Genetic and Metabolic Divergence within a *Rhizobium leguminosarum* bv. *trifolii* Population Recovered from Clover Nodules[†]

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Rhizobia are able to establish symbiosis with leguminous plants and usually occupy highly complex soil habitats. The large size and complexity of their genomes are considered advantageous, possibly enhancing their metabolic and adaptive potential and, in consequence, their competitiveness. A population of *Rhizobium leguminosarum* bv. *trifolii* organisms recovered from nodules of several clover plants growing in each other's vicinity in the soil was examined regarding possible relationships between their metabolic-physiological properties and their prevalence in such a local population. Genetic and metabolic variability within the *R. leguminosarum* bv. *trifolii* strains occupying nodules of several plants was of special interest, and both types were found to be considerable. Moreover, a prevalence of metabolically versatile strains, i.e., those not specializing in utilization of any group of substrates, was observed by combining statistical analyses of Biolog test results with the frequency of occurrence of genetically distinct strains. Metabolic versatility with regard to nutritional requirements was not directly advantageous for effectiveness in the symbiotic interaction with clover: rhizobia with specialized metabolism were more effective in symbiosis but rarely occurred in the population. The significance of genetic and, especially, metabolic complexity of bacteria constituting a nodule population is discussed in the context of strategies employed by bacteria in competition.

The soil bacterium *Rhizobium leguminosarum* bv. *trifolii* is capable of symbiotic interaction with the host plant *Trifolium* spp. (clover). The symbiotic process involves an exchange of chemical signals between both organisms, resulting in the expression of specific bacterial and plant genes. In response to flavonoid signals from legumes, bacterial lipochitooligosaccharides (Nod factors) are synthesized and in turn trigger the expression of plant genes and root nodule formation (9). Rhizobia invade the root nodules and differentiate into bacteroids that fix nitrogen (14, 16, 21, 36, 37). Atmospheric dinitrogen converted into ammonia is further transported and assimilated by the plant, which, reciprocally, provides photosynthates (42, 43, 50). The range of plant benefits varies and depends on the effectiveness of the bacterial strains as well as the legume plant genotype (8).

A common feature of rhizobial genomes is the complexity and diversity of genomic organization, with a single chromosome and large plasmids ranging in size from ca. 100 kb up to 2 Mb (34). The genes encoding symbiotic functions usually constitute independent replicons known as symbiotic plasmids (pSym), or symbiotic islands when incorporated into the chromosome (25). The plasmids constitute a pool of accessory genetic information (18, 53) and contribute to the plasticity and dynamic state of the genome commonly observed among members of the *Rhizobiaceae* family (4, 25, 28, 34). Rhizobia occupy highly complex soil habitats, and their

large and multipartite genomes, which encode many potentially useful metabolic traits, might be advantageous, enhancing their adaptive potential (33). Local populations of rhizobia may differ significantly on both the genetic and physiological levels. The diverse metabolic capacities of different strains and species of rhizobia might be important in their adaptation and survival in the rhizospheres of host plants. Plant root exudates contain a great number of chemical compounds, comprising sugars, amino acids, amines, aliphatic and aromatic acids, phenols, and others (2, 3, 15, 38, 49), thus potentially influencing the structure of the bacterial community in the rhizosphere. It was demonstrated that more metabolically versatile strains of *R. leguminosarum* were better competitors (51). Several studies showed that the nutritional diversity of soil habitats and the rhizosphere influences the number of rhizobia and that competition for root nodule colonization can take place even inside the infection threads, occupied, in some cases, by more than one strain (32, 38, 47). Up-to-date research on the diversity and competition of rhizobia focused on strains colonizing the soil or particular species of legume plants (8, 12, 24, 31, 35). Comprehensive analyses of the genetic and, especially, metabolic variability in rhizobia that occupy a spatially restricted area, for instance, all the nodules of a legume plant root system coexisting in one place, are still lacking.

In this work, we investigated the degree of genetic and metabolic variability within the *R. leguminosarum* bv. *trifolii* strains occupying a spatially restricted area—the nodules of several clover plants—focusing on estimation of possible interconnections between the metabolic-physiological properties of strains and their frequency of occurrence.

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MATERIALS AND METHODS

Rhizobium leguminosarum bv. *trifolii* strains. One hundred twenty-nine *Rhizobium leguminosarum* isolates were obtained from nodules of red clover (*Trifolium pratense* L. cv. Dajana) growing in sandy loam (N:P:K, 0.157%:0.014%:0.013%). This soil contained relatively large numbers of *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*, and *R. leguminosarum* bv. *phaseoli* organisms, namely, 4.2×10^3 , 9.2×10^3 , and 1.5×10^3 bacteria/g of soil, respectively, as determined by the most-probable-number (MPN) method (27). Plants were grown on a 1-m² plot for 6 weeks between May and June 2008. Ten randomly chosen clovers growing in each other's vicinity were harvested, the nodules were surface sterilized and crushed, and their contents were plated on 79CA medium (48). Pure cultures were used in further experiments.

Plasmid profiles and PCR fingerprinting. Analyses of the plasmid contents of the 129 isolates were performed as described by Eckhardt (10). Estimation of plasmid size was performed using Bio-Profil V11.01 (Vilber-Lourmat, France), using *R. leguminosarum* bv. *viciae* strain 3841 (53) as the plasmid standard.

PCR assays of the 16S-23S rRNA internal transcribed spacer (ITS) region were carried out using genomic DNAs isolated from all 129 *R. leguminosarum* bv. *trifolii* isolates as templates and primers FGPS1490 (5'-TGCGGCTGGATCAC CTCCTT-3') and FGPL132 (5'-CCGGGTTTCCCCATTCGG-3') (23). PCR amplicons were digested with the BsuRI and TaqI restriction enzymes, and restriction fragments were separated by 3% agarose gel electrophoresis.

For the subset of 27 *R. leguminosarum* bv. *trifolii* isolates differing significantly in plasmid profile and/or 16S-23S ITS PCR-restriction fragment length polymorphism (PCR-RFLP) profile, a 308-bp fragment of the 16S rRNA gene was amplified from DNA by using primers Y1 (5'-TGGCTCAGAACGACGCTG GCGGC-3') and Y2 (5'-CCCACTGCTGCTCCCGTAGGAGT-3') (52). The PCR products were sequenced using a BigDye Terminator kit (Applied Biosystems) and an ABI Prism 310 sequencer.

Physiological tests. The urease, catalase, phosphatase, peroxidase, and nitrate reductase activities and indole-3-acetic acid (IAA) production of *Rhizobium leguminosarum* isolates were examined by the methods described by Cowan and Steel (6) and Minamisawa and Fukai (29). The utilization of different carbon and energy sources by *Rhizobium* isolates was assessed using a Biolog GN2 microplate (Gram-negative bacterial identification test panel) containing 95 carbon sources, including sugars, amino acids, and organic acids, as described earlier (51). Briefly, bacteria growing overnight at 28°C on TY agar medium (41) were collected and washed twice with sterile water. After that, the pellet was diluted in water to an initial optical density at 550 nm (OD₅₅₀) of 0.1 (approximately 10^8 cells ml⁻¹), and a 150-μl suspension of rhizobia was inoculated into each well of a Biolog microplate. The cells were incubated for 24 h at 28°C, and the results were recorded using a Benchmark Plus microplate reader (Bio-Rad Laboratories).

Assays of growth kinetics. For assays of bacterial growth kinetics, the isolates were grown overnight at 28°C in 5 ml TY liquid medium. The cultures were then diluted to an OD₅₅₀ of 0.2, and the suspensions were used for inoculation (1:100 [vol/vol]) of 79CA, TY, and M1 liquid media, with the latter supplemented with 1 μg/ml thiamine, 0.5 μg/ml biotin, and 1 μg/ml pantothenate. The cultures (each in triplicate) were grown in 79CA and TY media for 48 h and in M1 medium for 72 h at 28°C. Every 24 h, the OD₅₅₀ of each culture was measured.

Plant tests. Red clover seeds (*Trifolium pratense* L. cv. Dajana) were surface sterilized, germinated, and grown on nitrogen-free Fåhræus medium (48). Clover seedlings were inoculated with 0.2 ml of *R. leguminosarum* bv. *trifolii* cell suspension at an approximate density of 1.0×10^9 cells/ml and were grown (one per tube) in a greenhouse under natural light supplemented with artificial light (14-h day–10-h night at 24 and 19°C, respectively). After 5 weeks, the plants were harvested, the nodules were counted, and fresh masses of shoots and roots were estimated. For each strain, 30 clover plants were used in three independent experiments.

Data statistical analysis. The relationship between genetic classification of isolates and their capability of using metabolic substrates (quantities of different substrates) was analyzed using the median test with the Yates correction (11), which is a nonparametric alternative to one-way analysis of variance (ANOVA). The studied feature was the number of different metabolic substrates used by strains, while genetic classification based on PCR-RFLP analysis of the 16S-23S ITS region (at four levels) and the isolate frequencies of occurrence in the population (at three levels) were the grouping factors. The test was applied separately for PCR-RFLP and for isolate frequency classification.

For cluster analysis and principal component analysis (PCA), the results of the Biolog test were coded in the binary system. The cluster analysis was used to define metabolic similarity profiles of rhizobia, which were calculated by a simple

TABLE 1. *R. leguminosarum* bv. *trifolii* isolates assigned to PCR-RFLP groups and their occurrence in the population and in individual plant nodules

PCR-RFLP genotype	No. of isolates	Occurrence of isolates in population (%)	No. of colonized plants	Classification
A	33	25.6	10	Frequent
B	38	29.5	7	Frequent
C	34	26.4	9	Frequent
D	17	13.2	6	Intermediate
E	3	2.3	2	Rare
F	1	0.8	1	Rare
G	2	1.6	2	Rare
H	1	0.8	1	Rare

matching coefficient, following which clustering was performed by the unweighted-pair group method using average linkages (UPGMA).

PCA with varimax rotation (30) was used to analyze the bacterial capability of utilizing particular substrates or groups of substrates. Factor loadings in PCA (see Table 2) are the correlation coefficients between the original variables (metabolic substrates) and the obtained factors, named PC1, PC2, and PC3. In this manner, the PCA method allowed us to transform the numerous variables (utilization of individual substrates), possibly correlated as well, into small groups of uncorrelated factors (utilization of groups of substrates) as well as to interpret the defined PCA factors. Moreover, the particular cases (strains) were plotted in the space generated by the factor axes to classify them into categories according to their position in the coordinate system.

To compare the growth of bacteria belonging to different PCR-RFLP groups (A to D), ANOVA was used, taking into account the time of growth as a repeated measure. Groups E, F, G, and H were omitted due to their low levels of representation in the studied population. Although there were no significant differences between mean values of bacterial growth on M1 and 79CA media, differences in growth were observed on TY medium, and the initial hypothesis assuming equal mean values of the studied feature among the strains was rejected. As a consequence, a post hoc Tukey unequal *N* honestly significant difference (HSD) test was applied to analyze the bacterial growth on TY medium (46).

The results of plant tests were initially subjected to the Shapiro-Wilk *W* test of normality. This test indicated that the distribution of the three studied features (i.e., wet mass of shoots, wet mass of roots, and nodule number) was significantly different from a normal distribution. Therefore, the median test was used to analyze nodule number as a noncontinuous trait and Kruskal-Wallis ANOVA by ranks was used to analyze continuous traits (shoot and root wet masses). The second test is highly recommended (13), but in the case of noncontinuous traits, it may be replaced by the median test. All of the described analyses were performed with Statistica software.

Nucleotide sequence accession numbers. GenBank accession numbers GQ131693 to GQ131719 were given to the nucleotide sequences determined in this study.

RESULTS

Genetic and metabolic variability among clover nodule isolates. One hundred twenty-nine isolates obtained from nodules of 10 clover plants growing in each other's vicinity were characterized by plasmid patterns and PCR-RFLP analyses of the 16S-23S rRNA ITS region. RFLP analysis of the PCR-amplified 16S-23S rRNA ITS region was performed for all strains, and eight genetically distinct PCR-RFLP groups were identified (Table 1). Three of them (A, B, and C) appeared most frequently, and members of these groups were found in the nodules of all (A and B) or almost all (C) clover plants, allowing their classification as "frequent" in the nodule population. The frequency of appearance of the D genotype was denoted "intermediate," whereas the remaining four genotypes, E, F, G, and H, were classified as "rare" because their frequencies of

FIG. 1. Colonization of individual clover plants by *R. leguminosarum* bv. *trifolii* isolates classified into different PCR-RFLP genetic groups.

occurrence in the nodules did not exceed a few percentage points (Table 1; Fig. 1). The plasmid profiles showed great variability: each of 129 isolates contained 2 to 5 plasmids, ranging in size from approximately 200 kb to 1 Mb (see Fig. S1 in the supplemental material). Overall, the nodules of clover plants growing in the vicinity of each other in cultivated soil were occupied by distinct rhizobia possessing different genomic organizations and, presumably, different genetic contents, suggesting potential phenotypic differences.

All 129 strains were screened for urease, catalase, phosphatase, peroxidase, and nitrate reductase activities and for IAA production. Such simple physiological tests are routinely employed in polyphasic taxonomy and are preliminary indicators of bacterial phenotypic diversification (40).

A dendrogram based on the enzyme activities, constructed by UPGMA clustering, demonstrated the diversification of isolates with respect to these selected metabolic traits. Only small subgroups were distinguishable, with no apparent large clusters. Strains belonging to groups A, B, and C were less diversified than those in “rare” groups. They were often clustered inside the same subgroups of strains, with >95% similarity, whereas most of the “rare” ones were located outside these subgroups (see Fig. S2 in the supplemental material).

The growth of the 129 strains on different media (M1 medium, 79CA medium [mineral-rich medium], and TY medium [complete medium]) was compared to further differentiate these strains. In most cases, irrespective of the culture medium, the strains genetically classified as “frequent” or “intermediate” grew better than the “rare” ones (data not shown). Moreover, when ANOVA and the post hoc Tukey unequal *N* HSD test were employed in relation to PCR-RFLP classification (this analysis could be applied only for groups composed of numerous strains), a statistically significant difference ($P < 0.01$) in growth of isolates belonging to classes A, B, and C versus those in class D was observed for TY medium (details not shown). Such significant differences were not seen when strains were grown on M1 and 79CA media.

Selection of representative strains for metabolic and symbiotic assays and sampling validation. A subset of 27 *R. leguminosarum* bv. *trifolii* isolates was selected from the large number of initially recovered strains for further analyses in which we retained genetically distinct strains which differed substantially in plasmid profiles and belonged to distinct PCR-RFLP groups. In the chosen subset, “rare” isolates were overrepresented and constituted 15% of the population, in comparison to 5% of the initial population. “Intermediate” strains constituted 13% of the population before and 26% after the selection.

Sampling conditions for the 27 representatives were further validated with the data on different enzyme activities and bacterial growth under various nutritional conditions. According to enzyme activities, the 27 selected strains did not form a single cluster on a dendrogram based on these enzyme activities (see Fig. S2 in the supplemental material). Instead, they were dispersed to different branches and thus might be considered metabolically diverse representatives of the population. Moreover, when ANOVA and the post hoc Tukey unequal *N* HSD test were applied to growth data concerning the four most abundant (A, B, C, and D) PCR-RFLP classes for both the selected pool of 27 and all 129 recovered strains (data not shown), similar results were obtained. This demonstrates that strains belonging to group D differ significantly ($P < 0.01$) in growth on TY medium from strains of groups A, B, and C, irrespective of the number of strains in a particular group.

We concluded that the 27 selected strains (5 from group A, 3 from group B, 8 from group C, 7 from group D, and 1 each from groups E, F, G, and H) could serve as a representation of the entire recovered population.

Next, the 16S rRNA genes of the 27 selected strains were partially sequenced. Almost no diversification of this sequence was found, despite substantial differences in plasmid patterns and PCR-RFLP profiles for 16S-23S rRNA: only two clusters could be distinguished using the UPGMA algorithm, with one predominating, demonstrating the taxonomic homogeneity of the selected subset (details not shown).

Symbiotic characteristics of clover nodule isolates. The 27 representative strains were assessed for their nodulation and plant growth promotion abilities on clover (Fig. 2) to investigate a possible correlation between the PCR-RFLP genotype of the isolates and the shoot mass of the infected plants. In the case of nodule number, there were no significant differences between medians of the analyzed groups (chi-square = 8.775; $P = 0.269$). Kruskal-Wallis ANOVA by ranks (i.e., multiple comparisons of mean ranks) revealed that both shoot and root wet masses differed significantly among the studied groups (Fig. 3), allowing application of multiple comparisons of mean ranks. A highly significant difference ($P < 0.01$) in shoot wet mass was observed between groups F and H and groups A, B, C, D, and E. Moreover, when PCR-RFLP groups composed of multiple strains were compared, significant differences ($P < 0.05$) were observed in shoot wet mass between group A and groups C and D and in root wet mass between group A and groups B and C.

Since the mass of plant shoots may be considered a determinant of a strain’s symbiotic efficiency, the distribution of mean shoot masses was analyzed. Because the distribution was, to some extent, asymmetric (Fig. 4), the Shapiro-Wilk *W* test

FIG. 2. Numbers of nodules and wet masses of clover plants inoculated with selected *R. leguminosarum* bv. *trifolii* strains.

of normality was applied, indicating that the distribution of the studied feature was significantly different from normal if P values of <0.01 were considered significant but not if P values of <0.05 were considered significant. It is worth noting that the strains highly efficient in plant growth promotion were not as abundant as the less-efficient ones. It was demonstrated that less-efficient strains prevailed in this rhizobial population.

Correlation between genotypes and metabolic capabilities of isolates. We assumed that the isolates that had different plasmid profiles and were classified into different genetic groups might also vary in their metabolic properties, which may have been associated further with the adaptive and competitive abilities of *R. leguminosarum* bv. *trifolii* strains (51). Metabolic profiling of 27 representative isolates by using a commercial Biolog GN2 microplate test was applied. The test revealed that even though the number of different carbon and energy sources metabolized by the individual isolates varied from 31 (strain 4.15) to 60 (strain 4.13), quantitative differences in the numbers of utilized substrates between various PCR-RFLP genotypes, as well as between the “frequent,” “intermediate,” and “rare” groups, were not found. The results of the median tests (detailed data not shown) indicated that there also were no significant differences in medians between PCR-RFLP classes ($P = 0.994$) and between groups distinguished on the basis of frequency in the population ($P = 0.904$).

It was concluded that the number of various carbon and energy sources that were metabolized by the isolates was not directly correlated with the frequency of strain occurrence in the recovered nodule population. On the other hand, when the Biolog results were analyzed by UPGMA, most strains belonging to genotypes A, B, C, and D (“frequent” and “intermediate”) were grouped in a single branch of the tree, with the similarity level exceeding 85%. All “rare” strains, belonging to PCR-RFLP groups E, F, G, and H, were clustered in the other

FIG. 3. Shoot and root wet masses of clover plants infected with selected *R. leguminosarum* bv. *trifolii* strains belonging to different PCR-RFLP genetic groups.

branch, indicating that their metabolic profiles were distinct (Fig. 5A). The clustering patterns of the strains, based on the Biolog test results and the data from the enzymatic assays described above, were compared (Fig. 5A and B). Despite the

FIG. 4. Distribution of mean wet masses of plant shoots infected with *R. leguminosarum* bv. *trifolii* strains classified into groups with respect to frequencies of PCR-RFLP genotypes in the population.

FIG. 5. Cluster analysis-based dendrograms (UPGMA method) showing diversity of selected *R. leguminosarum* bv. *trifolii* strains with respect to utilization of carbon sources from a GN2 microplate (Biolog Inc.) (A) and enzyme activities (urease, catalase, phosphatase, peroxidase, and nitrate reductase) and IAA production (B). Symbols for groups and numbers of strains are shown on the left. The scale bar represents percentages of similarity.

different levels of diversity, with a higher level for enzyme activity-based clustering, grouping of most of the “frequent” and “intermediate” strains remained generally similar for these two dendrograms.

Since cluster analysis did not allow identification of qualitative metabolic differences between the clover nodule isolates, the results of the Biolog test were subjected to PCA. A total of 95 carbon and energy sources used in the test were divided arbitrarily into nine groups: monosaccharides (S), complex saccharides (cS), modified saccharides (mS), nonmodified acids (A), modified acids (mA), sugar acids (sA), nonmodified amino acids (AA), modified amino acids (mAA), and others (with amines predominating) (O). PCA enabled us to group all mentioned carbon and energy sources into three factors explaining most of the total variance: principal component 1 (PC1), composed of A, mS, AA, O, and sA; principal component 2 (PC2), composed of S, cS, and mAA; and principal component 3 (PC3), including mA (Table 2). Taking into account the significant prevalence (>90%) of nonsugar substrates, PC1 was interpreted as “utilization of nonsugar compounds” and PC2 was interpreted as “utilization of sugar substrates.” There were differences in utilization capabilities for these two groups of carbon sources for the tested rhizobia. Individual strains were able to utilize 49 to 89% (mean value, 79%) of “sugar substrates” (35 different compounds), and thus the PC2 group may also be called “commonly utilized sub-

strates.” Consequently, the PC1 group (55 different compounds) may be named “seldom-utilized substrates,” because the individual strains were able to use only 13 to 53% (mean value, 30%) of them (detailed data not shown).

PCA also allows investigation of the metabolic similarities between individual representative strains, which is useful for establishing metabolic preference patterns for groups of isolates. The most interesting observations regarded PC1 and PC2, because 90 of 95 tested carbon sources were assigned to groups of substrates belonging to these principal components.

TABLE 2. PCA variables and correlation coefficients between PCA factors and variables

Substrate group	No. of substrates	Description	Factor loading correlation coefficient		
			PC1	PC2	PC3
S	17	Monosaccharides	0.175	0.805	-0.131
cS	13	Complex saccharides	0.195	0.913	0.125
mS	6	Modified saccharides	0.749	0.270	-0.375
A	18	Acids	0.862	0.075	0.005
mA	5	Modified acids	-0.109	0.019	0.927
sA	6	Sugar acids	0.532	0.092	-0.009
AA	16	Amino acids	0.638	0.374	-0.253
mAA	5	Modified amino acids	-0.156	0.528	-0.363
O	9	Others	0.633	0.070	0.157

FIG. 6. Scatter plots for 27 selected *R. leguminosarum* bv. *trifolii* strains, using the PC1 and PC2 coordinate system. (A) Strains classified into particular PCR-RFLP genotypes; (B) strains classified into groups with respect to frequencies of PCR-RFLP genotypes in the studied population. Numbers located close to the symbols are individual strain designations.

Using the PCA method, the calculated values concerning the metabolic potential of particular strains were plotted in a coordinate system using the first two principal components (Fig. 6A and B). The positions of the individual strains on scatter plots were related to the number of substrates belonging to PC1 and PC2 that were metabolized by a given isolate. Strains were dispersed in all quarters of the scatter plot, which suggests that different PCR-RFLP types comprising the local nodule population varied significantly in the ability for substrate

utilization. However, we also observed that strains grouped into the D genotype showed similarity in utilization of the "sugars," whereas "nonsugars" were or were not utilized. On the other hand, other PCR-RFLP genotypes, especially genotype C, clustered strains which were very divergent metabolically (Fig. 6A).

PCA may also be applied for simultaneous grouping of strains with respect to their PCR-RFLP genotype and their occurrence in the population (Fig. 6B). We found that most strains classified as "rare" were characterized by common utilization of "nonsugar" substrates, such as acids and amino acids, but rather seldom use of "sugars." In contrast, strains classified as "intermediate" in the population commonly utilized "sugars" but variably used the "nonsugar" substrates. An essentially different pattern of substrate utilization was observed in the case of "frequent" strains, which were characterized by common utilization of either "sugars" or "nonsugars" or, rarely, both (Fig. 6B). Thus, it may be assumed that "frequent" strains in a local nodule population were not "specialized" in utilizing any group of substrates and that utilization of "sugars" and "nonsugars" as carbon and energy sources was balanced. On the other hand, groups of isolates defined as "intermediate" and "rare" demonstrated some preferences for either the "sugar" or "nonsugar" substrates.

Detailed analysis of individual substrate utilization did not allow determination of any particular substrate(s) that was utilized or not utilized by all strains belonging to any group. Nevertheless, two groups of compounds which were diversely utilized by defined groups of strains could be distinguished: one comprising mainly sugars (*N*-acetyl-D-glucosamine, *i*-erythritol, gentiobiose, D-raffinose, D-galactonic acid lactone, and L-pyrogutamic acid) and another comprising acids and amino acids (D-galacturonic acid, γ -hydroxybutyric and γ -aminobutyric acid, L-asparagine, L-serine, and inosine). The former group of substrates was utilized predominantly by "frequent" and "intermediate" strains (69 to 100% of "frequent" and "intermediate" strains used them), with the latter group being utilized rarely (up to 19% of strains utilized them).

DISCUSSION

The structure and biological activity of the bacterial soil population are dependent on numerous factors, such as soil type, organic matter content, and management practices (17, 35), as well as plants' activities (26, 31, 39). All of these factors contribute to the nutritional complexity of the rhizosphere and influence the activity of a microbial population.

In this study, different approaches were used to examine the genetic and metabolic structure of a rhizobial population occupying the nodules of clover plants grown in each other's vicinity in the soil. The range of genetic diversity within this population, measured as the number of different 16S-23S ITS PCR-RFLP classes among recovered strains, was comparable with what has been described for populations originating even from distinct soils (24). The dynamic state of rhizobial genomes and/or lateral transfer events could influence the diversity of strains (1, 5, 19, 44, 45). The strains isolated from clover nodules also differed in their frequency of occurrence in the total population. It was hypothesized that if the prevalence of particular strains in the nodule population and, consequently,

in the rhizosphere depends on their genotypes, it should *ipso facto* mirror an advantageous metabolic pattern that allows bacteria to thrive better in the environment. We found that strains prevalent in the recovered population (“frequent” and “intermediate”) were grouped together in a distinct branch in the UPGMA analyses, based both on Biolog results and on the tested enzymatic activities (Fig. 5A and B). This suggested that the abundance of particular strains in the studied rhizobial population was in fact correlated with their metabolic profiles. PCA of the Biolog data revealed that strains belonging to a particular PCR-RFLP group displayed various metabolic profiles, except for group D, where some preference for “sugar” compound utilization was found (Fig. 6A). One explanation for this phenotypic strain diversification could be additional genetic variability stemming from their different plasmid contents (7, 32, 44).

In our previous work (51), we found a correlation between the metabolic capabilities and competitiveness of strains belonging to three biovars of *R. leguminosarum*, i.e., bv. *trifolii*, *viciae*, and *phaseoli*, originating from three different soils. Almost all strains utilized a relatively large number of sugar substrates, but the best competitors also utilized a relatively large number of nonsugar substrates. The strains utilizing a wider range of substrates were more competitive (51). The results presented in this work are in agreement with previously reported findings. The most important groups of substrates were “sugars” and “nonsugars,” with the “sugars” utilized more commonly. PCA supports the notion that a balanced utilization of sugar and nonsugar substrates characterizes groups of strains that occur frequently in a nodule population. Moreover, strains from groups A, B, and C (“frequent”) grew better than strains of group D (“intermediate”) on TY complete medium (containing mainly tryptone and yeast extract), which presumably better satisfies their versatile metabolic properties.

Finally, in an investigation of the putative relationship between PCR-RFLP genotypes and the symbiotic effectiveness of strains, we found that the “rare” strains, i.e., those belonging to groups G and H, could be considered highly effective in plant growth promotion but constituted only 2.4% of the studied population (Table 1). On the other hand, strains less efficient in symbiosis and more versatile in nutritional requirements were prevalent in the clover nodules. It is possible that their versatile metabolic capabilities, which enable them to access a wider range of nutrients present in the soil and the plant rhizosphere, might be the most successful long-term strategy for thriving in the soil. The great variety of compounds (i.e., sugars, acids, amino acids, amines, and many low-molecular-weight substances) secreted by plants (3, 15, 20, 22) makes this hypothesis plausible.

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REFERENCES

- Bailly, X., I. Olivieri, B. Brunel, J. C. Cleyet-Marel, and G. Bena. 2007. Horizontal gene transfer and homologous recombination drive the evolution of the nitrogen-fixing symbionts of *Medicago* species. *J. Bacteriol.* **189**:5223–5236.
- Bais, H. P., T. L. Weir, L. G. Perry, S. Gilroy, and J. M. Vivanco. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* **57**:233–266.
- Bertin, C., X. Yang, and L. A. Weston. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* **256**:67–83.
- Brom, S., A. García-de los Santos, L. Cervantes, R. Palacios, and D. Romero. 2000. In *Rhizobium etli* symbiotic plasmid transfer, nodulation competitiveness and cellular growth require interaction among different replicons. *Plasmid* **44**:34–43.
- Brom, S., A. García-de los Santos, M. de Lourdes Girard, G. Davilla, R. Palacios, and D. Romero. 1991. High-frequency rearrangements in *Rhizobium leguminosarum* bv. *phaseoli* plasmids. *J. Bacteriol.* **173**:1344–1346.
- Cowan, S. T., and K. J. Steel. 1965. Manual for the identification of medical bacteria. University Press, Cambridge, United Kingdom.
- Crossman, L. C., S. Castillo-Ramírez, C. McAnnula, L. Lozano, G. S. Venikos, J. L. Acosta, Z. F. Ghazoui, I. Hernández-González, G. Meakin, A. W. Walker, M. F. Hynes, J. P. W. Young, J. A. Downie, D. Romero, A. W. B. Johnston, G. Dávila, J. Parkhill, and V. González. 2008. A common genetic framework for a diverse assembly of plasmids in the symbiotic nitrogen fixing bacteria. *PLoS One* **7**:e2567. doi:10.1371/journal.pone.0002567.
- Depret, G., and G. Laguerre. 2008. Plant phenology and genetic variability in root and nodule development strongly influence genetic structuring of *Rhizobium leguminosarum* biovar *viciae* populations nodulating pea. *New Phytol.* **179**:224–235.
- D’Haeze, W., and M. Holsters. 2002. Nod factor structures, responses and perception during initiation of nodule development. *Glycobiology* **12**:79–105.
- Eckhardt, T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1**:584–588.
- Ferguson, G. A., and Y. Takane. 1989. Statistical analysis in psychology and education. McGraw-Hill, New York, NY.
- Flores, M., L. Morales, A. Avila, V. González, P. Bustos, D. García, Y. Mora, X. Guo, J. Collado-Vides, D. Piñero, G. Dávila, J. Mora, and R. Palacios. 2005. Diversification of DNA sequences in the symbiotic genome of *Rhizobium etli*. *J. Bacteriol.* **187**:7185–7192.
- Freidlin, B., and J. L. Gastwirth. 2000. Should the median test be retired from general use? *Am. Stat.* **54**:161–164.
- Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing bacteria during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* **68**:280–300.
- Gaworzewska, E. T., and M. J. Carlile. 1982. Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J. Gen. Microbiol.* **128**:1179–1188.
- Gibson, K. E., H. Kobayashi, and G. C. Walker. 2008. Molecular determinants of a symbiotic chronic infection. *Annu. Rev. Genet.* **42**:413–441.
- Girvan, M. S., J. Bullimore, J. N. Pretty, A. M. Osborn, and A. S. Ball. 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. *Appl. Environ. Microbiol.* **69**:1800–1809.
- Gonzalez, V., R. I. Santamaría, P. Bustos, I. Hernández-González, A. Medrano-Soto, G. Moreno-Hagelsieb, S. C. Janga, M. A. Ramírez, V. Jiménez-Jacinto, J. Collado-Vides, and G. Dávila. 2006. The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. *Proc. Natl. Acad. Sci. U. S. A.* **103**:3834–3839.
- Guo, X., M. Flores, P. Mavingui, S. I. Fuentes, G. Hernandez, G. Davila, and R. Palacios. 2003. Natural genomics design in *Sinorhizobium meliloti*: novel genomic architectures. *Genome Res.* **13**:1810–1817.
- Jaeger, C. H., S. E. Lindow, W. Miller, E. Clark, and M. K. Firestone. 1999. Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Appl. Environ. Microbiol.* **65**:2685–2690.
- Jones, K. M., H. Kobayashi, B. W. Davies, M. E. Taga, and G. C. Walker. 2007. How symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature* **5**:619–633.
- Knee, E. M., F. C. Gong, M. Gao, M. Teplitski, A. M. Jones, A. Foxworthy, A. J. Mort, and W. D. Bauer. 2001. Root mucilage from pea and its utilization by rhizosphere bacteria as a sole carbon source. *Mol. Plant Microbe Interact.* **14**:775–784.
- Laguerre, G., P. Mavingui, M. R. Allard, M. P. Charnay, P. Louvrier, S. I. Mazurier, L. Rigottier-Gois, and N. Amarger. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol.* **62**:2029–2036.
- Laguerre, G., P. Louvrier, M. R. Allard, and N. Amarger. 2003. Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar *viciae* for nodulation of host legumes. *Appl. Environ. Microbiol.* **69**:2276–2283.
- MacLean, A. M., T. M. Finan, and M. J. Sadowsky. 2007. Genomes of the symbiotic nitrogen-fixing bacteria of legumes. *Plant Physiol.* **144**:615–622.
- Martinez-Romero, E. 2009. Coevolution in *Rhizobium*-legume symbiosis? *DNA Cell Biol.* **28**:361–370.
- Martyniuk, S., A. Woźniakowska, M. Martyniuk, and J. Oroń. 2000. A new

- sand pouch-plant infection technique for enumeration of rhizobia in soil. *Acta Soc. Bot. Pol.* **69**:257–261.
28. Mavingui, P., M. Flores, X. Guo, G. Dávila, X. Perret, W. J. Broughton, and R. Palacios. 2002. Dynamics of genome architecture in *Rhizobium* sp. strain NGR234. *J. Bacteriol.* **184**:171–176.
 29. Minamisawa, K., and K. Fukai. 1991. Production of indole-3-acetic acid by *Bradyrhizobium japonicum*: a correlation with genotype grouping and rhizobiotoxine production. *Plant Cell Physiol.* **32**:1–9.
 30. Morrison, D. F. 1990. Multivariate statistical methods, 3rd ed. McGraw-Hill, New York, NY.
 31. Mutch, L. A., and J. P. W. Young. 2004. Diversity and specificity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes. *Mol. Ecol.* **13**:2435–2444.
 32. Oresnik, I. J., L. A. Pacaryuk, S. H. P. O'Brien, C. Yost, and M. F. Hynes. 1998. Plasmid-encoded catabolic genes in *Rhizobium leguminosarum* bv. *trifolii*: evidence for a plant-inducible rhamnose locus involved in competition for nodulation. *Mol. Plant Microbe Interact.* **11**:1175–1185.
 33. Oresnik, I. J., S. L. Liu, C. K. Yost, and M. F. Hynes. 2000. Megaplasmid pRme2011a of *Sinorhizobium meliloti* is not required for viability. *J. Bacteriol.* **182**:3582–3586.
 34. Palacios, R., and W. E. Newton (ed.). 2005. Genomes and genomics of nitrogen-fixing organisms. Springer, Dordrecht, Netherlands.
 35. Palmer, K. M., and J. P. W. Young. 2000. Higher diversity of *Rhizobium leguminosarum* biovar *viciae* populations in arable soils than in grass soils. *Appl. Environ. Microbiol.* **66**:2445–2450.
 36. Peoples, M. B., D. F. Herridge, and J. K. Ladha. 1995. Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? *Plant Soil* **174**:3–28.
 37. Perret, X., C. Staehelin, and W. J. Broughton. 2000. Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* **64**:180–201.
 38. Prell, J., and P. Poole. 2006. Metabolic changes of rhizobia in legume nodules. *Trends Microbiol.* **14**:161–168.
 39. Rangin, C., B. Brunel, J. C. Cleyet-Marel, M. M. Perrineau, and G. Bena. 2008. Effects of *Medicago truncatula* genetic diversity, rhizobial competition, and strain effectiveness on the diversity of a natural *Sinorhizobium* species community. *Appl. Environ. Microbiol.* **74**:5653–5661.
 40. Rossello-Mora, R., and R. Amann. 2001. The species concept for prokaryotes. *FEMS Microbiol. Rev.* **25**:39–67.
 41. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 42. Schulze, J. 2004. How are nitrogen fixation rates regulated in legumes? *J. Plant Nutr. Soil Sci.* **167**:125–137.
 43. Simms, E. L., and D. L. Taylor. 2002. Partner choice in nitrogen-fixation mutualism of legumes and rhizobia. *Integr. Comp. Biol.* **42**:369–380.
 44. Slater, F. R., M. J. Bailey, A. J. Tett, and S. L. Turner. 2008. Progress towards understanding of the fate of plasmids in bacterial communities. *FEMS Microbiol. Ecol.* **66**:3–13.
 45. Souza, V., T. T. Nguyen, R. R. Hudson, D. Pinero, and R. E. Lenski. 1992. Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: evidence for sex? *Proc. Natl. Acad. Sci. U. S. A.* **89**:8389–8393.
 46. Spjotvoll, E., and M. R. Stoline. 1973. An extension of the T-method of multiple comparisons to include the cases with unequal sample sizes. *J. Am. Stat. Assoc.* **68**:976–978.
 47. Stuurman, N., C. Pacios Bras, H. R. M. Schlaman, A. H. M. Wijnjes, G. Bloembergen, and H. P. Spaink. 2000. Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. *Mol. Plant Microbe Interact.* **13**:1163–1169.
 48. Vincent, J. M. 1970. A manual for the practical study of root nodule bacteria. International biological program handbook no. 15. Blackwell Scientific Publications Ltd., Oxford, United Kingdom.
 49. Walker, T. S., H. P. Bais, E. Grotewold, and J. M. Vivanco. 2003. Root exudation and rhizosphere biology. *Plant Physiol.* **132**:44–51.
 50. White, J., J. Prell, E. K. James, and P. Poole. 2007. Nutrient sharing between symbionts. *Plant Physiol.* **144**:604–614.
 51. Wielbo, J., M. Marek-Kozaczuk, A. Kubik-Komar, and A. Skorupska. 2007. Increased metabolic potential of *Rhizobium* spp. is associated with bacterial competitiveness. *Can. J. Microbiol.* **53**:957–967.
 52. Young, J. P., H. L. Downer, and B. D. Eardly. 1991. Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *J. Bacteriol.* **173**:2271–2277.
 53. Young, J. P., L. C. Crossman, A. W. Johnston, N. R. Thomson, Z. F. Ghazoui, K. H. Hull, M. Wexler, A. R. Curson, J. D. Todd, P. S. Poole, T. H. Mauchline, A. K. East, M. A. Quail, C. Churcher, C. Arrowsmith, I. Cherevach, T. Chillingworth, K. Clarke, A. Cronin, P. Davis, A. Fraser, Z. Hance, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabinowitsch, M. Sanders, M. Simmonds, S. Whitehead, and J. Parkhill. 2006. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biol.* **7**:R34.