Classification of Austrian Rhizobia and the Mexican Isolate FL27 Obtained from *Phaseolus vulgaris* L. as *Rhizobium gallicum*

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The phylogenetic positions of four rhizobial strains obtained from nodules of common bean plants (*Phaseolus vulgaris* L.) grown in an Austrian soil and of the Mexican bean isolate FL27 are described. Analysis of the 16S rRNA genes revealed sequences almost identical to that of the *Rhizobium gallicum* type strain, R602sp, with a maximum of two nucleotide substitutions. Comparison of the 16S rRNA gene sequences with those from other bacteria indicated highest similarity to *Rhizobium* sp. strain OK-50, *Rhizobium leguminosarum* IAM 12609, and *Rhizobium etli*. DNA homology determined by DNA-DNA hybridization was high among the Austrian isolates and R602spT (45 to 90%) and ranged from 21 to 65% with FL27, but hybridization analysis revealed very low homology to the recognized common bean-nodulating species, *R. leguminosarum* bv. phaseoli, *R. etli*, and *Rhizobium tropici*. Ribosomal gene organization was studied by Southern hybridization with the 16S rRNA gene and temperature gradient gel electrophoresis, indicating identical organizations and the presence of three identical 16S rRNA copies in the genome of this species. The six strains investigated showed different plasmid profiles based on their geographical origins. We propose that the Austrian isolates and the Mexican strain FL27 are members of the species *R. gallicum*.

Bacteria of the genus *Rhizobium* that are able to nodulate common bean plants (*Phaseolus vulgaris* L.) have been traditionally classified as *Rhizobium leguminosarum* bv. phaseoli (13) on the basis of the host plant they infect. Strains belonging to the other subdivisions of this species, *R. leguminosarum* bv. viciae and bv. trifolii, nodulate peas and clovers, respectively, and their symbiotic plasmids carry genes with different host specificities. Nevertheless, rhizobia from common bean plants have been found to be phylogenetically diverse based on different criteria, such as protein profiles (32), multilocus enzyme electrophoresis patterns (6, 28), results of DNA relatedness analysis (16, 37, 44), and differences in their 16S rRNA gene (rDNA) sequences (9, 16, 44). In addition to *R. leguminosarum* bv. phaseoli, two new species, *Rhizobium etli* (38) and *Rhizobium tropici* (20), have been described. Both *R. leguminosarum* bv. phaseoli and *R. etli* carry multiple copies of the nitrogenase reductase gene (*nifH*) on their symbiotic plasmids, while they have different 16S rRNA sequences (17, 30, 38). In contrast, *R. tropici* maintains only a single *nifH* gene copy on its symbiotic plasmid (20). *R. etli* and *R. tropici* show a broad host range, but they nodulate different hosts (9, 20). Several new species among bean-nodulating strains, including *Rhizobium gallicum* and *Rhizobium giardinii*, which comprise the French isolates (1, 16), as well as *Rhizobium sp. (Phaseolus)* RCR 3618D of unknown geographical origin, have been proposed (44). The partial 16S rDNA sequence of the *R. gallicum* type strain, R602sp, was found to be identical to that of strain FL27 (16), a Mexican isolate from the common bean which does not fix N2 well (28). In general, strains of *Rhizobium* that nodulate bean plants are of American origin, as is their host plant. For a long time it was believed that in European, *R. leguminosarum* bv. phaseoli was the only microsymbiont of common bean plants, but recently several species, including *R. etli*, *R. tropici*, *R. gallicum*, and *R. giardinii*, were found in European soils (1, 2, 16, 41).

Rhizobial strains recovered from common bean nodules from an Austrian soil showed high similarity to *R. gallicum* R602spT by PCR analysis with repetitive primers and PCR-restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (41). In addition, the *nifH* profiles and the nodulation phenotypes were identical (41). The aim of this study was to confirm that the Austrian isolates are members of the recently described species *R. gallicum*. As the Mexican isolate FL27 exhibited several similarities to *R. gallicum*, it was included in this investigation. Phylogenetic analysis was done by phenotypic characterization as well as sequence analysis of the 16S rRNA gene, analysis of the copy numbers and heterogeneity of ribosomal genes, analysis of plasmid profiles, and DNA-DNA hybridization.

MATERIALS AND METHODS

Bacterial strains, phenotypic characterization, and nodulation host range. Four strains, CHS-1, CHS-3, CHS-17, and CHS-18, were isolated from common bean plants grown in soil from fields around the Seibersdorf laboratory in Austria and characterized previously (41). *R. gallicum* R602sp, the Mexican isolate FL27, and reference strains were obtained either from G. Laguerre, Dijon, France, or from the culture collection at the Seibersdorf laboratory. All rhizobial strains were maintained on yeast extract-mannitol (YM) medium (46), and FL27, R602spT, CHS-1, CHS-3, CHS-17, and CHS-18 were tested for growth on Luria-Bertani medium (21) and on peptone-yeast extract medium (22). In order to test substrate utilization, modified minimal B&D medium (48) was amended with the following carbon sources at a concentration of 1 g/liter: D-glucose, starch, maltose, urea, fructose, L-lactate, L-sorbitol, L-asparagine, L-asparagine, L-arginine, L-lysine, L-methionine, L-cysteine, L-glutamate, L-glutamine, L-histidine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-tryptophan, L-3-hydroxy-L-proline, and L-tryosine, and L-valine were each tested as a sole carbon and nitrogen source at a concentration of 1 g/liter. Growth at different temperatures (20, 25, 30, 35, 37, and 40°C), growth on YM medium containing ampicillin (10 μg/ml), chloramphenicol (30 μg/ml), kanamycin (30 μg/ml), tetracycline (10 μg/ml), nalidixic acid (10 μg/ml), spectinomycin (10 μg/ml), or streptomycin (10 μg/ml), was tested as a sole carbon and nitrogen source at a concentration of 1 g/liter.
medium containing different concentrations of NaCl (1.0, 1.5, and 2% NaCl), and growth at different pH values (pHs 4.5, 6, 7, 8, 9, and 10) were determined in liquid culture.


**Plasmid profiles, DNA isolation, and DNA hybridization.** Plasmid profiles of *CbS-1*, *CbS-3*, *CbS-17*, *CbS-18*, *R602sp*, FL27, and CIAT 899T were investigated as described by Hynek et al. (12). Total genomic DNA was prepared as described elsewhere (3) with omission of the ClI purification step. Genomic DNA was digested with HindIII, and the resulting fragments were separated by electrophoresis with a 0.7% agarose gel that was blotted onto a Hybond-N membrane (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom). A probe containing the 16s rRNA gene from strain *R602sp* was prepared by PCR with the primers rD1 and fD1 (47) as described previously (41).

After isolation of the resulting fragment from a 1% agarose gel with a Gene-Clean II Kit (Bio 101, Inc., La Jolla, Calif.), it was labeled with [α-32P]dATP by using the Multiprime DNA labeling system (Amersham International) according to the manufacturer's protocol. Hybridization was carried out at 65°C for 2 h in Rapid-Hyb buffer (Amersham International) and washing and autoradiography were done as described elsewhere (39).

**DNA-DNA hybridization.** Dot blot hybridizations were performed as described previously (15) with 25-μl samples containing 400 ng of genomic DNA. In addition, 400 ng of denatured calf thymus DNA was transferred onto the membrane. The membranes were hybridized with 4 μg of genomic DNA previously digested with Alul and labeled with [α-32P]dATP by using the Multiprime DNA labeling system per dot. Hybridization was carried out under relaxed conditions at 55°C for 2 h in Rapid-Hyb buffer. The filters were washed under stringent conditions at 60°C in a solution containing 0.03 M NaCl, 0.003 M sodium citrate, and 1% sodium dodecyl sulfate. Membranes were cut, and 9- by 9-mm pieces were counted with a liquid scintillation counter (Tri Carb 2200CA; Packard). The amount of radioactive activity associated with calf thymus DNA was subtracted, and the percentages of DNA relatedness were determined relative to the signal found in the homologous hybridization.

**Analysis of the 16s rRNA genes.** The 16s rRNA genes of *CbS-1*, *CbS-3*, *CbS-17*, *CbS-18*, and *R602sp* were amplified by using the primers FPGS6-63 (with a BglII site) (16) and P1501HIII (with a PstI site) (23) and a standard protocol (41). The amplified fragments were digested with BglII and PstI and then cloned into pUC18Not that had been previously cut with BarnHI and PstI. As the 16s rRNA gene of strain FL27 showed an internal HindIII restriction site, the gene was amplified by using the primers FPGS6-63 (16) and P1501HIII equipped with a HindIII site (5'-GTGAGCCTGTTCACTTGGTTACGACT-3'), where the HindIII site is italicized. The resulting fragment was digested with BglII and HindIII and then cloned into BglII- and HindIII-cut pUC18Not, with *Escherichia coli* D50a as the recipient. DNA sequence analysis of the cloned 16s rRNA gene fragments was done by using the dideoxy-chain termination method (35) with a model LI-COR 4000L automated sequencer.

For the temperature gradient gel electrophoresis (TGGE), total genomic DNA was used as the template to amplify a fragment of ca. 440 bp comprising the V6 to V8 variable region of the 16s rRNA gene. The primers used, PCR and TGGE, were previously described by Nübel et al. (24).

**Sequence alignment and phylogenetic analysis.** Gene banks were searched by using the FASTA tool (27), and alignments of selected 16s rDNA sequences were made with the program multiple-alignment program (10). ClustalW (42) was used for the sequence alignments. Owing to uncertain alignment were excluded from further analyses. Calculation of evolutionary distances was done with the Jukes and Cantor model (14). Phylogenetic trees based on the neighbor-joining method (34) were constructed with 100 bootstrap replicates by using the TREEDIC software package (45). A maximum parsimony phylogenetic tree was created with the program PAUP3.1 (43).

**Nucleotide sequence accession numbers.** The 16s rDNA gene sequences of the strains analyzed have been deposited in GenBank under the accession no. AF008126 (CbS-1), AF008127 (CbS-3), AF008128 (CbS-17), AF008129 (CbS-29), AF008129 (FL27), and AF008130 (R602sp).

**RESULTS**

**Phenotypic characterization.** The strains *CbS-1*, *CbS-3*, *CbS-17*, *CbS-18*, *R602sp*, and FL27 showed identical characteristics. They could not utilize the following compounds: starch, urea, methanol, ethanol, L-alanine, L-arginine, L-asparagine, l-cysteine, L-phenylalanine, and L-tryptophan. The strains were not able to grow on medium or on YM medium supplemented with high (above 1%) concentrations of NaCl, but they could be cultivated on peptone-yeast extract medium. All strains were able to grow on D-glucose, maltose, fructose, D-lactose, D-sorbitol, D-xylene, D-mannose, arabinose, D-ribose, myo-inositol, melibiose, raffinose, D-flrheose, L-aspartic acid, L-glutamate, L-glutamine, L-histidine, L-leucine, L-methionine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine. The strains were able to grow on plates supplemented with ampicillin, chloramphenicol, and nalidixic acid. The optimum pH range was from 6 to 8, while no growth occurred at pHs 4, 9, and 10. All isolates were able to grow at a temperature up to 37°C but did not grow at 40°C. Among the host plants tested, FL27 was able to nodulate common bean plants, cowpea plants, *Leucaena*, and *Gliricidia* spp.

**Plasmid profiles.** Plasmid analysis showed that the Austrian isolates *CbS-1*, *CbS-3*, *CbS-17*, and *CbS-18* carried three plasmids identical in size but that the French strain R602sp harbored two plasmids. Strain FL27 and *R. tropici* CIAT 899T also carried three plasmids but had patterns different from each other and from those of the Austrian isolates. The largest plasmids of the Austrian isolates and of R602sp and FL27 appeared to have the same size (Fig. 1).

**Ribosomal gene organization.** Southern hybridization with the 16s rDNA gene of R602sp as a probe showed that isolates *CbS-1*, *CbS-3*, *CbS-17*, *CbS-18*, R602sp, FL27, *R. leguminosarum* bv. *phaseoli* H131, and *R. etli* CFN 42 contained at least three copies of the 16s rRNA gene. Both *R. tropici* CIAT 899T and *R. tropici* CFN 299 contained one copy of the 16s rDNA gene. The Austrian isolates, R602sp and FL27 showed identical patterns with three hybridizing HindIII fragments of 4.6, 11.0, and 12.9 kb. TGGE analysis of PCR-amplified segments of 16s rDNAs resulted in single band profiles for each tested strain (data not shown). This result suggested that the sequences in those strains bearing more than one copy of the gene are identical.

**DNA-DNA hybridization.** Isolates *CbS-1*, *CbS-3*, *CbS-17*, *CbS-18*, and R602sp showed high levels of DNA relatedness ranging from 45 to 90%. Homologies among the European isolates and FL27 ranged from 52 to 65% when FL27 was used as the probe and from 21 to 40% when the same strain was used as the template (Table 1). The average values of the percentages when FL27 was used as the probe and when it was used as the template ranged from 41 to 48% (data not shown). The DNA relatedness values of the Austrian strains, R602sp, and FL27 with *R. leguminosarum*, *R. tropici*, and *R. etli* were very low (Table 1).

**Analysis of the 16s rRNA gene sequence.** The determined 16s rDNA sequences of strains *CbS-1* and *CbS-3* were identical and differed by only one nucleotide from those of strains *CbS-18* and R602sp. Two substitutions at different positions were found when these four sequences were compared with the sequences of strains *CbS-17* and FL27. FASTA analysis
indicated several members of the alpha subclass of Proteobacteria as having the most related 16S rDNA sequences. All further comparisons were based on 1,431 nucleotides comprising more than 93% of the 16S rRNA gene. *Rhizobium* sp. strain OK-50 (26), *R. leguminosarum* IAM 12609 (26), and *R. etli* had the highest homology values, namely, 97.9, 97.9, and 97.6%, respectively. Phylogenetic dendrograms constructed by both the distance and the parsimony method showed essentially the same topology and similar evolutionary distances; the latter method yielded only one most parsimonious tree. The neighbor-joining phylogenetic tree (Fig. 2) showed a well-defined and compact cluster comprising *R. gallicum* R602spT; the Austrian isolates CbS-1, CbS-17, and CbS-18; and the Mexican isolate FL27. This cluster was clearly separated from other well-recognized rhizobial lineages and supported by high bootstrap values.

**DISCUSSION**

High relatedness among common bean-nodulating strains isolated in Austria (CbS-1, CbS-3, CbS-17, and CbS-18) and the French *Rhizobium* strain R602spT was demonstrated by using several approaches targeting symbiotic and chromosomal regions of the genome as well as the nodulation phenotype (41). In a recent study, strain R602spT as well as other isolates obtained from French soils was classified as a new species, *R. gallicum* strain R602spT; the Austrian isolates CbS-1, CbS-17, and CbS-18; and the Mexican isolate FL27. This cluster was clearly separated from other well-recognized rhizobial lineages and supported by high bootstrap values.

**TABLE 1. Levels of DNA relatedness between CbS-1, CbS-3, CbS-17, CbS-29, R602spT, FL27, and other common bean-nodulating *Rhizobium* species**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>CbS-1</th>
<th>CbS-3</th>
<th>CbS-17</th>
<th>CbS-18</th>
<th>R602spT</th>
<th>FL27</th>
<th>H131</th>
<th>CIAT 899T</th>
<th>CFN 299T</th>
<th>CFN 42T</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbS-1</td>
<td>100</td>
<td>76</td>
<td>75</td>
<td>69</td>
<td>90</td>
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<td>80</td>
<td>87</td>
<td>83</td>
<td>65</td>
<td>7</td>
<td>5</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>CbS-17</td>
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<td>83</td>
<td>100</td>
<td>88</td>
<td>82</td>
<td>62</td>
<td>6</td>
<td>5</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>CbS-18</td>
<td>45</td>
<td>68</td>
<td>86</td>
<td>100</td>
<td>87</td>
<td>52</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. strain R602spT</td>
<td>55</td>
<td>82</td>
<td>79</td>
<td>74</td>
<td>100</td>
<td>61</td>
<td>5</td>
<td>3</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>R. leguminosarum bv. phaseoli H131</td>
<td>26</td>
<td>21</td>
<td>40</td>
<td>30</td>
<td>35</td>
<td>100</td>
<td>5</td>
<td>14</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>R. tropici CIAT 899T</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>11</td>
<td>100</td>
<td>5</td>
<td>14</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>R. tropici CFN 299T</td>
<td>5</td>
<td>26</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>100</td>
<td>5</td>
<td>14</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>R. etli CFN 42T</td>
<td>7</td>
<td>12</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>6</td>
<td>18</td>
<td>4</td>
<td>7</td>
<td>100</td>
</tr>
</tbody>
</table>

a DNA used as a template.

b DNA used as a probe. DNA relatedness values of strains CbS-1, CbS-3, CbS-17, CbS-18, R602spT, and FL27 are mean values obtained from the results of two independent experiments.

Although ribosomal gene sequences play an important role in the description of new species, in many cases there is only a limited correlation between DNA relatedness and 16S rDNA homology. This finding was reported for *Rhizobium* (25) but also for other bacterial genera, such as *Aeromonas* and *Plesiomonas* (18). Recently, van Berkum et al. (44) suggested that although the level of 16S rDNA sequence similarity among bean rhizobia is high, the DNA relatedness data indicate different species. Stackebrandt and Goebel (42) demonstrated that the correlation between 16S rDNA homology and DNA-DNA reassociation is not necessarily linear, indicating that distinct species can show high 16S rDNA sequence similarities. Assessment of DNA relatedness was proposed as an important criterion for the description of new species of root- and stem-nodulating bacteria (8). The strains tested in the present study had very low DNA homology with the recognized common bean-nodulating species, i.e., *R. leguminosarum*, *R. tropici*, and *R. etli*. This result is in agreement with the work of Martinez-Romero (19), who found low DNA relatedness between *R. etli* and FL27. High DNA homology was found among the European isolates. The Mexican isolate FL27, however, had lower DNA relatedness to the European strains. It is not clear why generally higher values were obtained when a particular strain was used as a probe than when the same strain was used as a template. However, the average values of the percentages when FL27 was used as a probe and when it was used as a template in combination with the 16S rDNA similarities are within the possible range suggested by Stackebrandt and Goebel (42). One explanation for the lower DNA relatedness of FL27 to the other *R. gallicum* strains may be the presence of plasmids in FL27 showing little homology with those of the European isolates. These plasmids may carry up to 25% of the genetic information in *Rhizobium* (29), and plasmids are prone to losses or alterations. High-frequency plasmid-borne rearrangements, including sequence amplification, deletion, cointegration, and loss, have been particularly observed in *R. etli* CFN 42T plasmids (4, 33). In addition, exchange of plasmids among *Rhizobium* populations has been reported (31, 36). Interestingly, the Mexican, French, and Austrian strains showed plasmid profiles that reflected their geographical origins.

Data obtained by Martinez-Romero et al. (20) suggested that the RFLPs of rRNA in *Rhizobium* operons are species
specific but that riboprobing based on pulsed-field gel electrophoresis fingerprints resulted in strain-specific patterns among *Rhizobium galeae* strains (11). The identical 16S rRNA orga-

nizations in FL27, R602spT, and the Austrian isolates is an

ATCC 10004), U29388

three copies or a single copy of the

fragment resulted in hybridization with a fragment of the same

operons is common among rhizobia. Previously, Southern

analysis of R602spT and the Austria isolates with a

additional factor indicating the close relationship of these

strains. Our study as well as results obtained by Huber and

Selenkova-Pobell (11) suggests that the presence of three rRNA

operons is common among rhizobia. Previously, Southern

analysis of R602spT and the Austria isolates with a *nifH* gene

fragment resulted in hybridization with a fragment of the same

size, indicating the presence of one copy of the *nifH* gene and

related *nif* gene organization (41). Based on differences in the

symbiotic plasmid and nodulation host range, *R. gallicum* was
divided into two biosvars, phaseoli and gallicum, containing either

two copies or a single copy of the *nifH* gene (1). No differ-

ences in the results of phenotypic analysis were found among the

Austrian strains, R602spT, and FL27, and the results corre-

lated well with those reported by Amarger et al. (1). In a

former study the Austrian strains and R602spT showed the

same nodulation host range for nodulating not only bean plants

but also cowpea plants, *Leucaena*, and *Gliricidia* (41). Although

isolated from a very different geographical region, FL27 exhibited

the same nodulation host range, indicating the presence of the

same or a related symbiotic plasmid among the

European strains and FL27.

From our analyses it is evident that the Austrian isolates are

members of *R. gallicum* bv. gallicum, and we also propose that

FL27 belongs to this species. As Mesoamerica, where FL27

was isolated, is one center of origin of the common bean plant

(7), it is possible that *R. gallicum* has been imported to Europe

as a seed contaminant. Bean rhizobia belonging to species

originating in the Americas, such as *R. tropici* and *R. etli*,
have been found in Europe (2, 41). In addition, Martinez-Romero

(19) suggested that *R. leguminosarum* bv. phaseoli strains are a

result of plasmid transfer in historic times because of the high

similarity of their symbiotic plasmids with that from *R. etli*. The

origin of *R. giardinii*, a recently described species that nodu-
lates bean plants and that was obtained from France, is un-

known (1). However, two biosvars, *R. giardinii* bv. giardinii

and bv. phaseoli, have been described and it was suggested that *R.

giardinii* bv. phaseoli as well as *R. gallicum* bv. phaseoli received

their symbiotic plasmids from *R. leguminosarum* (1). On the

other hand, FL27 was isolated from bean plants grown in a

Leucaena field and was demonstrated to be a poor N₂ fixer in

common bean nodules (28), and therefore it is uncertain whether
the common bean plant is the true host of *R. gallicum* bv. gallicum.
Nevertheless, the Austrian isolates possess high

ability in nodulating bean plants in a soil where they

adapted well (40, 41). Further studies will be needed to

understand the origin of bean plant-nodulating rhizobia in

Europe as well as the role and frequency of plasmid transfer in

their evolution.

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