isolated from each plant line was tested by PCR sequencing on

- All isolates were characterized by PCR-restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Table 1) with the restriction enzyme RsaI, which differentiates the two \(S.\) \(melloti\) species.

- Genepop 3.4 (28). Significant departures from equal proportions of the two species in both trapping and interspecific-competition experiments were tested with a chi-square test using Statistica 6.

- Bacterial diversity. A total of 225 \(S.\) \(meliloti\) isolates were obtained, with 52 to 59 individuals from each of the four \(M.\ truncatula\) lines. The proportion of \(S.\) \(medicae\) was significantly higher than the proportion of \(S.\ meliloti\) (Fig. 1) \((\chi^2 = 169.27; df = 7; P < 0.001)\).

- Based on the single-inoculation experiment results, we tested the effects of plant lines, bacterial species, bacterial genotypes nested within species, and the two plant line-bacterial species and plant line-bacterial genotype interactions using an analysis of variance performed with JMP 7.0.1 (SAS Institute Inc.). Based on the two intraspecific competitions (the strain mixture for each species), we used an analysis of variance to test the effects of plant lines, bacterial species, and their interaction. Least-significant-difference Fisher post hoc tests were performed to compare the plant biomasses induced by the mixtures to those of the single-strain inocula.

- RESULTS

- Bacterial diversity. A total of 225 \(S.\) \(meliloti\) isolates were obtained, with 52 to 59 individuals from each of the four \(M.\ truncatula\) lines. The proportion of \(S.\) \(medicae\) was significantly higher than the proportion of \(S.\ meliloti\) (Fig. 1) \((\chi^2 = 169.27; df = 7; P < 0.001)\).

- Plant fitness and nodule formation ability analyses. Based on the single-inoculation experiment results, we tested the effects of plant lines, bacterial species, bacterial genotypes nested within species, and the two plant line-bacterial species and plant line-bacterial genotype interactions using an analysis of variance performed with JMP 7.0.1 (SAS Institute Inc.). Based on the two intraspecific competitions (the strain mixture for each species), we used an analysis of variance to test the effects of plant lines, bacterial species, and their interaction. Least-significant-difference Fisher post hoc tests were performed to compare the plant biomasses induced by the mixtures to those of the single-strain inocula.

- Nucleotide sequence accession numbers. All sequences have been deposited in the GenBank database under the following accession numbers: \(IGS\_\beta\-\gamma\-\protection\) AM991101 to AM991108; \(tphA\), AM991109 to AM991113; \(virB11\), AM991116, AM992458, AM992459, and AM993155; and \(avhB11\), AM991114 and AM991115.

- \(S.\) \(medicae\) and \(S.\) \(meliloti\) are differentiated by the expression of \(avhB11\) and \(virB11\) genes. As in the sequenced \(S.\) \(medicae\) strain, we detected only \(virB11\) in each \(S.\) \(medicae\) isolate. Within \(S.\) \(meliloti\) strains, we detected either two genes (\(avhB11\) and \(virB11\)), only the \(virB11\) gene (as in the sequenced \(S.\) \(medicae\) strain, WSM419), or only the \(avhB11\) gene (as in the sequenced \(S.\) \(meliloti\) strain, Sm2011).

- Using all loci, 13 different multilocus genotypes were detected, four and nine, respectively, for \(S.\) \(medicae\) and \(S.\) \(meliloti\) (Table 2). At least for the bacterial populations isolated from plant lines F83-005 and PRT180-21, \(S.\) \(meliloti\) diversity appeared larger than that of \(S.\) \(medicae\) in terms of allelic richness and genotypic distribution. Nucleotide divergence among strains was low for each locus, especially for \(S.\) \(medicae\).
Table 3. Tajima’s D value for the different loci of S. meliloti did not depart from a neutral hypothesis of evolution. In S. medicae, the most negative D value was found for IGSNO, deviating significantly from neutrality.

Among the four genotypes found within S. medicae species, the genotype md1 was dominant, representing more than 92% of the isolates in the four plant line populations (Table 4). For the DZA315-16 and JemalongA17 plant populations, for which more than one S. meliloti strain was recovered, the proportion of each S. meliloti genotype ranged from 12.5 to 40%.

Bacterial population differentiation among plant lines. The overall genotypic differentiation among the four S. medicae populations was barely significant (G-like test, P < 0.02). Conversely, we could not detect any significant differentiation among S. meliloti populations (G-like test, P = 0.62), which might result from the very small sample size of each population. Furthermore, the proportions of S. medicae and S. meliloti differed among the four plant lines, but not strongly (G-like test, P = 0.04).

Intraspecific competition with all species genotypes. Each of the 13 different bacterial genotypes was tested independently for nodulation. Only the S. meliloti ml9 strain could not renodulate any of the four plant lines (Fig. 2). This strain might be an opportunistic rhizobium (coinfecting a nodule), which might not harbor all the symbiotic genes.

When the four S. medicae genotypes were inoculated on each plant line in an equal mixture, only two genotypes (md2 and md4) were reisolated from the 138 nodules genotyped (Table 4), with genotype md4 being significantly dominant (χ² = 1.2951).

Table 2. Genotypes of S. medicae and S. meliloti isolates from soil trapping

<table>
<thead>
<tr>
<th>Species</th>
<th>Multilocus genotype</th>
<th>No. of individuals in the soil trapping</th>
<th>Reference isolate</th>
<th>Allele at each locus</th>
<th>No. of sequenced isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. medicae</td>
<td>md1</td>
<td>202</td>
<td>STM 5484</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>6 6 23</td>
</tr>
<tr>
<td></td>
<td>md2</td>
<td>2</td>
<td>STM 5465</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>md3</td>
<td>3</td>
<td>STM 5474</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>md4</td>
<td>2</td>
<td>STM 5477</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>2</td>
</tr>
<tr>
<td>S. meliloti</td>
<td>ml1</td>
<td>2</td>
<td>STM 5480</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>2 2 2 2</td>
</tr>
<tr>
<td></td>
<td>ml2</td>
<td>3</td>
<td>STM 5457</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>3 3 2 2</td>
</tr>
<tr>
<td></td>
<td>ml3</td>
<td>1</td>
<td>STM 5482</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td></td>
<td>ml4</td>
<td>1</td>
<td>STM 5466</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td></td>
<td>ml5</td>
<td>1</td>
<td>STM 5463</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td></td>
<td>ml6</td>
<td>1</td>
<td>STM 5473</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td></td>
<td>ml7</td>
<td>4</td>
<td>STM 5472</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>4 4 2 2</td>
</tr>
<tr>
<td></td>
<td>ml8</td>
<td>1</td>
<td>STM 5478</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td></td>
<td>ml9</td>
<td>1</td>
<td>STM 5481</td>
<td>16S IGSNO typA virB11 avhB11</td>
<td>1 1 1 1</td>
</tr>
</tbody>
</table>

* Total numbers are as follows: genotypes, 13; individuals, 224; alleles, 2 (16S), 8 (IGSNO), 5 (typA), 5 (virB11), and 3 (avhB11).

* NA, not amplified. Allele names were given randomly.
TABLE 4. Percentages of genotypes of *S. medicae* and *S. meliloti* isolates from trapping and intraspecific competitions in four plant lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Soil trapping</th>
<th>Intraspecific competition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DZA315-16</td>
<td>PRT180-21</td>
</tr>
<tr>
<td><em>S. medicae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>md1</td>
<td>100</td>
<td>96.4</td>
</tr>
<tr>
<td>md2</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>md3</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>md4</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>ml1</td>
<td>20</td>
<td>0</td>
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<td>ml5</td>
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<td>ml7</td>
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<td>0</td>
</tr>
<tr>
<td>ml8</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>ml9</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

The numbers in boldface indicate the highest proportion found for each bacterial species-plant line combination in the two experiments. The numbers of isolates were as follows: *S. medicae*, 51 (DZA315-16, F83-005, and JemalongA17) and 56 (PRT180-21) for soil trapping and 36 (DZA315-15), 30 (PRT180-21 and F83-005), and 42 (JemalongA17) for intraspecific competition and *S. meliloti*, 5 (DZA315-16), 1 (PRT180-21 and F83-005), and 8 (JemalongA17) for soil trapping and 31 (DZA315-15), 33 (PRT180-21), 32 (F83-005), and 34 (JemalongA17) for intraspecific competition.

363.48; df = 15; \( P < 0.001 \). It is noteworthy that, conversely, the most frequent genotype, *md4*, had been recovered at a very low frequency in the trapping experiment.

Seven of the nine different *S. meliloti* genotypes isolated from the soil-trapping experiment were recovered from the 130 nodules genotyped after plant inoculation with an equal mixture of the nine strains (Table 4). Two genotypes (*ml2* and *ml9*) were not isolated in this experiment. The chi-square tests could not rule out the hypothesis of equal frequencies among strain genotypes in DZA315-16 (\( \chi^2 = 8.69; \) df = 5; \( P = 0.12 \)), whereas one genotype was found significantly more frequently in other plant lines (data not shown). Finally, the four *S. meliloti* populations showed highly significant genotypic differentiation (G-like test, \( P < 0.001 \)).

**Interspecific competition with two species genotypes.** Genotype *md4* for *S. medicae* was systematically used and mixed with either *S. meliloti* genotype *ml8* (for the F83-005 and JemalongA17 lines) or *ml5* (for the PRT180-21 and DZA315-16 lines). One hundred and twenty-one nodules were genotyped. *S. meliloti* strains were recovered significantly more frequently than *S. medicae* strains in each of the four populations (\( \chi^2 = 23.99; \) df = 7; \( P < 0.001 \) (Fig. 1).

**Plant fitness statistical analyses.** Each plant line was inoculated with each single bacterial genotype isolated, with each intraspecific mixture, or with the mixture of the two dominant strains of each bacterial species. The means and variances of the aerial biomasses and nodule numbers are shown in Fig. 2. Measurements of DZA315-16 plants inoculated with genotype *md4* are absent, due to technical problems.

(i) Single inoculants. Direct estimation of plant line-bacterial genotype (species) interaction was not possible due to the nonequilibrated data we obtained because of the absence of the combination DZA315-16/ *md4*. Therefore, we included in our data virtual data corresponding to this missing combination. We tested several virtual conservative measures (of the nodule number and aerial biomass), i.e., the mean of the *md4* strain, the mean of the DZA315-16 line, and the mean of all *S. medicae* strains. The final results were independent of the choice of the mean (data not shown), suggesting the reliability of such an approach to compensate for missing data.

For both the dry aerial biomass and nodule number measures of all single genotypes, the plant line, bacterial species, bacterial genotype (species), and plant line genotype (species) interaction effects were highly significant. Plant line-bacterial species interaction was found to be barely significant for the aerial biomass and not significant for the nodule number (Table 5, all single genotypes).

Post hoc tests were performed to rank the various parameters within each effect studied: *S. meliloti* induced a greater dry biomass than *S. medicae* (\( P = 0.02 \)) on each of the four plant lines. Within *S. medicae*, genotype *md2* induced a better biomass than the three other *S. medicae* genotypes (\( P < 0.005 \)), while the eight *S. meliloti* genotypes were grouped in five clusters differing from each other significantly in their nitrogen fixation levels (data not shown).

*S. meliloti* species induced a higher nodulation rate than *S. medicae* species (\( P = 0.01 \)).

(ii) *S. medicae* mixture versus *S. meliloti* mixture effects. When plant biomass means between *S. medicae* and *S. meliloti* mixtures were compared, a slight bacterial species effect was detected (\( P = 0.023 \) (Table 5, two intraspecific mixtures), with a higher biomass for plants inoculated with the mixed inoculant of *S. medicae* species.

(iii) Intraspecific mixtures versus single inoculants. Within each species, dry aerial biomasses obtained with *S. meliloti* and *S. medicae* mixtures were either equivalent to or significantly higher than the mean of each single-strain inoculation (Fig. 2 and Table 5, *S. medicae* and *S. meliloti*). The *S. medicae* mixture and the *md2* genotype induced similar plant biomasses, in contrast to the other three bacterial genotypes (Table 6). Furthermore, post hoc tests showed that the *S. meliloti* mixture
induced greater aerial biomass than five genotypes (ml1, ml2, ml4, ml5, and ml7), whereas no significant difference was detected between the mixture and ml3 or ml6 for DZA315-16 and F83-005 and ml3 or ml8 for JemalongA17 (Table 6).

**DISCUSSION**

As previously found in several studies by studying the genetic diversity of rhizobia at five loci (11, 40), *S. medicae*

![FIG. 2. Mean dry aerial biomass (± standard error) (a) and mean nodule number (± standard error) (b) obtained for each plant line-bacterial genotype combination. The four *S. medicae* (md1 to md4) and the eight *S. meliloti* (ml1 to ml8) strains were inoculated separately on each *M. truncatula* line. “*S. medicae*” and “*S. meliloti*” indicate the results of the two mixtures including all the strains for each species, and “interspecific” is the result of a mixture including one strain of each species (see the text). “control −” is the negative control (no inoculation), and “control +” is the positive control (5 mM nitrogen).](http://aem.asm.org/)

![TABLE 5. Analyses of variance for dry aerial biomasses and nodule numbers of plants](http://aem.asm.org/)
Such variations imply that symbiotic partners differently. Specific levels, suggesting that the plant lines might select their among the four plant lines at both interspecific and intraspe-

\[ S. \text{ meliloti} \] (19). In our study, the proportions of the two bacterial species in the soil.

served in our study certainly reflect a major disequilibrium between the two bacterial species in the soil. At least in this local population, we may con-

FIGS 3-5) of the two species are estimated with Lamarc software, \( S. \text{ medicae} \) has a much higher value (\( G_{\text{estimated}} = 6,200 \)) than \( S. \text{ meliloti} \) (\( G_{\text{estimated}} = 11 \)), implying that \( S. \text{ medicae} \) may have had a much more rapid expansion rate than \( S. \text{ meliloti} \). Such demographic expansion would also fit with the much higher proportion in the soil observed for \( S. \text{ medicae} \) than for \( S. \text{ meliloti} \).

Nucleotidic polymorphism was observed for the two homol-

ogous genes implicated in translocation and conjugation, \( \text{virB}11 \) and \( \text{avhB}11 \), between the two species, but also within \( S. \text{ meliloti} \). Our results support the hypothesis that the common ancestor of the two \( \text{Sinorhizobium} \) species harbored the two copies \( \text{virB}11 \) and \( \text{avhB}11 \). As several authors (2, 5) have sug-

gested that \( S. \text{ medicae} \) might have emerged from a population of \( S. \text{ meliloti} \), the emerging species \( S. \text{ medicae} \) would have kept one ortholog, whereas \( S. \text{ meliloti} \) harbored either the two copies or only one. The role of each gene in \( S. \text{ meliloti} \) and between the two species merits more study.

In the trapping, we observed a high predominance of \( S. \text{ medicae} \) strains compared to \( S. \text{ meliloti} \). In previous literature (2), no marked symbiotic preference for \( S. \text{ medicae} \) was de-

scribed for any \( M. \text{ truncatula} \) line. Thus, the proportions ob-

served in our study certainly reflect a major disequilibrium between the two bacterial species in the soil.

\( \text{Medicago} \) species display a wide variability of specificity (4). Such variations imply that \( \text{Medicago} \) species might play a role in bacterial selection in the soil, as demonstrated by Jebara et al. (19). In our study, the proportions of the two bacterial species (\( S. \text{ meliloti} \) and \( S. \text{ medicae} \)) were slightly different among the four plant lines at both interspecific and intraspe-

fic levels, suggesting that the plant lines might select their symbiotic partners differently. \( M. \text{ truncatula} \) plant lines thus display differences in their specificities against bacterial popu-

lations, although the presence or absence of rare strains might influence this differentiation. Consequently, \( M. \text{ truncatula} \) ge-

netic diversity in a natural ecosystem might, at least in part, have an influence on rhizobial genetic diversity in the soil, because viable bacteria could be released into the plant rhizo-

sphere (25).

Both intraspecies competition results and soil-trapping re-

sults showed one genotype highly predominant for \( S. \text{ medicae} \) (but not the same genotype) and a large genetic diversity for \( S. \text{ meliloti} \). Thus, a strong competition might exist among the different \( S. \text{ medicae} \) genotypes for nodulation, leading to one predominant genotype being recovered, whereas this inter-

strain competition is apparently much weaker in \( S. \text{ meliloti} \), for which several genotypes were recovered in similar frequencies. We have no clue to explain such proportion differences in the competition outputs between the two species, even if it is not an intrinsic specificity of each species, as Bailly et al. (3) did not observe this unequal distribution in similar trapping experiments. Moreover, in \( S. \text{ medicae} \), the most competitive strain (\( \text{md}4 \)) in the competition experiment was not the genotype found to be dominant in soil trapping (\( \text{md}1 \)). We suspect that the \( \text{md}1 \) genotype is quantitatively dominant in the soil used but is not the most competitive for plant nodulation under the tube conditions tested, because competition among strains seems to be dependent on the experimental culture conditions, as previously demonstrated in the \( \text{Bradyrhizobium japonicum}-\text{soybean} \) model (15).

At an interspecies level, the competition experiment re-

sulted in a higher proportion of \( S. \text{ meliloti} \), whereas trapping from soil resulted in more than 90% \( S. \text{ medicae} \) isolated. The initial unbalanced proportions obtained in soil trapping most certainly resulted from a “population effect” rather than a methodological bias or a much higher competitiveness of \( S. \text{ medicae} \) in soil. At least in this local population, we may con-

sider \( S. \text{ meliloti} \) the best \( M. \text{ truncatula} \) symbiont for nodulation. To generalize this hypothesis, other competition analyses should be done between the two bacterial species trapped from other natural populations.

In \( \text{Sinorhizobium} \) spp., several authors have shown variation in the ability to fix nitrogen among strains originating from different geographical areas for \( S. \text{ meliloti} \) (22, 27) or for \( S. \text{ medicae} \) (17). In this study, we demonstrated high polymor-

phism in terms of nitrogen fixation effectiveness and nodula-

<table>
<thead>
<tr>
<th>Inoculant</th>
<th>Bacterial genotype</th>
<th>Biomass comparison for plant line*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S. \text{ medicae} ) intraspecific mixture</td>
<td>( \text{md}1 )</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>( \text{md}2 )</td>
<td>0.9299</td>
</tr>
<tr>
<td></td>
<td>( \text{md}3 )</td>
<td>0.2018</td>
</tr>
<tr>
<td></td>
<td>( \text{md}4 )</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>( S. \text{ meliloti} ) intraspecific mixture</td>
<td>( \text{ml}1, \text{ml}2, \text{ml}4, \text{ml}5, \text{ml}7 )</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>( \text{ml}3 )</td>
<td>0.9607</td>
</tr>
<tr>
<td></td>
<td>( \text{ml}6 )</td>
<td>0.2547</td>
</tr>
<tr>
<td></td>
<td>( \text{ml}8 )</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Based on Fisher post hoc tests (\( P \) values are shown). The numbers in boldface indicate that the genotypes were not significantly different from the bacterial mixtures considered. ND, not determined.
tion ability within a single soil, at both the interspecies and the intraspecies levels, even though weak genotypic and nucleotidic divergence were observed among strains.

Within each bacterial species, we detected a significant bacterial genotype-plant line interaction on the aerial biomass and nodule number data. Previous studies showed that the fixation effectiveness of three S. meliloti strains (27) and two S. medicae strains (17) was dependent on the M. truncatula lines and can be expressed even when they are put in contact with a single soil. We showed that the partner choice in the M. truncatula-Sinorhizobium association is controlled by the plant lines and by the bacterial competition for nodulation and not strictly by the fixation efficiencies of the bacterial genotypes. However, it is relevant to keep in mind that bacterial adaptation to the environment is essential for symbiosis.

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REFERENCES


