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Assessment of Competitiveness of Rhizobia Infecting *Galega orientalis* on the Basis of Plant Yield, Nodulation, and Strain Identification by Antibiotic Resistance and PCR

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Competition between effective and ineffective *Rhizobium galegae* strains nodulating *Galega orientalis* was examined on the basis of plant growth, nodulation, antibiotic resistance, and PCR results. In a preliminary experiment in Leonard's jars, ineffective *R. galegae* strains HAMBI 1207 and HAMBI 1209 competed in similar manners with the effective strain *R. galegae* HAMBI 1174. In a pot experiment, soil was inoculated with 0 to 10^5 HAMBI 1207 cells per g before *G. orientalis* was sown. Seeds of *G. orientalis* were surface inoculated with 2×10^4 and 2×10^5 cells of HAMBI 1174 per seed (which represent half and fivefold the commercially recommended amount of inoculant, respectively). Plant yield and nodulation by the effective strain were significantly reduced, with as few as 10^2 ineffective rhizobia per g of soil, and the inoculation response was not improved by the 10-fold greater dose of the inoculant. Bacteria occupying the nodules were identified by antibiotic resistance and PCR with primers specific for *R. galegae* HAMBI 1174, *R. galegae*, and genes coding for bacterial 16S rRNA (bacterial 16S rDNA). Sixty-two large nodules examined were occupied by the effective strain HAMBI 1174, as proven by antibiotic resistance and amplification of the strain-specific fragment. From 20 small nodules, only the species-specific fragment could be amplified, and isolated bacteria had the same antibiotic resistance and 16S PCR restriction pattern as strain HAMBI 1207. PCR with our strain-specific and species-specific primers provides a powerful tool for strain identification of *R. galegae* directly from nodules without genetic modification of the bacteria.

Despite the continual demand to improve legume productivity in the field by inoculation with root nodule bacteria, problems often arise from the inability of the inoculum strains to establish themselves in soil occupied by indigenous rhizobia. The inoculation response of the plant is dependent on various environmental and genetic factors (5, 28, 29), and therefore the performance of different bacterial strains under particular agricultural conditions should be evaluated case by case.

The symbiosis between *Galega orientalis* (goat's rue) and *Rhizobium galegae* has recently been studied extensively because of the promising agricultural usefulness of *G. orientalis* as a forage legume (30) and because of the interesting extreme host specificity of its rhizobia (17). In Finnish soils, no indigenous *R. galegae* organisms have been found, and seeds must be inoculated to ensure nodulation of the plant. However, once inoculated, *R. galegae* persists in soil in densities comparable to those of other rhizobia even after ploughing down of the host crop (10a, 16). Competition of different *R. galegae* strains for nodulation has not, to our knowledge, been tested previously.

Recent development of molecular methods has made it possible to construct genetically engineered strains in order to improve the ability of rhizobia to compete for nodule occupancy or to introduce genetic markers for the tracing of strains released into the environment. These investigations, followed by actual releases of genetically modified microorganisms into agroecosystems (21, 26, 34), also necessitate better understanding of competition. Development of fast and convenient methods for monitoring of inoculated strains, preferably based on indigenous characteristics of the investigated bacteria rather than genetically engineered markers, is also needed.

The aim of the present work was to investigate competitiveness of rhizobia infecting *G. orientalis*. In a pot experiment we wanted to mimic realistic agricultural situations with different, known levels of indigenous, ineffective rhizobia in the soil and different seed inoculum levels. We also describe the development of *R. galegae* species-specific PCR primers. Along with antibiotic resistance, PCR with the species-specific primers and previously described strain-specific primers (27) was used to identify bacteria occupying the nodules.

MATERIALS AND METHODS

Bacterial strains. *R. galegae* strains HAMBI 1207 and HAMBI 1209 are streptomycin (1,000 μg/ml) resistant and form effective nodules on *Galega officinalis* and ineffective nodules on *G. orientalis*. Strain HAMBI 1174 is effective in symbiosis with *G. orientalis* and ineffective with *G. officinalis*, and it is streptomycin (1,000 μg/ml) and spectinomycin (500 μg/ml) resistant (13). These three *R. galegae* strains were used in the competition experiments. Other bacterial strains used in the PCR primer specificity studies are listed in Table 1.

Strains were grown and maintained on yeast extract-mannitol (YEM) medium (0.5 g of K_2HPO_4 per liter, 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter, 3 g of yeast extract per liter, 10 g of mannitol per liter, 15 g of agar per liter) at 28°C. Bacteria from nodules were isolated on YEM medium containing 250 μg of Actidione per ml and amended with antibiotics when appropriate.

Plant. Seeds of goat's rue (*G. orientalis* Lam. cv. Gale) were obtained from the Estonian Institute of Agriculture and Land Improvement, Saku, Estonia.

Leonard's jar experiment. Plants were grown in a greenhouse in modified Leonard's jars (31) filled with sterile sand. *Galega* seeds were sterilized and germinated as described previously (23). Quarter-strength Jensen's solution (31), containing 10 mg of NH_4NO_3 per liter as starter nitrogen, was used as a nutrient solution. After 4 weeks, plants were thinned to four per jar.

Seven days after being sown, the jars were inoculated with 20 ml of a suspension of rhizobia containing 2×10^{10} bacteria of either the effective strain alone

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TABLE 1. Bacterial strains used in this study and specificities of the HAMBI 1174- and *R. galegae*-specific PCR primers

Strain[a]	Geographical origin, description, or source (reference)[b]	PCR with the following primer[c]:	
		HAMBI 1174 specific	*R. galegae* specific
R. galegae (host, *G. orientalis*)			
HAMBI 540^T (ATCC 43677^T)	Finland (13)	+	+
HAMBI 1174 (gal 1261R)	Sm^r and Spc^r derivative of HAMBI 540^T	+	+
HAMBI 1461 (gal 302)	Russia (13)	−	+
HAMBI 1428 (K-092)	Russia (13)	−	+
gal 740	Estonia	−	+
913	Estonia	−	+
842	Caucasus	−	+
843	Caucasus	−	+
847	Caucasus	−	+
848	Caucasus	−	+
849	Caucasus	−	+
CIAM 0707	Russia	−	+
CIAM 0703	Russia	−	+
912	Estonia	−	+
R. galegae (host, *G. officinalis*)			
HAMBI 1141 (gal 1)	New Zealand (13)	−	+
HAMBI 1207 (gal 1s)	Sm^r derivative of HAMBI 1141	−	+
HAMBI 1143 (gal 3)	New Zealand (13)	−	+
HAMBI 1146 (gal 14)	New Zealand (13)	−	+
HAMBI 490 (gal B7i)	Finland (13)	−	+
HAMBI 1209 (gal B7is)	Sm^r derivative of HAMBI 490	−	+
HAMBI 503 (59A2)	United States (13)	−	+
HAMBI 1183(G6)	United Kingdom (13)	−	+
HAMBI 1185(G9)	United Kingdom (13)	−	+
HAMBI 1144 (gal 7)	New Zealand (13)	−	+
HAMBI 1184 (G8)	United Kingdom (13)	−	+
HAMBI 1145 (gal 12)	New Zealand (13)	−	+
HAMBI 1151 (NZP 5068)	New Zealand (13)	−	+
NBIMCC 2246 (BG1)	Bulgaria	−	+
NBIMCC 2247 (BG2)	Bulgaria	−	+
NBIMCC 2248 (BG5)	Bulgaria	−	+
NBIMCC 2249 (BG6)	Bulgaria	−	+
NBIMCC 2250 (BG7)	Bulgaria	−	+
NBIMCC 2251 (BG8)	Bulgaria	−	+
R. meliloti			
HAMBI 1463 (Rm1021)	F. M. Ausubel	−	−
HAMBI 1318 (Rm D44d)	F. M. Ausubel	−	−
HAMBI 470 (MEL 2)	Finland	−	−
HAMBI 1212 (Rm1027)	F. M. Ausubel	−	−
HAMBI 1213 (Rm1126)	F. M. Ausubel	−	−
Rhizobium leguminosarum			
HAMBI 1125 (NZP 5262; CB596)	United Kingdom (35)	−	−
HAMBI 464 (HT3)	Finland (14)	−	−
HAMBI 499 (175 F 1)	United States (35)	−	−
MPI 7001	United Kingdom (15)	−	−
HAMBI 458 (502; 60B)	Finland (35)	−	−
HAMBI 1142 (NZP 5486; 3HOg2)	United States (14)	−	−
Rhizobium loti			
HAMBI 1129 (NZP 2213)	New Zealand (35)	−	−
MPI 4001	New Zealand (35)	−	−
Rhizobium fredii HAMBI 1337 (USDA 191)	China	−	−
Rhizobium tropici HAMBI 1163 (CIAT 899)	Colombia	−	−
B. japonicum HAMBI 1269 (DES 122)	H. Evans	−	−
Escherichia coli HAMBI 1272 (DSM 2840)		−	−
Agrobacterium tumefaciens HAMBI 1217 (AC58)	F. M. Ausubel	−	−
Erwinia carotovora subsp. *atroseptica* HAMBI 1429 (549)	J. Kankila	−	−
Pseudomonas syringae AD111	D. Bamford	−	−
Klebsiella pneumoniae AS	K. Haahtela	−	−

[a] Alternative names are given in parentheses. HAMBI, culture collection at the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland; MPI, culture collection at Max-Planck Institute, Cologne, Germany; CIAM, collection of root nodule bacteria in the Institute for Agricultural Microbiology, St. Petersburg, Russia; NBIMCC, National Bank for Industrial Microorganisms and Cellular Cultures, Sofia, Bulgaria.

[b] Sm^r, streptomycin resistant; Spc^r, spectinomycin resistant.

[c] +, amplification of the fragment; −, no amplification.

TABLE 2. Amounts of *R. galegae* HAMBI 1207 inoculated into soil and densities in the soil after 6 weeks[a]

Inoculation level (aim)	No. of bacteria inoculated	Plate count
0	0.00	0.00
2	2.05	2.14
3	3.00	2.86
4	3.79	3.31
5	4.86	4.07

[a] Data are expressed as $\log_{10}$ bacteria per g of soil.

or a mixture of the effective strain and one of the ineffective strains, prepared as follows. Rhizobial cultures grown in YEM media to stationary phase were gently centrifuged, washed, and resuspended in sterile 0.05 M phosphate buffer (pH 6.5) to a density of 10^9 bacteria per ml. The cell density was adjusted by turbidimetry (using a Klett-Summerson photoelectric colorimeter 800-3 [Klett Manufacturing Co., Inc., New York, N.Y.] with a 400- to 465-nm filter) according to previously determined strain-specific calibration curves. Appropriate amounts of suspensions containing 10^9 bacteria of both the effective strain (HAMBI 1174) and one of the ineffective strains (HAMBI 1207 or HAMBI 1209) per ml were mixed at the following volume ratios: 0:100, 1:99, 10:90, 50:50, 90:10, 99:1, and 100:0.

Uninoculated plants served as negative controls. There were five replicates for each treatment.

The plants were harvested after 9 weeks. The two biggest plants were collected from each jar for further analysis. The shoots were separated from the roots, oven dried, and weighed. Acetylene reduction by the roots was measured according to the method of Lindström (11, 12), and the roots were stored in 20% (vol/vol) glycerol at −20°C until nodules were analyzed. The number of large and small nodules was counted, and bacterial strains were isolated from the nodules. Before isolation, the roots were surface sterilized by being washed for 20 min in 6% hydrogen peroxide containing detergent (0.1% [vol/vol] Deconex 15 PF; Borer Chemie AG, Zuchwil, Switzerland) and dried between sterile papers. Then 6 small nodules and at most 20 big nodules were picked from each root, crushed in a microtiter dish, and transferred onto YEM agar plates containing 25 μg of Congo red (Merck GmbH., Darmstadt, Germany) per ml and appropriate amounts of streptomycin and spectinomycin.

Preparation of soil for the pot experiment. Agricultural soil from the Partala Research Station for Ecological Agriculture, Juva, Finland, was used. It is coarse silt soil (Finnish classification [7]) with high humus content (6% organic C; pH 6.2; levels of exchangeable nutrients [determined according to the method of Vuorinen and Mäkitie {32}] in milligrams per liter: Ca, 1,200; Mg, 198; K, 128; P, 6.2). No indigenous *R. galegae* could be detected.

The soil was passed through a 15-mm-perforation-size sieve. For each 2.5-kg soil lot, corresponding to one pot, 9.4 g of Siilinjärvi biotite rock powder (ground phlogopite and biotite containing 10% Mg–7% Ca–5% K solubilized in 10 M HNO_3; Kemira Chemicals, Siilinjärvi, Finland) and 0.63 g of commercial superphosphate (18% P) were added as fertilizers. The soil and fertilizers were mixed together in a concrete mixer, to which *R. galegae* (HAMBI 1207) was also added. Bacteria were appropriately diluted in sterile water from a peat-based inoculant, and 50 ml of bacterial suspension was sprayed into the soil during mixing to reach 0, 10^2, 10^3, 10^4, and 10^5 cells per g of dry soil. Pots (3.5 liters) were filled with 2.5 kg of soil (wet weight), loosely covered, and left to equilibrate for 6 weeks at ambient temperature (10 to 25°C).

Prior to the sowing, the size of the *R. galegae* HAMBI 1207 population in the soils was determined by plate counts on YEM agar containing streptomycin (1,000 mg/liter), Actidione (250 mg/liter), Congo red (25 mg/liter), and crystal violet (0.5 mg/liter) (Table 2).

Plant growth. Nonsterile seeds of *G. orientalis* were inoculated with *R. galegae* HAMBI 1174 in peat-based inoculant by the inoculant pellet technique (4). The inoculation rates were 2×10^4 and 2×10^5 cells per seed, which corresponded to 0.5 and 5 times the recommended rate, respectively. Uninoculated seeds were treated with sterile inoculant peat. Ten seeds per pot were sown, and after 3 weeks, the seedlings were thinned to four per pot. The plants were grown in a greenhouse for 14 weeks. The temperature fluctuated from 15 to 25°C. Artificial illumination was provided to ensure a 16-h light period per day. Pots were routinely watered three times a week and to constant weight every second week, to ensure constant moisture content in the pots. There were six replicates for each treatment.

At harvest, the shoots were excised, oven dried (105°C overnight), and weighed. The roots were examined for nodulation. Small white nodules were classified as ineffective, and their estimated number was scored on a four-step scale as follows: no small nodules, less than 30 small nodules per plant, 30 to 100 small nodules per plant, and more than 100 small nodules per plant. Pink nodules were classified as effective and were counted.

Collection of nodules for identification. Nodules were picked from the roots, rinsed with 70% ethanol, surface sterilized as described for the roots in the Leonard's jar experiment, and air dried laminar in a hood. Big nodules were aseptically cut into two halves, one of which was used for antibiotic resistance analysis and the other of which was stored in a sterile microtiter dish at −20°C for later PCR analysis. Small nodules were also collected, sterilized as described above, and kept frozen in microtiter dishes for later identification.

Antibiotic resistance tests. One half of each collected effective nodule was punctured with a sterile needle, which was thereafter stuck into duplicate YEM agar plates, both supplemented with 250 μg of Actidione per ml and one containing 500 μg of spectinomycin per ml. The plates were incubated at 28°C for 4 days. Isolates growing only without spectinomycin were identified as HAMBI 1207, and isolates growing also on plates with spectinomycin were identified as HAMBI 1174.

Rhizobia from the small nodules were isolated from nodules stored at −20°C. After thawing, nodules were crushed in 60 μl of H_2O. Ten microliters of the suspension was frozen again and stored at −20°C before PCR. The remaining 50 μl of the suspension was spread on YEM agar plates containing streptomycin (1,000 μg/ml) and Congo red. Streptomycin-resistant colonies with *Rhizobium* morphology were isolated, cultured in 3 ml of YEM broth (with 1,000 μg of streptomycin per ml) for DNA isolation, and tested for spectinomycin resistance on YEM agar plates with 500 μg of spectinomycin per ml.

Statistical methods. Data analysis was done by using the SURVO 84C statistical program (20) or the Statistix II program (NH Analytical Software) for microcomputers. For the analysis of variance, plant yield, acetylene reduction, and nodulation data were square-root transformed to equalize variances. Tukey's test was applied to compare the means, and the significance of the differences was tested at $P < 0.05$.

Molecular biological methods. Standard DNA methods were used as described by Ausubel et al. (2). Genomic DNA from pure bacterial cultures was purified by the CTAB (cetyltrimethylammonium bromide) method (2). DNA sequencing was done at the Institute of Biotechnology, University of Helsinki, by the Sanger dideoxy-chain termination method, using the T7 Sequencing Kit (Pharmacia, Uppsala, Sweden), fluorescein-labeled primers, and an automatic laser fluorescent sequencer (Pharmacia).

PCR primers. PCR primers were synthesized by the Institute of Biotechnology, University of Helsinki, Helsinki, Finland.

Strain-specific primers (2755 and 2756) were developed earlier by isolation of a 342-bp strain-specific fragment from *R. galegae* HAMBI 1174 by subtraction hybridization (27) (EMBL accession number S72496). Their nucleotide sequences are as follows: 2755, 5′-CCT TTC GCC TCG GTT CGG CTT C-3′, and 2756, 5′-CGA GGC CGG GAT GAT TTG CTA TGA G-3′. The amplified fragment is 264 bp long.

Species-specific primers (3181 and 3182) amplifying an approximately 850-bp fragment of *R. galegae* were designed during this work. Their sequences are as follows: 3181, 5′-CTG GGG CGA TGG GCG ATA ACT ACT G-3′, and 3182, 5′-TGC TTG GAG TCG GTG CAA GTT TGG T-3′.

The specificity of these two sets of primers was tested with purified genomic DNA isolated from 54 bacterial strains (Table 1).

Primers described by Weisburg et al. (33) were used for amplification of sequences of genes coding for 16S rRNA (16S rDNA). They were as follows: fD1, 5′-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3′, and rD1, 5′-CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC-3′.

PCR with crushed nodules. Nodules, collected freshly or stored at −20°C, were crushed with sterile glass rods in an Eppendorf tube and suspended in 200 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.5]). The suspension was shaken for 1 to 2 min and centrifuged for 1 min at 14,000 rpm in an Eppendorf centrifuge. A 1- to 2-μl portion of the supernatant was immediately used for PCR, and the rest of the preparation was kept at −20°C for later use. All PCRs were done twice.

The 25-μl PCR mixture contained 1× reaction buffer (10 mM Tris-HCl [pH 8.8], 50 mM KCl, 0.1% Triton X-100), 3.5 mM $MgCl_2$, 0.2 μM (each) the four primers (2755, 2756, 3181, and 3182), 160 μM (each) deoxynucleoside triphosphates (Pharmacia), 1 U of DNA polymerase (DynaZyme; Finnzymes Oy, Espoo, Finland), and 1 to 2 μl of the template. A hot start was performed by melting and cooling a small wax bead (Difco catalog no. 8825-19) on top of the reaction mixture before adding the enzyme. Finally, 25 μl of mineral oil was layered on top of the reaction mixture.

The amplification was performed in a MiniCycler machine (MJ Research, Inc.) with the following temperature profile: an initial denaturation at 94°C for 4 min, 30 cycles of a two-step amplification (94°C for 30 s and 72°C for 1 min), and a final extension at 72°C for 5 min. Amplified DNA was examined by gel electrophoresis in 1.5% agarose (Promega, Madison, Wis.) with 10-μl aliquots of the PCR products.

RFLP analysis of amplified 16S rDNA. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA sequences was performed according to the method of Laguerre et al. (10), using AmpliTaq DNA polymerase (Perkin-Elmer) with its own reaction buffer and TaqStart antibody (catalog no. 5400-1; CLONTECH Laboratories, Inc., Palo Alto, Calif.) for the hot start. PCR amplification was carried out in a 70-μl reaction volume containing 1 μl of template (purified bacterial DNA), polymerase reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM (each) nucleotides, 0.1 μM (each) primers fD1 and rD1, 2 U of AmpliTaq DNA polymerase, and 440 ng

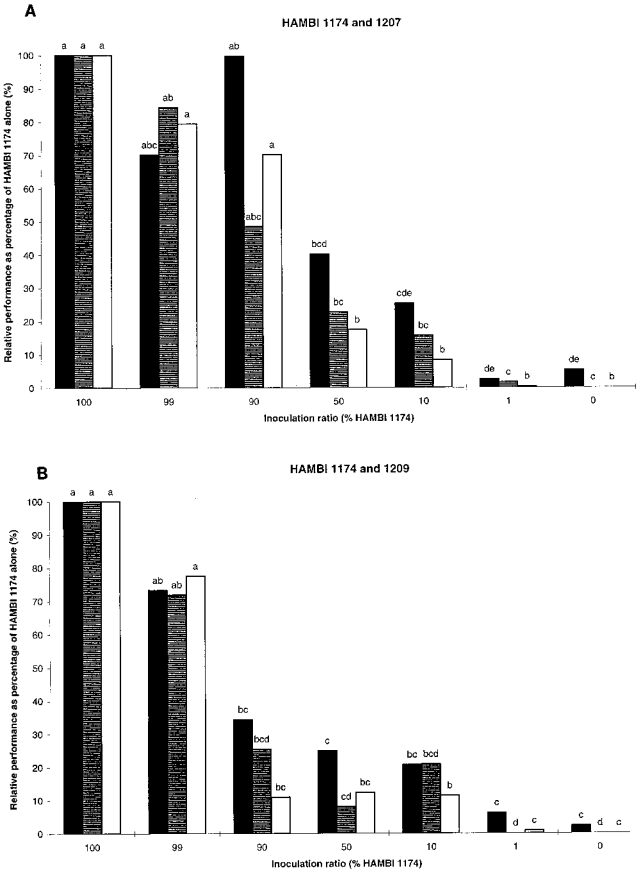

FIG. 1. Relative symbiotic performances of *R. galegae* HAMBI 1174 in mixtures with HAMBI 1207 (A) and HAMBI 1209 (B) inoculated onto *G. orientalis* in Leonard's jars. Plant yield (dry weight of shoots; black columns; 100% = 857 mg), acetylene reduction (stippled columns; 100% = 6.77 μmol h^{-1}), and the number of big nodules (white columns; 100% = 73) are expressed as percentages of the results obtained with the effective strain, HAMBI 1174, when it was applied alone. Values are means for 5 replicates (or 10 replicates for the 100% inoculation ratio). Columns with identical letters do not differ significantly at $P < 0.05$ (Tukey's test).

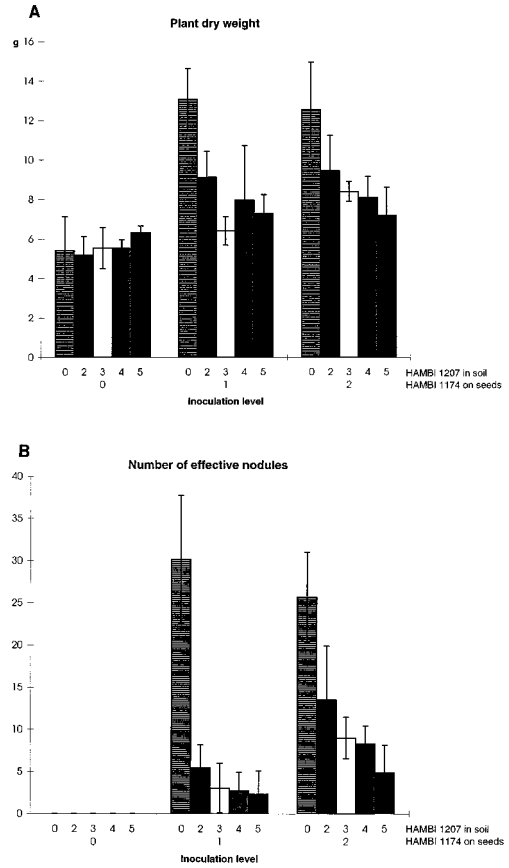

FIG. 2. Competition in the pot experiment between the ineffective strain HAMBI 1207 inoculated into the soil and the effective strain HAMBI 1174 inoculated onto seeds of *G. orientalis*. (A) Dry weights of the shoots; (B) numbers of effective nodules. The upper *x*-axis labels refer to the level of HAMBI 1207 in the soil (0, no inoculation; 2 through 5, 10^2, 10^3, 10^4, and 10^5 bacteria per g of soil, respectively). The lower *x*-axis labels show the HAMBI 1174 seed inoculant level (0, no inoculation; 1, 2×10^4 bacteria per seed; 2, 2×10^5 bacteria per seed). Data are means for six replicates with error bars showing standard deviations.

of TaqStart antibody (the molar ratio of antibody to polymerase was 28:1 according to the manufacturer's recommendation). PCR cycles were as follows: initial denaturation at 95°C for 3 min, 30 cycles of amplification (94°C for 30 s, 55°C for 1 min, and 72°C for 2 min), and final extension at 72°C for 3 min. Five-microliter portions of the PCR products were examined in 1% agarose, and 10-μl aliquots were digested with the following restriction endonucleases: *Cfo*I, *Hin*fI, *Msp*I, and *Rsa*I (Promega). Digested DNA was analyzed in a 5% agarose gel.

RESULTS

Competition between effective and ineffective *R. galegae* strains in Leonard's jars. The symbiotic performance of *G. orientalis* plants inoculated with a mixture of the effective strain, HAMBI 1174, and one of the ineffective strains, HAMBI 1207 or HAMBI 1209, was reduced with increasing proportions of each of the two ineffective strains (Fig. 1).

When the ineffective strain HAMBI 1207 was present at only a 1% proportion in the inoculant mixture, the plant yield decreased by 30%, the level of acetylene reduction decreased by 15%, and the number of big nodules decreased by 20%. When the proportion of the ineffective strain was 50%, the measured values decreased by 60 to 80% (Fig. 1A). The number of big nodules correlated with both the shoot dry weight ($r = 0.945$) and the level of acetylene reduction ($r = 0.973$).

When HAMBI 1209 was present at a 1% proportion, plant yield and the level of acetylene reduction decreased by 25%, whereas the number of big nodules decreased by 20%. When the ratio of the strains was 50%, plant yield decreased by more than 70%, the level of acetylene reduction decreased by 90%, and the number of big nodules decreased by 75% (Fig. 1B). The number of big nodules correlated again with both the shoot dry weight ($r = 0.962$) and the level of acetylene reduction ($r = 0.973$).

The number of small nodules did not vary with different inoculation proportions (data not shown) for either ineffective strain HAMBI 1207 or ineffective strain HAMBI 1209.

From the results we concluded that both ineffective strains are strong competitors against the effective strain HAMBI 1174. Since the abilities of the two ineffective strains to compete with the effective strain were similar, we chose HAMBI 1207 for further experiments.

Competition between soil-inoculated *R. galegae* HAMBI 1207 and seed-inoculated HAMBI 1174 in pots. The plant yields from the pot experiment are shown in Fig. 2A. When there was no competition between effective and ineffective bacteria, inoculation with the effective strain HAMBI 1174 doubled plant yield compared with that in uninoculated pots.

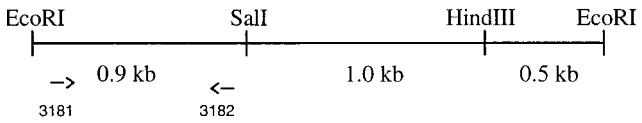

FIG. 3. Map of the 2.4-kb species-specific fragment isolated from the putative host specificity gene region of *R. galegae* HAMBI 1174. The arrows show PCR primers.

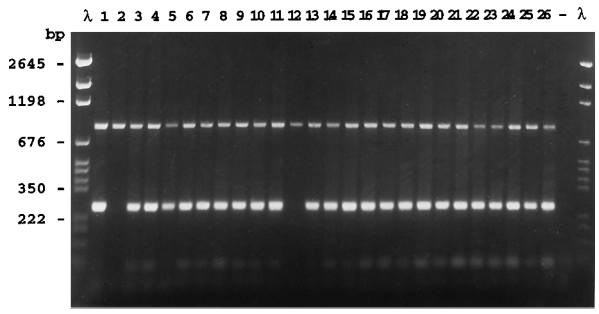

FIG. 4. PCR amplification of the 264-bp HAMBI 1174-specific fragment and the 850-bp *R. galegae*-specific fragment from crushed nodules. Nodules collected from the plant roots were surface sterilized and crushed as described in Materials and Methods. The positive controls (lanes 1 and 2) contained 1 ng of purified bacterial genomic DNA, and the negative control had no template DNA. Lanes: λ, phage lambda DNA digested with *Ava*II; 1, *R. galegae* HAMBI 1174 pure genomic DNA; 2, HAMBI 1207 pure DNA; 3 to 26, crushed nodules; −, no template DNA.

The plant response to inoculation decreased with increasing numbers of ineffective HAMBI 1207 bacteria in soil. As few as 10^2 ineffective HAMBI 1207 bacteria per g of soil significantly reduced plant yield, and in pots having 10^3 or more HAMBI 1207 bacteria per g of soil, plants inoculated with the effective strain did not grow significantly better than uninoculated ones. The growth of uninoculated plants was not affected by the density of ineffective HAMBI 1207 bacteria in soil. There was no significant difference between the plants with the two different seed inoculation rates.

As can be seen from Fig. 2B, nodulation data were in agreement with plant yield data: the greater the number of ineffective bacteria that were present in soil, the smaller the number of effective nodules that could be found after inoculation. The decrease in the number of effective nodules was significant already with 10^2 HAMBI 1207 bacteria per g of soil at both seed inoculation levels. At the lower inoculation level (2×10^4 HAMBI 1174 bacteria per seed), the pots with 10^2 to 10^5 HAMBI 1207 bacteria per g of soil did not differ significantly from each other. The higher level of seed inoculant (2×10^5 HAMBI 1174 bacteria per seed) resulted in significantly more effective nodules than did the lower seed inoculation level when soils had 10^2 to 10^4 HAMBI 1207 bacteria per g. At the higher seed inoculation level, significantly more nodules were formed in pots with 10^2 HAMBI 1207 bacteria per g of soil than in pots with 10^5 ineffective bacteria per g of soil, but results for pots with 10^3 and 10^4 HAMBI 1207 bacteria per g of soil did not differ from those for either of these groups.

In some of the uninoculated pots we found a few effective nodules. As they were concentrated around drainage holes of the pots, we concluded that they were contaminants and excluded these pots from the data analysis.

The density of small nodules on the roots was high already with 10^2 ineffective bacteria per g of soil, although it was lower when seeds were inoculated with the effective strain, and it did not vary very much with increasing numbers of ineffective bacteria in the soil (data not shown).

Generation of species-specific PCR primers. Species-specific primers were designed by analyzing the putative host specificity gene region of *R. galegae*, which was previously isolated and physically mapped (data not shown). A 2.4-kb *Eco*RI fragment of this region is highly conserved among *R. galegae* strains. It was monomorphic in RFLP analysis of all *R. galegae* strains studied but had no homology to DNA of any other bacteria (25). A 0.9-kb *Eco*RI-*Sal*I subfragment of this 2.4-kb region (Fig. 3) is also species specific as determined by dot blot hybridization experiments (data not shown). The 0.9-kb fragment hybridized with a 2.4-kb *Eco*RI fragment in Southern blots of total genomic DNA of different *R. galegae* strains, which corresponds to the original 2.4-kb probe (data not shown). The 0.9-kb fragment was sequenced from the ends, and specific PCR primers (3181 and 3182; Fig. 3) were designed to amplify the fragment. The amplified region is about 850 bp long. The primers were constructed in such a way that they had the same theoretical melting temperature, 72°C, as the strain-specific primers. This is also the optimal extension temperature for the polymerase enzyme. This allowed us to use stringent primer-annealing conditions and rapid two-temperature PCR cycles and made possible the concurrent amplification of the two fragments. The identities of the amplified fragments were confirmed by Southern hybridizations (data not shown). The specificities of the primers are shown in Table 1. The fragments could be amplified not only from purified bacterial genomic DNA but also from nodules of plants inoculated with *R. galegae* strains.

Identification of nodule-occupying bacteria by antibiotic resistance and PCR. In the pot experiment 62 nodules considered to be effective were cut into halves and examined both for antibiotic resistance markers and by PCR. Fifty-nine of these nodules contained strain HAMBI 1174, as proven by both methods (growth on spectinomycin plates and amplification of both the strain-specific fragment and the species-specific fragment), and one was determined to carry HAMBI 1207 by the antibiotic test (growth only on streptomycin). From two nodules bacteria could not be isolated at all on streptomycin plates, and no DNA could be amplified from three nodules. Figure 4 shows the results of one of our PCR experiments. A few small nodules were also crushed in TE buffer and run in a PCR together with the effective nodules. Only the 850-bp species-specific fragment was amplified (Fig. 4, lane 12).

Twenty small nodules were investigated in more detail. In direct PCR with crushed nodules we did not always achieve amplification after 30 PCR cycles. When 30 more PCR cycles were run, the 850-bp fragment was quite strong in all samples, but we also got some nonspecific fragments, possibly due to contamination. We decided to examine these nodules by isolating streptomycin-resistant bacteria from them and purifying genomic DNA of the isolated colonies for further PCR and 16S RFLP analysis. Streptomycin-resistant bacteria could be isolated from only 14 of the 20 nodules. None of these isolated bacteria grew on spectinomycin agar. One to three colonies per nodule were isolated, and genomic DNA was purified from 20 isolates. PCR with the HAMBI 1174- and *R. galegae*-specific primers was applied to the purified DNA. The 850-bp species-specific fragment was amplified from 14 isolates derived from nine nodules (Fig. 5). 16S rDNA was amplified from all 20 of the isolates. Restriction patterns with four different enzymes were identical for 12 of the 14 isolates that contained the *R. galegae*-specific fragment. Purified DNA of *R. galegae* HAMBI 1174 and HAMBI 1207 gave the same patterns. A *Rhizobium meliloti* strain, used as a control, gave patterns different from

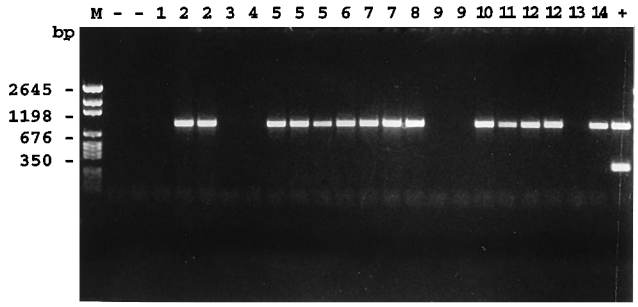

FIG. 5. PCR with genomic DNA purified from bacterial isolates from ineffective nodules using the *R. galegae*- and HAMBI 1174-specific primers. All lanes represent individual DNA samples from different bacterial isolates. Lanes assigned the same number correspond to different isolates from the same nodule. M, molecular weight marker (pGEM; Promega); −, no template DNA; +, purified DNA from HAMBI 1174.

those observed for *R. galegae* with enzymes *Hin*fI and *Msp*I, in agreement with the results of Laguerre et al. (10). The restriction patterns with *Msp*I are shown in Fig. 6. The other two isolates that seemed to be *R. galegae* according to the species-specific PCR showed slightly different patterns. All of the isolates without the species-specific fragment gave significantly different 16S RFLP patterns with most of the enzymes.

DISCUSSION

When a legume which already has a compatible indigenous *Rhizobium* population in soil is inoculated, inoculant bacteria have to compete with indigenous ones. Unfortunately, the nitrogen-fixing efficiency and competitive ability of a strain are not necessarily correlated (1). This was also demonstrated in our Leonard's jar experiments, in which infective but ineffective strains of *R. galegae* (HAMBI 1207 and HAMBI 1209) competed well with an effective strain (HAMBI 1174), resulting in poor plant growth.

The results of competition experiments performed under axenic laboratory conditions are difficult to interpret and extrapolate to practical inoculation conditions, under which soil populations and inoculant bacteria on the seed surface compete for nodule formation. Since *R. galegae* is not indigenous to Finnish soils, we could prepare soils having well-characterized but still naturalized populations of *R. galegae*. Application of diluted, mature peat inoculant to soil 6 weeks before the planting of seeds resulted in introduced "indigenous" bacteria in a realistic physiological state, in contrast to laboratory conditions, under which bacteria grown in rich nutrient broth are normally used. To our knowledge, this is the first time that quantitative, well-defined competition experiments have been performed under nonsterile, natural conditions.

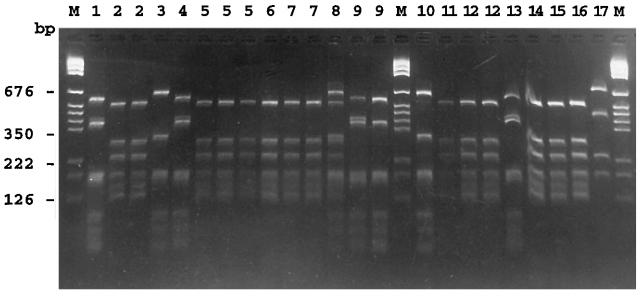

FIG. 6. Restriction patterns of PCR-amplified 16S rDNA fragments from bacterial isolates from ineffective nodules as explained in the legend to Fig. 5. The amplified fragments were digested with *Msp*I and run in a 5% agarose gel. The lane assignments 1 to 14 and M are the same as those for Fig. 5. Lanes 15 to 17, pure genomic DNA from HAMBI 1174 (lane 15), HAMBI 1207 (lane 16), and *R. meliloti* Rm1021 (lane 17), respectively.

Thies et al. (28) showed that indigenous rhizobia in relatively small numbers, i.e., fewer than 10^2 cells per g of soil, eliminated the inoculation response. They attributed this elimination to the few effective strains assumed to be always present in a natural soil populations. In our experiments the soil population was totally ineffective. Therefore, it was unexpected when as few as 10^2 ineffective rhizobia per g of soil significantly diminished the positive effect of inoculation on plant growth and nodulation.

The relatively large standard deviations in our results are probably due to high-level genetic variability of goat's rue (19). Goat's rue is cross-pollinating, and the cultivar Gale that we used in our experiments is actually inbred, selected from a natural population.

We have previously used light and electron microscopy to study the structures of effective and ineffective nodules formed on *G. orientalis* (18). We found that the ineffective nodules had an internal structure similar to that of the effective ones and even contained bacteroids. Thus, the small nodules are really ineffective nodules and not some other kind of cell proliferation, such as tumors induced by *Agrobacterium* spp. on plant roots. We also found earlier that in a test tube nodulation experiment, when *G. orientalis* plants were inoculated solely with an ineffective strain, big nodules were also formed, as if the plants were trying to develop structures for perfect symbiosis. These nodules, however, did not fix nitrogen. On the other hand, in the Leonard's jar experiment described here we did not find big nodules on plants inoculated solely with an ineffective strain. Plant growth and acetylene reduction of the roots correlated with the number of big nodules, suggesting that the big nodules are effective. We used this information in our pot experiment when we classified big nodules as effective and small nodules as ineffective. Resistance to antibiotics and the results of our PCR experiments were in agreement with conclusions based on nodule morphology. All these observations suggest that *G. orientalis* cannot distinguish between effective and ineffective strains at the infection stage but that it recognizes the effectiveness of the nodule-inducing bacteria at a rather late stage of development of the symbiosis.

Competitive ability of the inoculant strain has been recognized as an important property of a good inoculant (3). However, when a new plant and its microsymbiont are introduced into a new environment without appropriate indigenous populations, good competitive ability for nodule formation is not necessary. Instead, a good competitor may in the future prevent the replacement of the first strain by a new one, which turns out to be more optimal. For instance, many U.S. soybean fields are occupied by aggressive *Bradyrhizobium japonicum* strains (9). New inoculant strains with greater nitrogen-fixing capacity are unable to form nodules (6, 8), and nitrogen fertilization remains the only way to improve plant yields.

Therefore, we propose that the first inoculant strain for a new crop should be a weak competitor. High-level genetic stability to minimize the transfer of symbiotic genes to other, nonnodulating rhizobia (24) is also desirable.

One important aspect of competition experiments, especially those performed under natural conditions, is the identification of the bacterial strains involved. Antibiotic resistance and other markers, occasionally including monoclonal antibodies, are used for this purpose. We also used antibiotic-resistant strains to facilitate confirmation of our results, but we wanted to develop an identification method based on genetic markers

characteristic also for wild-type strains. We developed strain- and species-specific primers for amplification of specific fragments of the *R. galegae* genome and successfully applied PCR for identification of strains from the nodules.

One advantage of PCR-based identification over the use of antibiotic resistance markers is that PCR can be applied also to dead cells, while antibiotic resistance can be tested only for living and culturable cells. The amplification and RFLP analysis of 16S sequences constitute an effective and sensitive method for bacterial identification, and this method is widely used for taxonomic purposes (10, 22). It distinguishes species but usually cannot reveal differences between strains. We used this method to confirm that our isolates, from which the *R. galegae*-specific fragment could not be amplified, do belong to some other species. 16S RFLP analysis is slightly more time-consuming and expensive than direct amplification of taxon-specific fragments because of the required restrictions and running of more special gels. In addition, for 16S RFLP analysis and other fingerprinting methods it is important to use isolated bacteria, whereas specific fragments can be amplified also from mixed cultures, such as most environmental samples. Thus, PCR with specific primers is more powerful and could be the best choice for ecological studies.

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