APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1997, p. 1852–1860 Vol. 63, No. 5
0099-2240/97/$04.00+0

Metabolic and Genotypic Fingerprinting of Fluorescent Pseudomonads Associated with the Douglas Fir-*Laccaria bicolor* Mycorrhizosphere

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Received 1 November 1996/Accepted 24 February 1997

A collection of 300 isolates of fluorescent pseudomonads was established from Douglas fir-*Laccaria bicolor* mycorrhizas and mycorrhizosphere and from adjacent bulk soil. These isolates were first phenotypically characterized with the Biolog method. Taxonomic identification assigned 90% of the isolates to the different biovars of *Pseudomonas fluorescens*, with inverted frequencies of biovars V and I from the bulk soil to the mycorrhizas, suggesting that the mycorrhizas exert a selective stimulation of the *P. fluorescens* bv. I and a counterselection of the *P. fluorescens* bv. V present in the soil. Multivariate analyses of the carbon source utilization data led to the definition of homogenous metabolic groups and to the identification of the most discriminating substrates for each group. The isolates from the mycorrhizosphere and from the mycorrhizas seem to preferentially utilize carbohydrates, in particular trehalose, which is the most abundant carbohydrate accumulated in the mycelium of *L. bicolor*. The results suggest that *L. bicolor* exerts a trehalose-mediated selection on the fluorescent pseudomonads present in the vicinity of the mycorrhizas. Isolates of *P. fluorescens* from the mycorrhizosphere and mycorrhizas were then genotypically characterized by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes and enterobacterial repetitive intergenic consensus-PCR DNA fingerprinting. Both methods revealed a high genetic polymorphism within the population studied, which was well correlated with the phenotypic characterization.

Some soil bacteria, particularly fluorescent pseudomonads, promote the establishment of the mycorrhizal symbiosis (7, 35). These bacteria were called MHB for mycorrhiza helper bacteria (13). Conversely, the establishment of mycorrhizal symbiosis qualitatively and quantitatively alters the exudation of carbohydrates by the roots (43), and thus perturbs the microbial equilibrium of the rhizosphere. This effect was called the mycorrhizosphere effect (12, 30, 40). For instance, the population densities of fluorescent pseudomonads were lower in the mycorrhizosphere of clover, maize, and grapevine than in the rhizosphere of the same nonmycorrhizal plants (36, 50). In contrast, the population densities of an MHB *Pseudomonas fluorescens* which promotes the symbiosis between Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] and the ectomycorrhizal fungus *Laccaria bicolor* (Maire) Orton are significantly greater in the mycorrhizosphere of Douglas fir associated with *L. bicolor* than in the rhizosphere of nonmycorrhizal seedlings (11).

Several phenotypic and/or genotypic characterizations of rhizospheric versus nonrhizospheric microbial communities have shown that the rhizosphere of different crops (15, 26, 28, 34) or tree species (16, 17) exerts a selection on the bacterial communities present in the soil. This selection was mainly attributed to the nature of the root exudates. However, most of these studies did not take into account the possible role of endo- or ectomycorrhizal fungi in the selection process of bacterial communities, although the roots of the plants studied were certainly mycorrhizal since they were grown in nonsterile soil.

The aim of the present study was to assess the effect of the mycorrhizosphere of Douglas fir seedlings mycorrhizal with *L. bicolor* on the phenotypic and genotypic traits of fluorescent pseudomonad populations from the soil. For this purpose, we collected 300 isolates of fluorescent pseudomonads from mycorrhizas, mycorrhizosphere, and bulk soil in a forest nursery of Douglas fir mycorrhizal with *L. bicolor*. All of these isolates were phenotypically characterized by the Biolog method. A subsample of isolates of *P. fluorescens* from the mycorrhizosphere and mycorrhizas was then genotypically characterized by restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rRNA genes (rDNAs) and enterobacterial repetitive intergenic consensus (ERIC)-PCR.

MATERIALS AND METHODS

Collection of bacterial strains. Bacterial strains were collected in a Douglas fir experimental nursery in Champenoux (France) containing a sandy soil (pH 5.4; 7.5% organic matter, 31 ppm P [extracted in 0.5 M $NaHCO_3$], 0.3% N). The nursery bench was divided into 0.5-m^2 plots, planted with 9-month-old Douglas fir seedlings, which had been inoculated with *L. bicolor* S238N (synonym *Laccaria laccata* S238N) at sowing. The planted plots were separated from each other with 0.5-m^2 unplanted plots of the same soil. The experimental design consisted of four of these plots (replicates), 6 m distant from each other in the nursery bench. Observation of the root systems with a stereomicroscope showed that the seedlings presented a high percentage (71%) of short roots mycorrhizal with *L. bicolor*. Bacteria were isolated from the following three compartments (one sample per compartment per plot): soil adherent to the root systems of Douglas fir seedlings, designated the mycorrhizosphere; mycorrhizas collected from these root systems; and nursery soil from unplanted plots adjacent to each planted plot sampled, designated the bulk soil.

Bulk soil suspensions were prepared by vigorously shaking 1 g of soil in 9 ml of sterile distilled water for 60 s. Mycorrhizosphere suspensions were prepared by vigorously shaking the entire root system with adhering soil in 100 ml of sterile distilled water for 60 s. From each root system, 10 mycorrhizas typical of *L. bicolor* were collected under a stereomicroscope. Mycorrhizas were washed 10

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times in 1 ml of sterile distilled water and crushed with a sterile pestle in a microtube containing 1 ml of sterile distilled water.

Suspensions from the three compartments were dilution plated on King's medium B (KB) (23) and incubated at 25°C for 48 h to detect isolates of fluorescent pseudomonads. Suspensions were also plated on 1/10-strength tryptic soy agar (TSA) medium (44) and incubated at 25°C for 7 days to quantify the total culturable bacterial community. The KB plates were examined under UV light, and the fluorescent colonies were selected. To collect bacterial isolates showing a similar level of dominance within each compartment, bacterial isolation from each compartment was always performed with samples diluted to the same level, i.e., 10-fold dilutions for the bulk soil and mycorrhizosphere suspensions and the undiluted suspension for the mycorrhizas. All of the fluorescent colonies appearing on one or several plates were collected to obtain about 30 isolates per sample for each compartment. A total of 300 fluorescent colonies were collected, i.e., 145 from the bulk soil, 123 from the mycorrhizosphere, and 32 from the mycorrhizas. Isolates were subjected to single-colony isolation and cryopreserved at −80°C in 20% glycerol.

Phenotypic fingerprinting of the isolates. The 300 fluorescent pseudomonad isolates were phenotypically characterized by the Biolog (Hayward, Calif.) system, which is based on the differential utilization of a large number of organic compounds by the test microorganisms (3). The Biolog GN microplates were inoculated as described in the manufacturer's recommendations and incubated at 30°C for 24 h. Formazan accumulation in the bacterial cells was measured as the optical density at 590 nm by an automatic microplate reader (Dynatech MR 5000) interfaced to an IBM compatible computer. The 300 isolates were identified by their patterns of utilization of the 95 substrates by using the Biolog Microlog software.

The quantitative data of carbon source utilization by each isolate were also compared by multivariate analyses (14, 16, 52). Principal-component analysis and correspondence analysis were used to visualize the relationships between the isolates. Classification of the isolates into homogeneous metabolic groups was achieved by use of the KMACL4 software, a new unsupervised classification method for building overlapping clusters of bacteria and determining the main characteristics of these classes (2, 27). This software was designed for marrying the benefits coming out of both cluster and factor representations.

PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S rDNA. The 16S rDNAs were amplified by PCR, with the primer pair fD1 and rD1 (51), and digested with 13 restriction endonucleases as described previously (25), except that the restriction endonuclease *Mse*I was replaced by its isoschizomer *Tru*9I.

ERIC-PCR analysis. ERIC-PCR consists of the use of primers corresponding to conserved motifs in bacterial repetitive elements referred to as enterobacterial repetitive intergenic consensus elements (20). The method employed was adapted from de Bruijn (6). Bacterial cells were grown at 25°C for 24 h in liquid Luria-Bertani medium (44), harvested by centrifugation (12,000 × *g*, 10 min), and washed twice with sterile ultrapure water (Millipore-Q reagent water). The cell suspension was adjusted to an optical density at 600 nm of 0.5. Amplification was performed in 25-μl reaction volumes containing 1.25 mM (each) deoxynucleoside triphosphates, 6 mM $MgCl_2$, 67 mM Tris-HCl, 16.6 mM ammonium sulfate, 6 μM EDTA, 30 mM β-mercaptoethanol, bovine serum albumin at 170 μg ml^{-1}, dimethyl sulfoxide at 10%, 2 μM (each) primers ERIC 1-R and ERIC 2 (6), 1.5 U Goldstar DNA polymerase (Eurogentec, Seraing, Belgium), and 10 μl of bacterial suspension. PCR mixtures were overlaid with 1 drop of mineral oil (Sigma). The PCRs were performed in an automated thermal cycler (Eurogentec) with an initial denaturation (94°C, 7 min) followed by 30 cycles of denaturation (94°C, 1 min), annealing (52°C, 1 min), and extension (65°C, 8 min), with a final extension (65°C, 16 min) and a final soak at 5°C. Products of PCR amplification were separated by horizontal electrophoresis with a 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer (44) at 5 V cm^{-1} over 3 h. The 1-kb DNA ladder (Gibco BRL Life Technologies, Cergy Pontoise, France) was used as a molecular weight marker. Gels were stained with ethidium bromide and photographed under UV illumination with Polaroid type 665 positive-negative film. PCR amplifications were performed in duplicate with 10 μl of undiluted bacterial suspension (optical density at 600 nm, 0.5) and with 10 μl of 10-fold-diluted bacterial suspension (optical density at 600 nm, 0.05). Similar band patterns were obtained. The presence or absence of a particular DNA fragment was visually determined and converted into binary data, and the resulting matrix was analyzed with the PAUP software (48).

RESULTS

Total culturable bacterial and fluorescent pseudomonad densities in the three compartments. The total culturable bacterial populations, determined by plating suspensions on TSA medium, were significantly higher in the mycorrhizosphere (4.69×10^7 CFU g [dry weight] of $soil^{-1}$) than in the bulk soil (3.34×10^6 CFU g [dry weight] of $soil^{-1}$), according to the paired *t* test ($T = 6.83$; $df = 3$; $P = 0.007$) calculated on log-transformed data. The fluorescent pseudomonad populations, as determined by plating the suspensions on KB medium and counting under UV illumination, were also significantly higher in the mycorrhizosphere (8.91×10^4 CFU g [dry weight] of $soil^{-1}$) than in the bulk soil (2.40×10^4 CFU g [dry weight] of $soil^{-1}$), according to the paired *t* test ($T = 3.93$; $df = 3$; $P = 0.03$) calculated on log-transformed data. In the mycorrhiza compartment, the total culturable bacterial populations were 3.26×10^4 CFU per mycorrhiza, and the fluorescent pseudomonad populations were 2.58 CFU per mycorrhiza.

TABLE 1. Identification of the 300 isolates of fluorescent pseudomonads by the Biolog method

Species and biovar	No. (%) of isolates in[a]:		
	Bulk soil (145 isolates)[b]	Mycorrhizosphere (123 isolates)	Mycorrhiza (32 isolates)
P. fluorescens bv. V	91 (62.8)	36 (29.3)	1 (3.1)
P. fluorescens bv. I	1 (0.7)	62 (50.4)	29 (90.6)
P. fluorescens bv. II	30 (20.7)	12 (9.8)	0 (0)
P. fluorescens bv. IV	4 (2.8)	3 (2.4)	0 (0)
P. fluorescens bv. III	1 (0.7)	0 (0)	0 (0)
P. putida bv. B1	9 (6.2)	2 (1.6)	0 (0)
P. corrugata	1 (0.7)	2 (1.6)	0 (0)
P. tolaasii	0 (0)	1 (0.8)	1 (3.1)
Not identified	8 (5.5)	5 (4.1)	1 (3.1)

[a] Total number (and percentage) of isolates of each species and biovar from each compartment.
[b] Total number of isolates is shown in parentheses.

Taxonomic identification with the Biolog system. Of the 300 fluorescent pseudomonad isolates collected, 270 isolates were assigned to the species *P. fluorescens* by the Biolog Microlog database (Table 1). Some isolates were assigned to other fluorescent *Pseudomonas* species, namely, *P. putida*, *P. corrugata*, and *P. tolaasii*. Fourteen isolates could not be identified by this method. Within *P. fluorescens*, the biotype classification proposed by Stanier et al. (47) and used by the Biolog system was replaced by the biovar classification (41) which is preferred by most authors: biotypes A, B, C, F, and G of *P. fluorescens* correspond to biovars I, II, III, IV, and V, respectively (41).

Biovars I and V were the most frequently isolated biovars of *P. fluorescens*. However, their respective frequencies showed a clear-cut difference between the bulk soil and the mycorrhizas, with an intermediate status for the mycorrhizosphere (Table 1). In the bulk soil, most isolates (62.8%) belonged to biovar V, and only one isolate belonged to biovar I. In the mycorrhizosphere, biovar I was more frequent (50.4%) than biovar V (29.3%). In the mycorrhiza compartment, most isolates (90.6%) were assigned to biovar I, and only one isolate was assigned to biovar V. Thus, the distribution frequencies of *P. fluorescens* bv. I and V presented inverted gradients from the bulk soil to the mycorrhizas. *P. fluorescens* bv. II isolates showed a distribution among the three compartments, quite similar to that of *P. fluorescens* bv. V, with much lower frequencies.

Multivariate analyses of the metabolic fingerprints. The quantitative data of carbon source utilization (95 data per isolate) provided by the Biolog system were subjected to different methods of multivariate analysis, to cluster the isolates sharing the same metabolic traits and to determine the characteristic traits of each cluster. Principal-component analysis and correspondence analysis were used to visualize the relationships between isolates. Both methods resulted in two-dimensional representations of the 300 isolates, which were consistent with the taxonomic classification. Nevertheless, as shown by Garland (14), principal-component analysis is influenced by small variations in the density of inoculum in the Biolog mi-

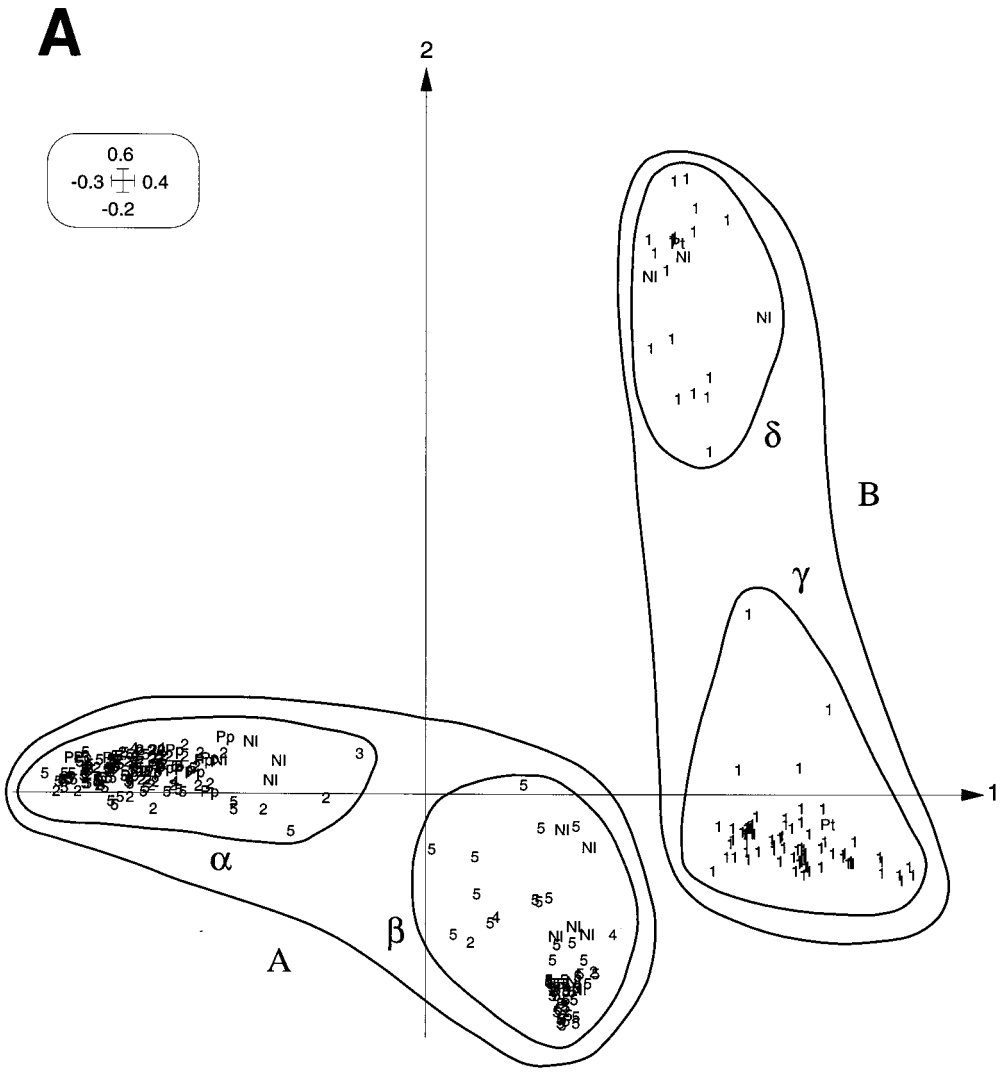

FIG. 1. Correspondence analysis of the metabolic profiles of the 300 fluorescent pseudomonad isolates. (A) Projection of the 300 isolates on the plane defined by the first two principal components. Numbers 1, 2, 3, 4, and 5 correspond to biovars I, II, III, IV, and V, respectively of *P. fluorescens*. Pp, *P. putida*; Pt, *P. tolaasii*; NI, not identified. (B) Projection of the 95 substrates from the Biolog GN microplates on the plane defined by the first two principal components. For legibility reasons, only the most characteristic substrates of groups A and B are identified. The positions of the other substrates are indicated by asterisks.

croplates. This bias is eliminated by normalizing data prior to ordination or by using an alternative ordination technique, like correspondence analysis (14). Therefore, correspondence analysis was retained for the treatment of the Biolog fingerprint data.

According to the correspondence analysis, the projection of the 300 isolates on the plane defined by the first two principal components, which account for 37% and 18%, respectively, of the total variance, reveals the presence of two major groups, named A and B (Fig. 1A). These two groups have been divided in four subgroups, named α, β, γ, and δ. All of the isolates identified as *P. fluorescens* bv. II and V by the Biolog system gathered in group A, whereas all the isolates identified as *P. fluorescens* bv. I gathered in group B. There was also a good correlation between the clustering shown in Fig. 1A and the origin of the isolates. Almost all isolates (99%) from the bulk soil gathered in group A, whereas most isolates (97%) from the mycorrhizas gathered in group B. Isolates from the mycorrhizosphere were equally distributed in group A (48%) and group B (52%).

The apparent clustering according to the correspondence analysis was confirmed by the use of the KMACL4 software. Analysis of the metabolic data with this software allowed the classification of the 300 isolates into two major groups, each divided in two subgroups, corresponding exactly to those described above. Furthermore, the KMACL4 software allowed the classification of the most discriminating substrates for each group and for each subgroup. The most characteristic substrates for group A (in decreasing order of importance) were putrescine, *N*-acetyl-D-glucosamine, α-ketovaleric acid, L-proline, mono-methyl succinate, glucyl-L-glutamic acid, urocanic acid, L-leucine, sebacic acid, and L-phenylalanine. The most characteristic substrates for group B were itaconic acid, adonitol, D-sorbitol, D-trehalose, *m*-inositol, *i*-erythritol, xylitol, L-histidine, D-glucuronic acid, DL-α-glycerol phosphate, D-galacturonic acid, L-rhamnose, and L-fucose. The most discrimi-

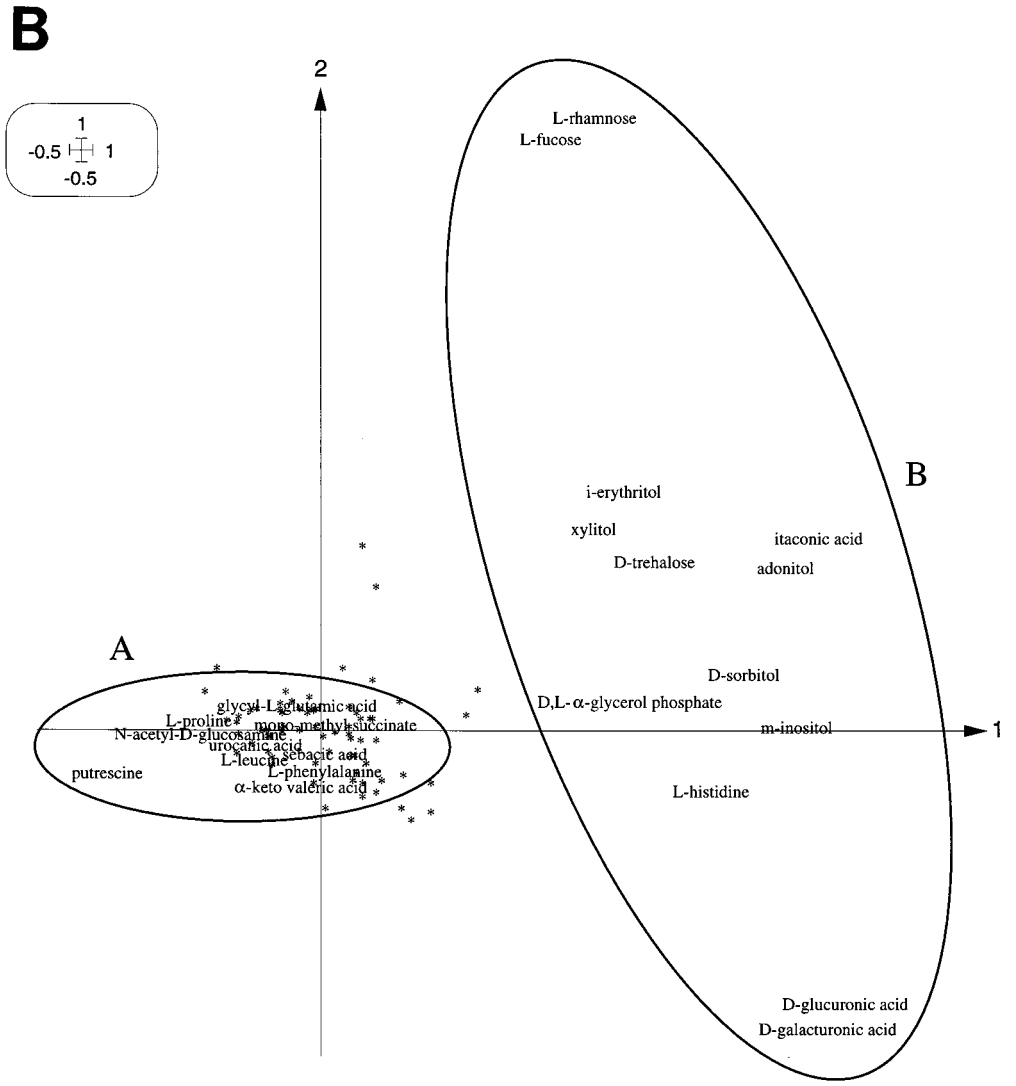

FIG. 1—*Continued.*

nating substrates for groups A and B were mapped on the factorial plane of the correspondence analysis (Fig. 1B). In consequence, group A isolates (mainly *P. fluorescens* bv. II and V) seem to preferentially utilize amino acids and some carboxylic acids, whereas group B isolates (mainly *P. fluorescens* bv. I) seem to preferentially utilize carbohydrates and other carboxylic acids.

PCR-RFLP analysis of the 16S rDNA. Since *P. fluorescens* bv. I isolates were mainly isolated from the mycorrhizosphere and from the mycorrhizas, the analysis of the genotypic diversity was focused on this biovar. Forty-eight isolates of *P. fluorescens* bv. I were subjected to PCR-RFLP of their 16S rDNAs, i.e., 1 isolate from the bulk soil, 28 isolates from the mycorrhizosphere, and 19 isolates from the mycorrhizas. Amplification of the 16S rDNA for each isolate yielded the expected approximately 1,550-bp PCR product corresponding to the full-length 16S rRNA gene. All the amplified 16S rDNAs were digested with each of the 13 restriction endonucleases. Depending on the restriction enzyme and the isolates, three to eight restriction fragments were recorded. As an example, results of digestion of the 16S rDNAs of six isolates with each of three restriction enzymes are shown in Fig. 2. Similar restriction patterns for each enzyme were designated by the same letter, according to the nomenclature defined by Laguerre et al. (25). Each isolate was assigned to a composite 16S rDNA type defined by the combination of the restriction patterns obtained with the 13 restriction endonucleases (Table 2). Among the 13 restriction endonucleases used, four (*Alu*I, *Dde*I, *Msp*I, and *Hin*fI) revealed polymorphism in the 16S rDNAs of the *P. fluorescens* bv. I isolates. The 48 isolates studied were assigned to four different 16S rDNA types, which were all different from the 20 16S rDNA types previously described by Laguerre et al. (25) and therefore designated 16S rDNA types 21 to 24. 16S rDNA type 22 was largely dominant, with 39 isolates, namely, 1 isolate from the bulk soil, 21 isolates from the mycorrhizosphere, and 17 isolates from the mycorrhizas. The other 16S rDNA types were represented only by a few isolates (Table 2).

A phylogenetic analysis was performed to evaluate the genetic relationships between the isolates from this study and a set of international reference strains of fluorescent pseudomonads and *Escherichia coli* (25). The data matrix of polymor-

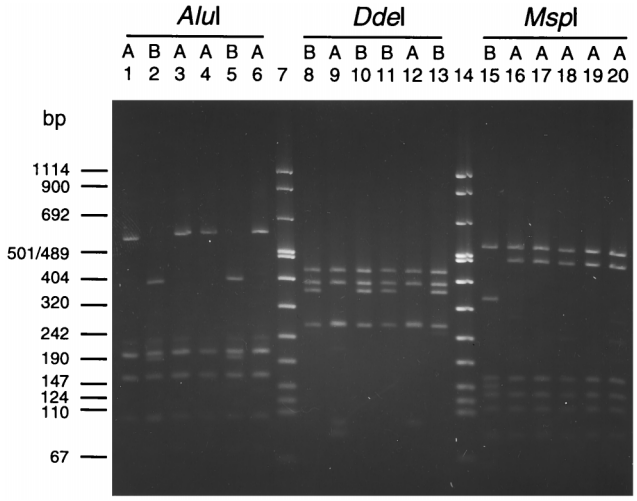

FIG. 2. Gel electrophoresis of PCR-amplified 16S rDNA fragments digested with three restriction enzymes. Similar restriction patterns for each enzyme were assigned the same letter. Lanes: 1 to 6, digestion with *Alu*I; 8 to 13, digestion with *Dde*I; 15 to 20, digestion with *Msp*I; 1, 8, and 15, strain PFX307; 2, 9, and 16, strain PFX308; 3, 10, and 17, strain PFX320; 4, 11, and 18, strain PFX323; 5, 12, and 19, strain PFX326; 6, 13, and 20, strain PFX332; 7 and 14, molecular weight markers.

phic restriction sites between 16S rDNA types of the reference strains (25) was completed with the data from the 16S rDNA types 21 to 24 (data not shown). A Dollo parsimony analysis (48) of the data matrix was performed with the PAUP software. Several equally parsimonious trees were obtained from a heuristic search. These trees showed few differences in topology. One of these trees is shown in Fig. 3. 16S rDNA types 22 and 24 are clustered with the *P. fluorescens* reference strains. 16S rDNA types 21 and 23, which are closely related, seem to form a different cluster which is equally distant from the *P. fluorescens* reference strains and from the *P. putida* bv. B strain. Interestingly, the genetic relationships among the *P. fluorescens* bv. I isolates are consistent with the metabolic fingerprinting analysis. Indeed, all the isolates assigned to 16S rDNA types 22 and 24 belong to the γ subgroup defined above, whereas all the isolates assigned to 16S rDNA types 21 and 23 belong to the δ subgroup.

ERIC-PCR fingerprinting and correlation with metabolic fingerprinting. Since the 16S rDNA type 22 is dominant in the mycorrhizosphere and in the mycorrhizas, genetic relationships between isolates within this 16S rDNA type were investigated by a more discriminating method, ERIC-PCR fingerprinting. Thirty-three of 39 isolates belonging to 16S rDNA type 22 were analyzed by ERIC-PCR, namely, 1 isolate from the bulk soil, 15 isolates from the mycorrhizosphere, and 17 isolates from the mycorrhizas. Each isolate produced a multiple-DNA band pattern. Among the 33 isolates, 16 different profiles were identified (Fig. 4). The binary matrix from the ERIC-PCR profiles was analyzed with the PAUP software. One of the trees obtained (Fig. 5) reveals a high genotypic diversity in the mycorrhizosphere (nine different profiles) and in the mycorrhizas (six different profiles) and the presence of 11 isolates from the mycorrhizas which seem to belong to the same clone.

Furthermore, a correspondence analysis of the Biolog data of the same 33 isolates led to the distinction of eight metabolic groups (data not shown). The correspondence between these metabolic groups and ERIC-PCR profiles is indicated in Fig. 5. Interestingly, there is a good correlation between ERIC-PCR and metabolic fingerprinting. However, there was no evident relationship between genotypic clusters and the origin of the isolates.

DISCUSSION

The total culturable bacterial densities and the fluorescent pseudomonad populations were 14- and 4-fold greater, respectively, in the mycorrhizosphere of Douglas fir than in the adjacent bulk soil. These results show that the mycorrhizosphere exerts a significant stimulatory effect on the populations of bacteria present in the soil, including the fluorescent pseudomonads. A similar stimulatory effect was observed in the mycorrhizosphere of hybrid larch, Sitka spruce, and sycamore (17) and in the mycorrhizosphere of hazel trees mycorrhizal with truffles (32). Besides a global effect on the size of the fluorescent pseudomonad populations, we demonstrated that the mycorrhizosphere of Douglas fir induced two contrasted dynamics of pseudomonad populations: (i) the *P. fluorescens* bv. I strains which were present in the bulk soil at a very low frequency were highly stimulated in the mycorrhizosphere and in the mycorrhizas, and (ii) on the other hand, *P. fluorescens* bv. V and II strains were counterselected in the mycorrhizosphere and in the mycorrhizas.

Multivariate analyses of the metabolic fingerprints resulted in the distinction of two major groups, with a good correlation with the origin of the isolates. Multivariate analyses of Biolog fingerprints have also been successfully applied to differentiate total microbial communities present in the rhizosphere of different plants (15–17). This approach clearly differentiated microbial communities from a bulk soil and from the rhizosphere of different tree species (17).

Furthermore, the analysis of the Biolog fingerprints with KMACL4 software resulted in the identification of the most discriminating substrates for the two major groups of isolates. Isolates from the bulk soil were characterized by the capacity

TABLE 2. Composite 16S rDNA types revealed by restriction analysis of PCR-amplified 16S rDNA genes and origin of the *P. fluorescens* bv. I isolates within each 16S rDNA type

16S rDNA type no.[a]	Restriction patterns of amplified 16S rDNA genes digested with enzymes[b]													No. of isolates from:		
	*Alu*I	*Cfo*I	*Dde*I	*Hae*III	*Hin*fI	*Tru*9I	*Msp*I	*Nci*I	*Nde*II	*Rsa*I	*Sau*96I	*Scr*FI	*Taq*I	Bulk soil	Mycorrhizosphere	Mycorrhizas
21	B	A	A	A	A	A	B	A	A	A	A	A	A	0	1	1
22	A	A	B	A	A	A	A	A	A	A	A	A	A	1	21	17
23	B	A	A	A	A	A	A	A	A	A	A	A	A	0	5	1
24	A	A	B	A	E	A	B	A	A	A	A	A	A	0	1	0

[a] 16S rDNA types numbered from 21 to 24 represent the combinations of restriction patterns obtained when PCR-amplified 16S rDNA was digested with 13 individual restriction enzymes.

[b] Groups of similar restriction patterns for each restriction enzyme are designated by the same letter, according to the nomenclature defined by Laguerre et al. (25).

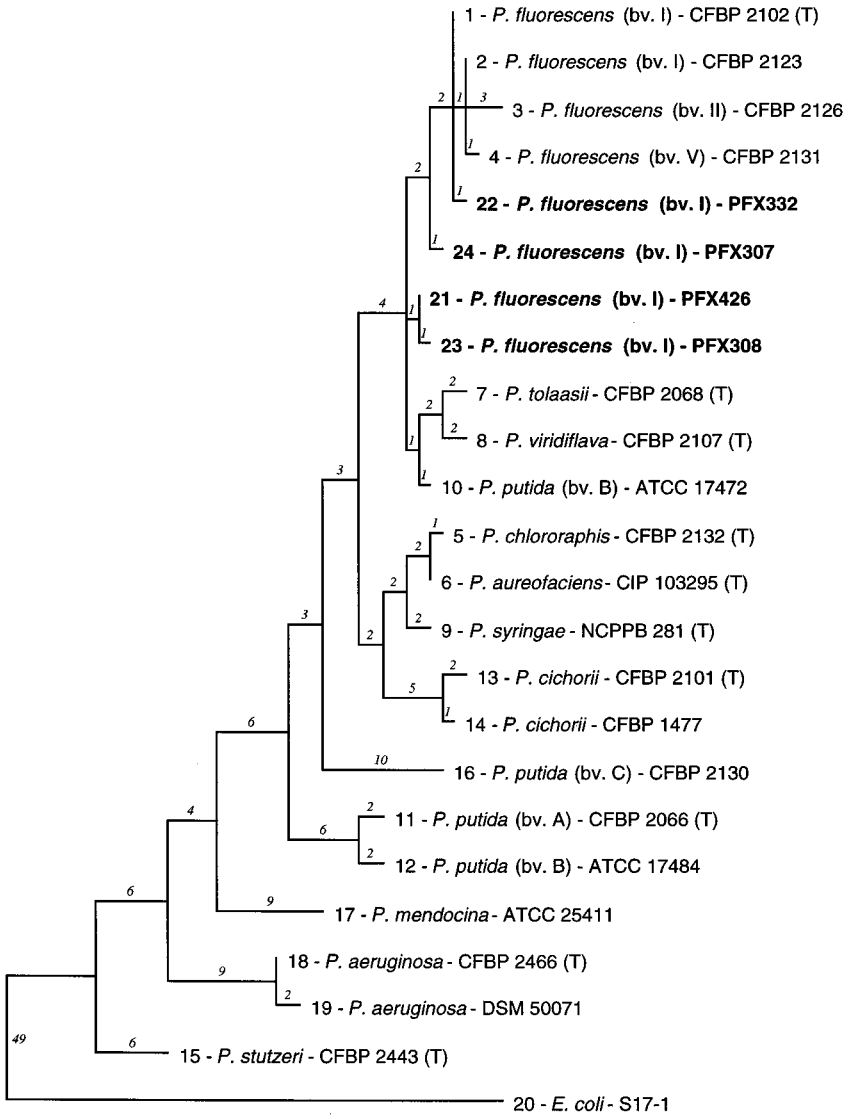

FIG. 3. Phylogenetic tree constructed by Dollo parsimony analysis with the PAUP software showing the relationships between the *P. fluorescens* bv. I isolates collected from the Champenoux nursery (in boldface type) and the reference strains of *Pseudomonas* spp. and *E. coli* studied by Laguerre et al. (25), based on PCR-RFLP analysis of the 16S rDNA. The horizontal branches are drawn proportionally to the number of restriction site changes. The numbers above the branches are the total number of changes assigned to each branch by PAUP. T, type strain of the species.

of preferentially utilizing aminoacids and some carboxylic acids. The second most discriminating substrate was *N*-acetyl-D-glucosamine. This compound could result from the hydrolysis of chitin, a cell wall polymer characteristic of higher fungi. This may suggest that the fluorescent pseudomonads present in the bulk soil could utilize *N*-acetyl-D-glucosamine resulting from the decomposition of dead fungal hyphae. In contrast, *P. fluorescens* bv. I isolates, which originated mainly from the mycorrhizosphere and from the mycorrhizas, were characterized by the capacity of preferentially utilizing carbohydrates and other carboxylic acids. Among these substrates, some were identified in similar studies as discriminating between bacterial isolates or communities originating from the rhizosphere of different plant species. These included the following: sorbitol (34); inositol, erythritol, and trehalose (28); D-galacturonic, D-glucuronic, and itaconic acids (16); and adonitol and fucose (15). Some of these carbohydrates may be exuded by the roots of Douglas fir. Many authors have hypothesized that the nature of the root exudates influence the composition of the microflora in the rhizosphere (15, 17, 26, 28, 42). However, there are very few studies on the nature of tree root exudates (29).

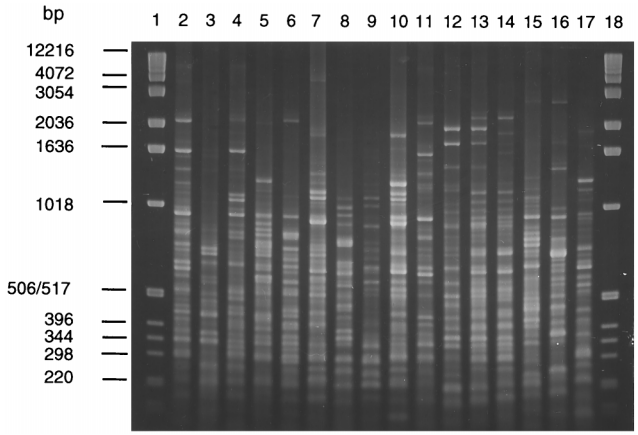

FIG. 4. ERIC-PCR profiles of 16 *P. fluorescens* bv. I isolates belonging to 16S rDNA type 22. Lanes: 1 and 18, molecular weight markers; 2, PFX184; 3, PFX207; 4, PFX223; 5, PFX264; 6, PFX266; 7, PFX318; 8, PFX320; 9, PFX323; 10, PFX332; 11, PFX361; 12, PFX402; 13, PFX405; 14, PFX413; 15, PFX428; 16, PFX430; 17, PFX432.

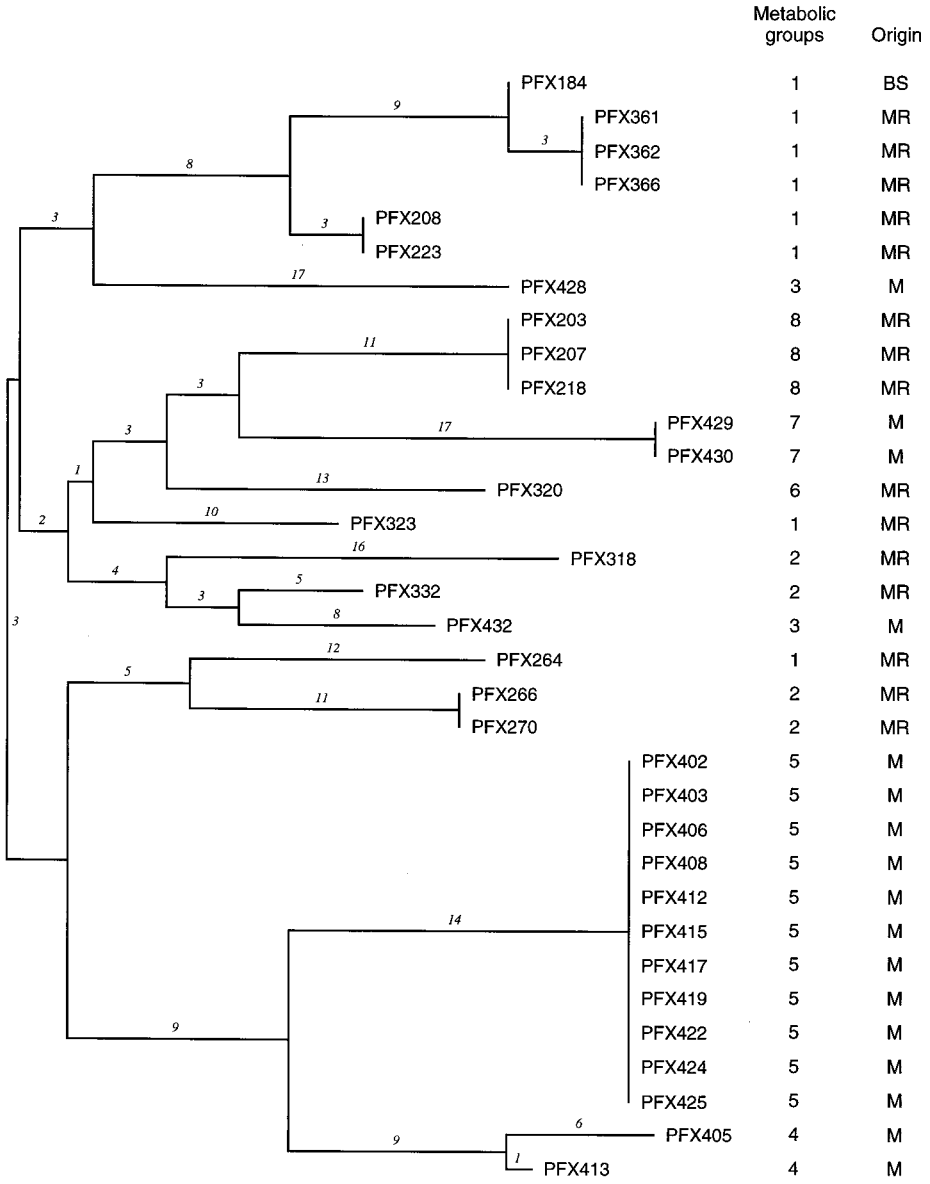

FIG. 5. Tree constructed by parsimony analysis with the PAUP software showing the relationships between 33 *P. fluorescens* bv. I isolates belonging to 16S rDNA type 22 based on ERIC-PCR analysis. The horizontal branches are drawn proportionally to the number of DNA band changes. The numbers above the branches are the total number of changes assigned to each branch by PAUP. Metabolic groups, as determined by correspondence analysis of Biolog data, and origins of the isolates are indicated on the right. BS, bulk soil; MR, mycorrhizosphere; M, mycorrhizas.

Trehalose was identified as one of the most characteristic substrates for *P. fluorescens* bv. I isolates. A detailed analysis of the Biolog fingerprints revealed that all of the *P. fluorescens* bv. I isolates were able to metabolize trehalose, whereas all the *P. fluorescens* bv. V isolates were unable to utilize this carbohydrate. Trehalose is produced by many fungi (49) and by some invertebrates. Most vascular plants, except for a few drought-resistant plants, are unable to produce trehalose (37). The presence of trehalose has been evidenced in all kinds of mycorrhizas, including vesicular and arbuscular endomycorrhizas (1, 46), arbutoid endomycorrhizas or orchids (19), and ectomycorrhizas (21, 33, 39). Furthermore, trehalose is the most abundant carbohydrate accumulated in the mycelium of *L. bicolor* in pure culture (4, 11). These observations strongly suggest that the *P. fluorescens* bv. I strains present in the soil are selectively stimulated in the vicinity of the mycorrhizas by the release of trehalose by *L. bicolor*. Trehalose-mediated selection was also suggested in a study of *P. fluorescens* strains associated with sporocarps of the ectomycorrhizal fungus *Cantharellus cibarius* (5).

However, this study did not allow us to definitely assess the relative contribution of each of the partners of the symbiosis in the selection exerted on the fluorescent pseudomonad community, since nonmycorrhizal control plants were not available. Indeed, in a forest nursery soil, all of the seedlings are always mycorrhizal, either with an introduced fungal strain or with other resident ectomycorrhizal fungi.

PCR-RFLP analysis of the 16S rDNA has been successfully developed to differentiate species within different genera (18, 22, 24, 25). This technique has been proposed for identification

and classification of members of the fluorescent pseudomonad group (25). We identified four newly described 16S rDNA types within the *P. fluorescens* bv. I isolates, which confirms the high genetic heterogeneity among *P. fluorescens* strains observed by Laguerre et al. (25). These results suggest that even among each biovar of *P. fluorescens*, several species could be described. Furthermore, our results revealed a strict correlation between the classification based on PCR-RFLP analysis of the 16S rDNA and the multivariate analysis of the Biolog fingerprints.

If we consider that the 33 isolates of 16S rDNA type 22 belong to the same fluorescent pseudomonad species, we demonstrated in this work that an intraspecific diversity can be evidenced by using the ERIC-PCR technique, since 16 different ERIC-PCR profiles were identified. Among fluorescent pseudomonad isolates collected from two different soils, Latour et al. (26) also found several REP-PCR profiles within each 16S rDNA type. These results confirm the value of rep-PCR methods (PCR with primers corresponding to ERIC, REP, or BOX elements) for diversity studies at the intraspecific level (6, 10, 31, 38, 45). In the present study, a good correlation was found between ERIC-PCR and metabolic fingerprintings, which concurs with previously published studies (26, 28).

Finally, the results of this study are also of practical importance in the context of the use of MHB to promote the establishment of the ectomycorrhizal symbiosis in commercial forest nurseries (8, 9). Indeed, a promising MHB strain isolated from a *L. bicolor* sporocarp was shown to be a *P. fluorescens* bv. I organism which is phenotypically and genotypically clustered with the isolates from the mycorrhizas in the present study (11). Work is in progress to evaluate the ability of some isolates from this study to promote the establishment of the symbiosis between *L. bicolor* and Douglas fir. Thus, the present study could provide some phenotypic and/or genotypic criteria for a rapid selection of new MHB strains.

ACKNOWLEDGMENTS

We are very grateful to Gisèle Laguerre for her help with the 16S rDNA restriction maps and the use of the PAUP software. We also thank Alain Lelu for his help with the KMACL4 software, Ludovic Vial for the multivariate analyses, and Philippe Lemanceau and Xavier Nesme for stimulating discussions.

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