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Effect of the Mycorrhizosphere on the Genotypic and Metabolic Diversity of the Bacterial Communities Involved in Mineral Weathering in a Forest Soil[▽]

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To date, several bacterial species have been described as mineral-weathering agents which improve plant nutrition and growth. However, the possible relationships between mineral-weathering potential, taxonomic identity, and metabolic ability have not been investigated thus far. In this study, we characterized a collection of 61 bacterial strains isolated from *Scleroderma citrinum* mycorrhizae, the mycorrhizosphere, and the adjacent bulk soil in an oak forest. The ability of bacteria to weather biotite was assessed with a new microplate bioassay that measures the pH and the quantity of iron released from this mineral. We showed that weathering bacteria occurred more frequently in the vicinity of *S. citrinum* than in the bulk soil. Moreover, the weathering efficacy of the mycorrhizosphere bacterial isolates was significantly greater than that of the bulk soil isolates. All the bacterial isolates were identified by partial 16S rRNA gene sequence analysis as members of the genera *Burkholderia*, *Collimonas*, *Pseudomonas*, and *Sphingomonas*, and their carbon metabolism was characterized by the BIOLOG method. The most efficient isolates belonged to the genera *Burkholderia* and *Collimonas*. Multivariate analysis resulted in identification of three metabolic groups, one of which contained mainly bacterial isolates associated with *S. citrinum* and exhibiting high mineral-weathering potential. Therefore, our results support the hypothesis that by its carbon metabolism this fungus selects in the bulk soil reservoir a bacterial community with high weathering potential, and they also address the question of functional complementation between mycorrhizal fungi and bacteria in the ectomycorrhizal complex for the promotion of tree nutrition.

Minerals are a reservoir of nutrients in the soil. The physical weathering, chemical weathering, and biological weathering of minerals play a major role in forest ecosystems as these processes release numerous nutrients required for tree growth (e.g., phosphorus, potassium, magnesium, calcium, and iron). Evidence which indicates that mineral weathering by soil microorganisms affects ion cycling and plant nutrition is accumulating (11, 26, 44, 51). However, to date, little is known about the taxonomic and metabolic diversity of the soil microorganisms involved in this process.

The ability to solubilize poorly soluble calcium phosphates, such as hydroxyapatite, or to weather silicates has been described for a range of bacterial genera belonging to the α-proteobacteria (*Agrobacterium* and *Rhizobium*), the β-proteobacteria (*Achromobacter* and *Burkholderia*), the γ-proteobacteria (*Acidithiobacillus*, *Aerobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, and *Pseudomonas*), and the gram-positive bacteria (*Bacillus* and *Micrococcus*) (38), as well as for a range of mycorrhizal (*Pisolithus*, *Paxillus*, *Trichoderma*, and *Suillus*) (1, 36, 51) and nonmycorrhizal (*Aspergillus* and *Penicillium*) (41, 45, 50) fungi. Phosphate-solubilizing bacteria have been isolated from various environments, such as eutrophic lakes (20), mangrove tree roots (48), the cactus rhizoplane (38), and tree rhizospheres (28). However, in only a limited number of studies have the workers taken into account the taxonomic identity, the niche, and the ability to solubilize minerals of a collection of bacterial isolates at the same time (19, 25).

Frey et al. (18) and Frey-Klett (19) studied the genotypic and functional diversity of fluorescent pseudomonads in a forest nursery soil and demonstrated that the Douglas fir-*Laccaria bicolor* ectomycorrhizal symbiosis is responsible for the genotypic and functional structure of the fluorescent pseudomonad communities. This probably results from the biochemical impact of the symbiosis on the surrounding soil, the so-called mycorrhizosphere effect (5, 13, 21, 39). As the mycorrhizosphere selects strains that are able to solubilize inorganic phosphate and efficiently mobilize iron in comparison with the fluorescent pseudomonad communities in the bulk soil, Frey-Klett et al. (19) suggested that the symbiosis has an indirect effect on plant nutrition via its selective pressure on bacterial communities. To confirm this hypothesis, further research is needed; notably, the impact of the mycorrhizosphere on the genotypic and metabolic diversity of the soil bacterial communities involved in mineral weathering, which has not been documented yet, should be examined.

In this study, we selected 61 bacterial strains from 140 strains isolated from the bulk soil, the soil-mycorrhiza interface (mycorrhizosphere), and the oak-*Scleroderma citrinum* symbiotic mantle in a 30-year-old oak stand. We characterized these strains genotypically by amplifying and sequencing a portion of

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the 16S rRNA gene. We also determined the metabolic potentials of these strains by the Biolog method and their mineral-weathering potentials using a microplate assay. Data were then analyzed statistically in order to determine whether there was a relationship between the weathering abilities of the bacterial isolates, their genotypic identities and/or their metabolic potentials, and their origins.

MATERIALS AND METHODS

Collection of bacterial strains. A total of 140 bacterial strains were obtained from oak (*Quercus petraea*)-*S. citrinum* ectomycorrhizae and bulk soil in the mineral soil horizon (depth, 5 to 10 cm) in an experimental forest site located at Breuil in the French Massif Central (11; C. Calvaruso, M. P. Turpault, E. Leclerc-Cessac, and P. Frey-Klett, submitted for publication). The bacterial isolates were obtained from three compartments: the bulk soil (PN isolates), the soil-mycorrhiza interface (i.e., mycorrhizosphere) (PML isolates), and the symbiotic fungal mantle (PMB isolates). In the present study, 61 isolates were selected randomly from the strains from the three compartments in order to obtain strains that were representative of the whole 140-isolate collection; these strains included 21 PN isolates, 20 PML isolates, and 20 PMB isolates. Two reference strains of *Collimonas fungivorans*, strains Ter6 and Ter331 (kindly provided by W. de Boer, Netherlands Institute of Ecology, Heteren, The Netherlands), were also surveyed. All bacterial isolates were cryopreserved at −80°C in 20% glycerol and then cultivated on 0.1× tryptic soy agar (3 g/liter tryptic soy broth [Difco], 15 g/liter agar) and incubated at 25°C.

Phenotypic and metabolic fingerprinting of the isolates. Bacterial strains were examined to determine the colony morphology. A Gram analysis was performed using the aminopeptidase test from Sigma. Cell morphology and motility were observed with a BX41 Olympus microscope at a magnification of ×1,000 and by using phase interference contrast with unstained living bacteria. The utilization of sole carbon sources by the isolates was analyzed with Biolog GN2 (gram-negative) microplates used according to the manufacturer's instructions. Formazan accumulation in the bacterial cells was measured by determining the optical density at 590 nm with an automatic microplate reader (Bio-Rad model 550).

The quantitative data for utilization of carbon sources by the isolates were compared by multivariate analysis. Principal-component analysis was used to visualize the relationships between the isolates using the Splus software (49). Since all the variables were in the same units, we analyzed the variance-covariance matrix instead of the correlation matrix to obtain better evidence of distances between the variables. The isolates were classified into homogeneous metabolic groups using the *K*-means method. First, initial groups were formed, and centroids were calculated as barycenters of the clusters. Then, the algorithm assigned each observation for the group to the closest centroid, and new centroids were calculated. These steps were repeated until the centroids no longer moved (34).

Mineral-weathering potentials of the isolates. Bacterial isolates were cultivated overnight in liquid LBm medium (47). One hundred milliliters of each culture was washed three times with sterile ultrapure water. The absorbance at 600 nm of each resulting suspension was then adjusted ca. 0.8 to 1. Twenty microliters of each suspension was transferred into a well of a sterile Multiscreen microplate (MAGVN22; pore size, 0.22 μm; Millipore) containing 10 mg of sterile biotite particles (diameter, 200 to 500 μm, which was convenient for the experimental procedure used) and 180 μl of Bushnell-Hass medium lacking iron [20 mg/liter KCl, 150 mg/liter $MgSO_4 \cdot 7H_2O$, 80 mg/liter $NaH_2PO_4 \cdot 2H_2O$, 90 mg/liter $Na_2HPO_4 \cdot 2H_2O$, 65 mg/liter $(NH_4)_2SO_4$, 100 mg/liter KNO_3, 20 mg/liter $CaCl_2$] buffered at pH 6.5 and supplemented with glucose or mannitol (2 g/liter). The biotite was obtained from Bancroft (Canada) and was a 2:1 phyllosilicate which is frequently present in acid soil, weathers relatively quickly, and holds K, Mg, and Fe nutrients. It is a pure homogeneous mineral, and its composition is as follows: 410.1 g/kg SiO_2, 109 g/kg Al_2O_3, 22.1 g/kg Fe_2O_3, 100.5 g/kg FeO, 2.7 g/kg MnO, 189 g/kg MgO, 4.1 g/kg Na_2O, 94.6 g/kg K_2O, 22.8 g/kg TiO_2, 44.2 g/kg F, and 0.8 g/kg Zn. Its structural formula is $(Si_3Al_1)(Fe^{3+}_{0.12}Fe^{2+}_{0.61}Mg_{2.06}Mn_{0.02}Ti_{0.13})$ and $K_{0.88}Na_{0.06}O_{10}(OH_{0.98}F_{1.02})$. Biotite and culture media were sterilized by autoclaving (20 min at 120°C). No effect of autoclaving on the biotite structure was observed. Glucose and mannitol (final concentration, 2 g/liter) were added after autoclaving. Multiscreen microplates containing 10 mg of biotite particles were sterilized by UV treatment for 30 min before they were filled with sterile liquid media.

For each bacterial strain, eight wells were filled with the same inoculum (four wells for pH determination and four wells for iron measurement). In each experiment, eight wells were used as a control without bacteria: these wells received only 200 μl of Bushnell-Hass medium lacking iron. Two bacterial strains, strains PN3(3) and PML1(4), were used as negative and positive controls, respectively, in each experiment.

The Multiscreen microplates were incubated at 25°C for 48 h with constant shaking (orbital; 350 rpm). After incubation, all of the liquid in each well was filtered with a 0.22-μm filter by centrifugation using MultiScreen microplates in order to eliminate bacterial cells and biotite particles. To assess biotite weathering, the filtrates were recovered in a new microplate containing 20 μl of ferrospectral (Merck) for quantification of iron or bromocresol green (1 g/liter; Sigma) for determination of the pH. The pH and the amount of total iron (Fe^{2+} and Fe^{3+}) released from biotite in the solution were both determined at A_{595} with a Bio-Rad model 550 microplate reader. Using the manufacturer's instructions, calibration curves were constructed to determine the relationship between the absorbance and the amount of protons and between the absorbance and the amount of iron released for the bromocresol green and ferrospectral dyes, respectively. To validate these relationships, the pH and the total iron content were also determined using a drop pH meter (Mettler TSDL25) and an induction-coupled plasma emission spectrophotometer (plasma torch JY180 ULTRACE) (data not shown). The average values for the four replicates for iron quantification and for pH measurements obtained by the colorimetric assays described above were used to determine the weathering potential of each isolate.

To investigate which mechanisms could explain the weathering abilities of the bacterial isolates in the collection, the same microplate weathering assay was performed with serial dilutions of citric acid (10^{-3} M; pH adjusted to 6 to 2) and hydrochloric acid (concentration adjusted to be at pH 6 to 2) in the absence of bacteria. Citric acid was used as a model chelating agent, and hydrochloric acid was used as a model acidifying molecule. Experiments were performed in triplicate as described above. The data obtained were used to construct two reference curves corresponding to the complexation and acidification reactions that occur during the weathering process.

Molecular analysis of bacterial isolates. *rrs* gene amplification was performed using universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′), which allowed amplification of almost the entire gene (15). PCRs were performed in 50-μl (total volume) mixtures containing 1× PCR Mastermix (Eppendorf), 0.1 μM primers, and 1 μl cell extract. Cell extract was prepared by adding one colony of each isolate to 100 μl of sterile water. The following temperature cycle was used: initial denaturation for 5 min at 95°C, followed by 30 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 56°C, and 1.5 min of extension at 72°C and then a final extension at 72°C for 10 min. PCR products were purified and concentrated using a Qiaquick gel extraction kit (QIAGEN). The sequencing reaction was performed using the multicapillary Beckman CEQ 8000XL automated sequencer system. The sequencing primer used was 518r (5′ATTACCGCGGATGCTGG-3′) (27). The sequences were compared with the sequences in the GenBank databases (www.ncbi.mlm.nih.gov.BLAST), using the BLAST program (2).

Phylogenetic analysis. The partial 16S rRNA gene sequences of the bacterial isolates were aligned with previously described 16S rRNA gene sequences of α-, β-, and γ-proteobacteria using Clustal X (version 1.8) (43). Phylogenetic algorithms were obtained and tree design (DNA-DIST, NEIGHBOR, and SEQBOOT) was performed using the PHYLIP package (version 3.65; J. Felsenstein, University of Washington, Seattle [http://evolution.genetics.Washington.edu/phylip.html]). A bootstrap analysis was based on 1,000 replicates.

Statistical analysis. The effects of the origin and of the sugar source (glucose or mannitol) on the weathering abilities of the bacterial isolates were determined by analysis of variance (ANOVA) at a threshold level of $P = 0.05$ and by the Bonferroni-Dunn test using the Superanova software (Abacus Concepts, Inc., Berkeley, CA). The correlation between pH and the amount of released iron was analyzed by performing a linear regression analysis at a threshold level of $P = 0.05$ with the Statview software (SAS Institute, Cary, NC).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession numbers EF194778 to EF194839.

RESULTS

Relationship between mineral-weathering potential and origin of the bacterial isolates. A microplate assay was developed to quantify the mineral-weathering potential of bacterial isolates by measuring the amount of iron and protons released in solution from biotite. The weathering efficacies of the soil-

mycorrhiza interface (PML) and symbiotic fungal mantle (PMB) isolates were significantly higher than those of the bulk soil (PN) isolates, as determined by a one-factor (origin) ANOVA ($P = 0.0001$) and the Bonferroni-Dunn test performed for the iron concentrations in the culture filtrates (Fig. 1A and B). The distribution according to the soil compartment of the 61 bacterial isolates belonging to three different weathering efficacy classes (<0.1, <0.8, and > 0.8 mg/liter of iron released) highlighted the fact that the frequency of the isolates that exhibited the greatest weathering efficiency was higher in the compartments associated with the mycorrhizosphere than in the bulk soil compartment (Fig. 1C).

Relationship between mineral-weathering potential and weathering mechanisms. Most of the bacterial isolates that released large amounts of iron (Fe) from biotite also produced high levels of protons (Fig. 1B). For example, the majority of the isolates that released ca. 1 mg/liter of iron acidified the solution to pH values between 3.64 and 3.09; the only exception was isolate PMB4(3) (pH 5.07). This trend was confirmed by a linear regression analysis between the amount of iron released from the biotite and the log of the pH ($y = 14.88 + 236.26x$; $R^2 = 0.75$; $P = 0.0001$). PMB3(1) was the most efficient isolate of the 61 isolates tested; it released ca. 2.9 mg/liter of Fe and acidified the solution to pH 3.09. The majority of the bacterial spots shown in Fig. 1B were distributed along the reference curve for acidification.

Relationship between taxonomy and origin of the bacterial isolates. The nucleotide sequence of the PCR-amplified region (ca. 400 b) of the *rrs* gene of each isolate revealed that the 61 bacterial isolates belonged to the α-, β-, and γ-proteobacterial lineages. The *rrs* sequences exhibited high levels of homology (95 to 100%) with the sequences of cultivated and uncultivated environmental bacteria belonging to the genera *Burkholderia*, *Collimonas*, *Pseudomonas*, and *Sphingomonas*. They were clustered into six phylogenetic groups (Fig. 2). Eighty-four percent of the 61 bacterial isolates (clusters C, D, and E) belonged to the genus *Burkholderia*. Clusters C and D comprised isolates obtained from the three soil compartments sampled. On the other hand, cluster E contained only isolates obtained from the mycorrhizosphere (PML isolates) and the symbiotic mantle (PMB isolates), and these isolates exhibited 97 to 100% sequence homology with *Burkholderia glathei*. Similarly, cluster B contained only isolates obtained from the mycorrhizosphere and the symbiotic mantle that belonged to the genus *Collimonas*. The *rrs* genes of isolates PML3(4), PML3(7), PML3(8), and PMB3(1) exhibited 99% sequence homology with the *rrs* gene of *C. fungivorans*. Clusters A and F contained only one *Sphingomonas* strain [PML4(2)] obtained from the mycorrhizosphere and two *Pseudomonas* strains [PN1(10) and PN2(8)] obtained from the bulk soil, respectively.

Relationship between taxonomy, mineral-weathering efficacy, and origin of the bacterial isolates. *Collimonas* isolates associated exclusively with mycorrhizae were the organisms that most actively weathered the biotite (1.49 ± 0.3 mg/liter Fe released) according to a one-factor (genus) ANOVA ($P = 0.0001$) and the Bonferroni-Dunn test. In contrast, the efficacy of the single *Sphingomonas* isolate was low (0.94 mg/liter Fe released). When the 51 *Burkholderia* isolates were examined, the isolates obtained from the soil-mycorrhiza interface (PML isolates; 0.83 ± 0.15 mg/liter Fe released) and from the sym-

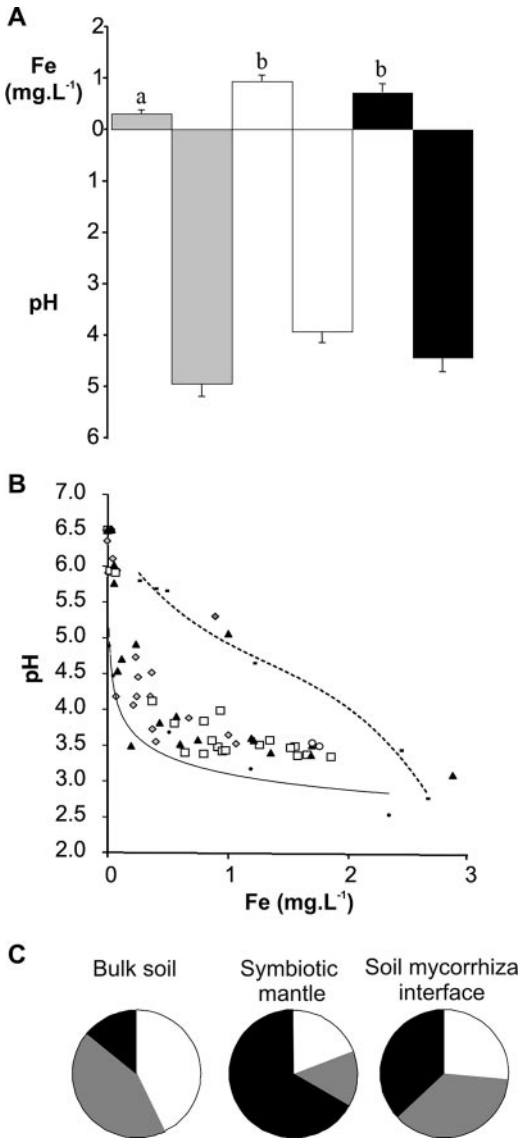
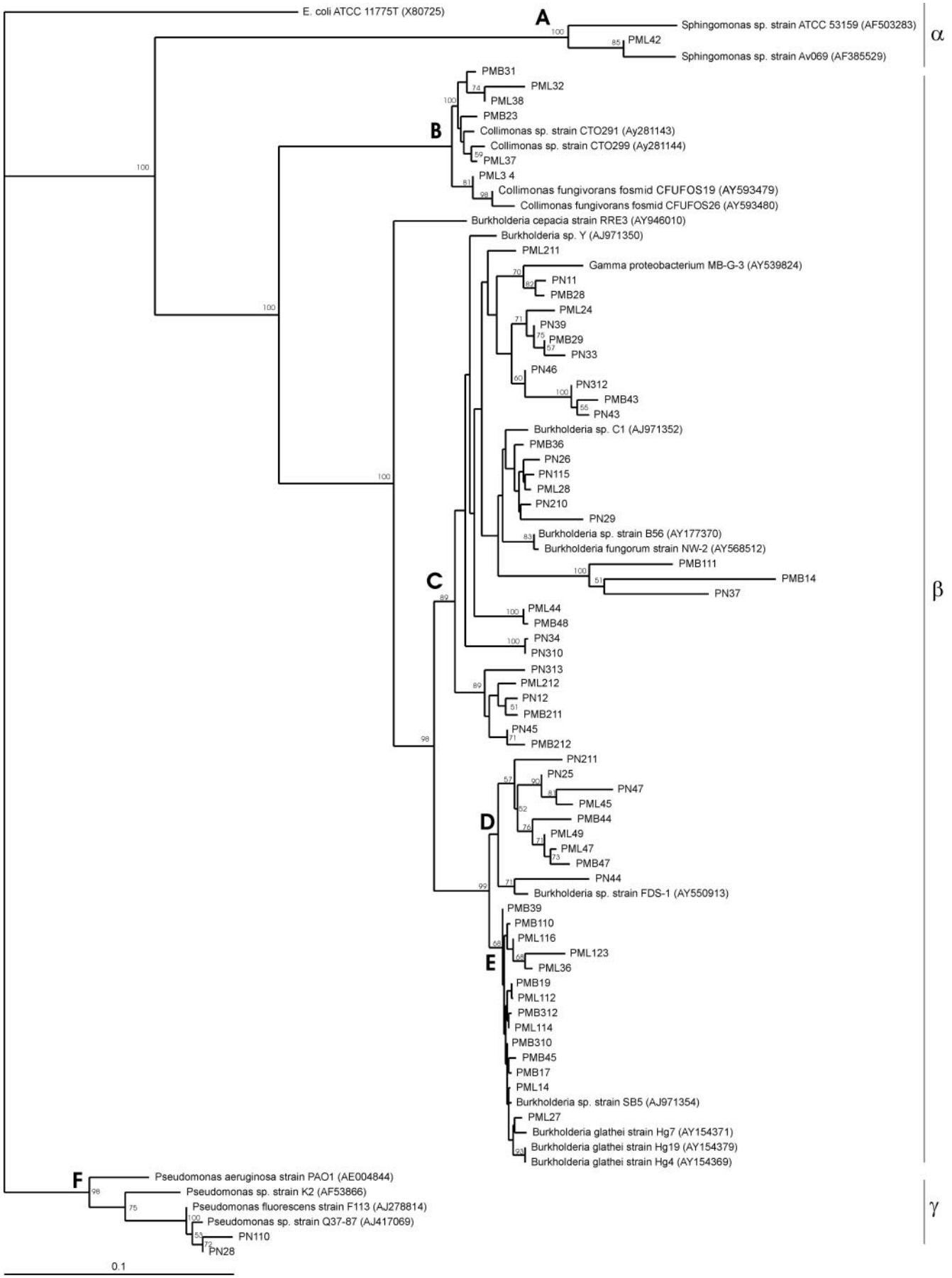


FIG. 1. Mineral-weathering potential of the bacterial isolates, as assessed by measurement of the pH and iron concentration in the microplate assay. (A) Relationship between the mean levels of iron released from the biotite, the mean pH values, and the three soil compartments from which the bacterial isolates were obtained, the bulk soil (PN isolates) (gray bars), the mycorrhizosphere (PML isolates) (open bars), and mycorrhizae (PMB isolates) (solid bars). The error bars indicate standard deviations. For the iron measurements, different letters above the bars indicate that the values are significantly different according to a one-factor (origin) ANOVA ($P = 0.0001$) and the Bonferroni-Dunn test. (B) Individual mineral-weathering potentials of the 61 bacterial isolates (pH versus Fe released). Each symbol represents a bacterial isolate from one of the three compartments analyzed, the bulk soil (PN isolates) (gray diamonds), the mycorrhizosphere (PML isolates) (open squares), and the mycorrhizae (PMB isolates) (solid triangles). The two reference strains, *C. fungivorans* Ter6 and Ter331, are indicated by open circles. Each symbol indicates the mean value of four replicates. The two curves indicate the mineral-weathering effect of a complexing agent (citric acid) (dashed line) and a strong acid (hydrochloric acid) (solid line). The relative distribution of the bacterial spots with respect to these two curves suggests mechanisms for the mineral-weathering effects of the bacterial isolates. (C) Distributions of the 61 bacterial isolates based on their origins (bulk soil [PN isolates], mycorrhizosphere [PML isolates], and mycorrhizae [PMB isolates]) and their membership in three mineral-weathering efficacy classes (<0.1 mg/liter of iron released [white areas], <0.8 mg/liter of iron released [gray areas], and >0.8 mg/liter of iron released [black areas]).


E. coli ATCC 11775T (X80725)
A
Sphingomonas sp. strain ATCC 53159 (AF503283)
PML42
Sphingomonas sp. strain Av069 (AF385529)
α
PMB31
PML32
PML38
PMB23
B
Collimonas sp. strain CTO291 (Ay281143)
Collimonas sp. strain CTO299 (Ay281144)
PML37
PML3 4
Collimonas fungivorans fosmid CFUFOS19 (AY593479)
Collimonas fungivorans fosmid CFUFOS26 (AY593480)
Burkholderia cepacia strain RRE3 (AY946010)
Burkholderia sp. Y (AJ971350)
PML211
Gamma proteobacterium MB-G-3 (AY539824)
PN11
PMB28
PML24
PN39
PMB29
PN33
PN46
PN312
PMB43
PN43
Burkholderia sp. C1 (AJ971352)
PMB36
PN26
PN115
PML28
PN210
PN29
Burkholderia sp. strain B56 (AY177370)
Burkholderia fungorum strain NW-2 (AY568512)
PMB111
PMB14
PN37
C
PML44
PMB48
PN34
PN310
PN313
PML212
PN12
PMB211
PN45
PMB212
PN211
PN25
PN47
PML45
D
PMB44
PML49
PML47
PMB47
PN44
Burkholderia sp. strain FDS-1 (AY550913)
PMB39
PMB110
PML116
PML123
E
PML36
PMB19
PML112
PMB312
PML114
PMB310
PMB45
PMB17
PML14
Burkholderia sp. strain SB5 (AJ971354)
PML27
Burkholderia glathei strain Hg7 (AY154371)
Burkholderia glathei strain Hg19 (AY154379)
Burkholderia glathei strain Hg4 (AY154369)
β
F
Pseudomonas aeruginosa strain PAO1 (AE004844)
Pseudomonas sp. strain K2 (AF53866)
Pseudomonas fluorescens strain F113 (AJ278814)
Pseudomonas sp. strain Q37-87 (AJ417069)
PN110
PN28
γ
0.1

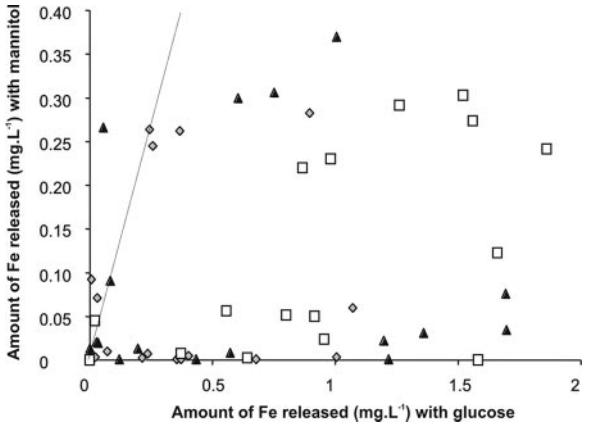


FIG. 3. Influence of the carbon source on the mineral-weathering potential. The mineral-weathering potentials of 20 bacterial isolates from bulk soil (PN isolates) (gray diamonds), 16 isolates from the mycorrhizosphere (PML isolates) (open squares), and 17 isolates from the symbiotic mantle (PMB isolates) (solid triangles) were assayed with either mannitol or glucose as the sole carbon source. Each symbol indicates the mean value for four replicates and represents the amount of iron (mg/liter) released from the biotite. For legibility, standard errors are not shown, and the straight line corresponds to equivalent amounts of Fe released (1:1 ratio) from biotite in the presence of glucose and in the presence of mannitol. Whatever the origin of the isolates, the mineral-weathering efficacies of the isolates were significantly higher in presence of glucose than in the presence of mannitol and could be classified as follows according to a two-factor ANOVA ($P = 0.0001$) and the Bonferroni-Dunn test: PML isolates > PMB isolates > PN isolates. With both carbon sources, the mycorrhizosphere isolates (PML isolates) were significantly more efficient than the bulk soil isolates (PN isolates), according to a one-factor ANOVA ($P = 0.0431$) and the Bonferroni-Dunn test.

biotic mantle (PMB isolates; 0.59 mg/liter ± 0.14 Fe released) weathered biotite significantly more efficiently than the PN isolates weathered biotite (0.31 ± 0.08 mg/liter Fe released), according to a one-factor ANOVA ($P = 0.0001$) and the Bonferroni-Dunn test.

Effect of the carbon substrate on the weathering efficacy. The potentials for mineral weathering discussed above were determined with glucose as the sole carbon substrate in the microplate assay. As sugar utilization varies among the soil bacteria and could directly or indirectly influence the weathering ability, utilization of mannitol was also tested for 20 PN isolates, 16 PML isolates and 17 PMB isolates (Fig. 3). This polyol, which was metabolized by ca. 94% of the bacterial isolates tested based on a Biolog analysis, is frequently detected in fungal exudates. In the presence of mannitol as a sole carbon source, the weathering efficacy of the PN isolates was significantly less than that of the PML and PMB isolates. In contrast, the weathering efficacies of the PML and PMB isolates were not significantly different, according to a one-factor ANOVA ($P = 0.0431$) and the Bonferroni-Dunn test. For isolates from all soil compartments, the weathering efficacies were significantly higher when the bacteria were incubated in the presence of glucose as a sole carbon source than when they were incubated in the presence of mannitol, as determined by a two-factor (carbon source, origin of the isolates) ANOVA ($P = 0.0001$) and the Bonferroni-Dunn test; the only exception was isolate PMB2(9), which was more efficient in the presence of mannitol (0.265 ± 0.018 mg/liter Fe released) than in the presence of glucose (0.058 ± 0.32 mg/liter Fe released). Conversely, four isolates obtained from the symbiotic mantle [PMB1(9), PMB1(11), PMB3(12), and PMB4(5)], one isolate obtained from the mycorrhizosphere [PML1(23)], and two isolates obtained from the bulk soil [PN2(10) and PN4(7)] were able to weather the biotite only in the presence of glucose. Finally, whatever carbon source was provided to the bacteria, the weathering efficacy of the PML strains was significantly greater than that of the PMB strains, which was significantly greater than that of the PN strains, as determined by the same two-factor ANOVA ($P = 0.0001$).

Multivariate analysis of the metabolic fingerprints. As carbon metabolism may influence biotite weathering, the 61 isolates were characterized by the Biolog method in order to determine if some metabolic traits could be related to the weathering efficacy of the bacterial isolates. Biolog GN2 microplates were used as the aminopeptidase test showed that all the bacterial isolates were gram negative. The quantitative data for carbon source utilization (95 data per isolate) were subjected to multivariate analysis in order to cluster the isolates having the same metabolic traits and to determine the characteristic traits of each cluster. Principal-component analysis was used to visualize the relationships between bacterial isolates. As several bacterial isolates exhibited high levels of sequence homology with the genus *Collimonas*, two reference strains, strains Ter6 and Ter331, were included in this study. According to this analysis, projection of the 61 isolates and the two reference strains on the plane defined by the first two principal components, which accounted for 42 and 10% of the total variance, respectively, revealed that most (ca. 86%) of the bacterial isolates from the bulk soil (PN isolates) were below the F1 axis, whereas 64% of the isolates from the mycorrhiza (PML isolates and PMB isolates) were above this axis (Fig. 4A). Using the *K*-means method, the data set was classified into three clusters, designated groups A, B, and C (number fixed a priori) (Fig. 4A). Isolates from the mycorrhizosphere (PML isolates) and from the symbiotic mantle (PMB isolates) were equally distributed in the three clusters, in contrast to the isolates from the bulk soil, which were concentrated in clusters A (ca. 33%) and C (62%).

The analysis of the metabolic data showed that the F1 axis was positively correlated with all the substrates except trehalose, L-glycyl-L-glutamic acid, and L-alanyl-glycine. This analysis allowed us also to determine the most discriminating sub-

FIG. 2. Neighbor-joining tree showing the phylogenetic relationships of the 61 bacterial isolates studied and reference strains, based on PCR sequencing of a portion of the 16S rRNA gene with primers pA and pH. A bootstrap analysis was performed with 1,000 repetitions, and only values greater than 50 are indicated at the nodes. The bootstrap analysis identified six clusters, clusters A, B, C, D, E, and F. The bacterial isolates used in this study were designated based on their origins, as follows: PN isolates, bulk soil; PML isolates, mycorrhizosphere; and PMB isolates, symbiotic mantle.

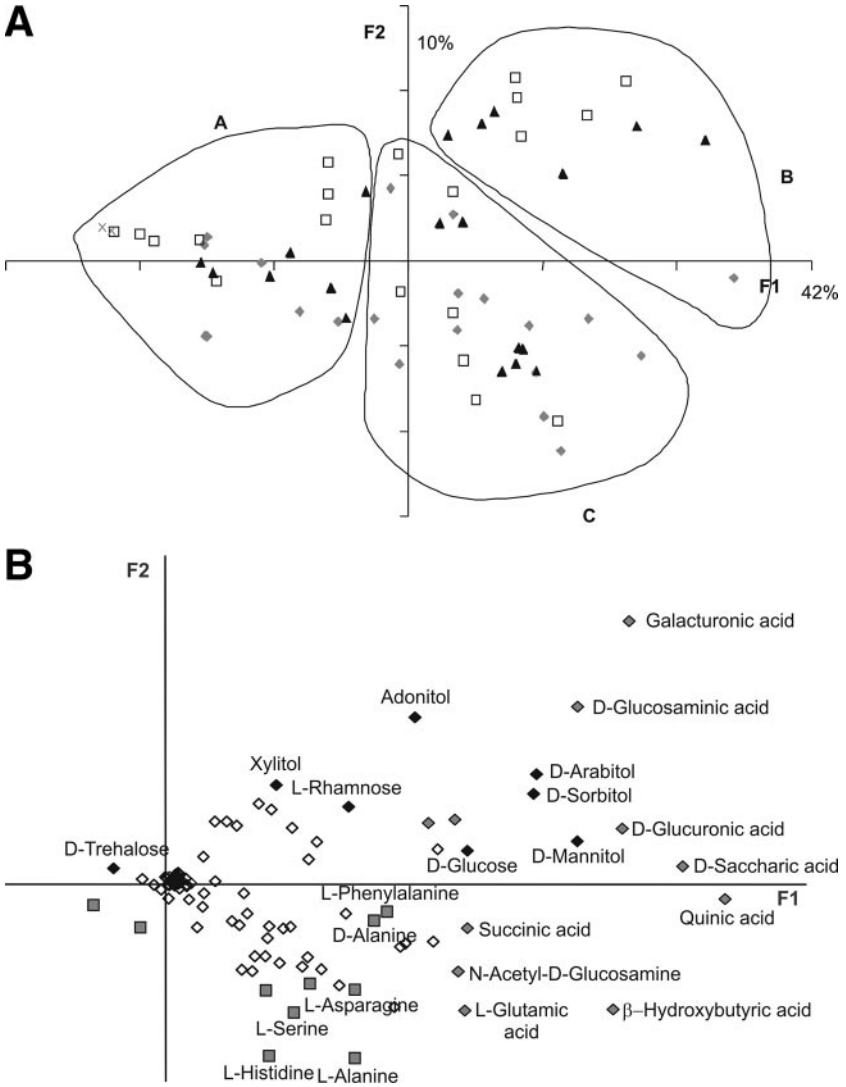


FIG. 4. Principal-component analysis of the metabolic profiles of the 61 bacterial isolates. (A) Projection of the 61 isolates on the plane defined by the first two principal components. The isolates were placed into three homogeneous metabolic groups designated groups A, B, and C using the *K*-means method. The origins of the bacterial isolates are indicated as follows: gray diamonds, bulk soil (PN isolates); open squares, mycorrhizosphere (PML isolates); and solid triangles, symbiotic mantle (PMB isolates). *Collimonas* reference strains Ter6 and Ter331 are indicated by gray multiplication signs. (B) Projection of the 95 carbon substrates from the Biolog GN microplates on the plane defined by the first two principal components. For legibility, only the most characteristic substrates of cluster A isolates (trehalose), cluster B isolates (glucose), and cluster C isolates (L-alanine) are identified (gray squares, amino acids; solid diamonds, sugars; gray diamonds, carboxylic acids). The positions of the other substrates are indicated by open diamonds.

strates for each group (Fig. 4B). Group A bacterial isolates seemed to use a limited number of substrates, in contrast to group B and C isolates. Trehalose, L-glycyl-L-glutamic acid, and L-alanyl-glycine were the preferentially catabolized substrates in group A isolates. Group B isolates seemed to preferentially use carbohydrates, in contrast to group C isolates, which seemed to prefer amino acids. The 10 most characteristic substrates for group B isolates, which included isolates from only the mycorrhizosphere and the symbiotic mantle, were D-saccharic acid, galacturonic acid, D-glucosaminic acid, D-glucuronic acid, D-mannitol, D-arabitol, D-sorbitol, α-D-glucose, L-aspartic acid, and bromosuccinic acid, and the 10 most characteristic substrates for group C isolates were quinic acid, β-hydroxybutyric acid, L-glutamic acid, *N*-acetyl-D-glucosamine, succinic acid, lactic acid, D-fructose, formic acid, L-phenylalanine, and D-alanine (in decreasing order of importance).

DISCUSSION

Soil colonization by microorganisms depends on their ability to access and metabolize the organic and inorganic nutrients present in the soil, as well as their ability to interact with other microorganisms, such as root-associated mycorrhizal fungi. In the rhizosphere of forest trees, ectomycorrhizal fungi, microfungi, and bacteria physically interact and form multitrophic ectomycorrhizal complexes that contribute to tree nutrition (19). So far, very few studies have addressed the question of the functional diversity of the different microbial components

of these assemblages in relation to mineral weathering, a central process in gross production and nutrient cycling. Our aim was to characterize the impact of the mycorrhizosphere structure on the genotypic and metabolic diversity of the soil culturable bacterial communities involved in mineral weathering. Our results confirmed that the ectomycorrhizosphere determines the structure of the functional diversity of culturable bacterial communities related to the mineral-weathering process. Similar results were obtained by Frey et al. (18) and Calvaruso et al. (Calvaruso et al., submitted), who used two chromoazurol S and tricalcic phosphate in vitro tests which are related to iron and phosphorus mobilization. As little is known about the possible relationship between genotype, metabolism, and mineral-weathering potential in soil bacterial communities, the 61 bacterial strains that we isolated were characterized by PCR sequencing of the 16SrRNA genes and by the Biolog method.

The 61 bacterial isolates studied belong to the genera *Burkholderia*, *Collimonas*, *Pseudomonas*, and *Sphingomonas*, and the genus that occurred most frequently was *Burkholderia*. Strains closely related to known species of the genera *Burkholderia* and *Pseudomonas* have been found previously to be the dominant cultivated bacteria associated with ectomycorrhizal fungi and forest soils (7, 14, 37). The presence of a majority of β-proteobacterial isolates in our bacterial collection could have resulted from our sampling procedure with diluted tryptic soy agar, which is commonly used for analysis of culturable communities in soils (6). However, analysis of a 16S rRNA clone library performed for the same oak-*Scleroderma* mycorrhizosphere showed that the α-, β-, and γ-proteobacteria accounted for ca. 9, 7, and 9%, respectively, of the total number of sequences (data not shown). This confirms that the β-proteobacterial lineage is a representative lineage in the bacterial communities of the mycorrhizosphere. Previously, *Collimonas* strains were characterized as organisms that grow at the expense of living fungi (12), but their mineral-weathering ability was unknown. In our study, all the *Collimonas* strains in our collection and in the collection of W. de Boer (Heteren, The Netherlands) (strains Ter6 and Ter331) (12) were among the strains that were most efficient for mineral weathering. Interestingly, these strains were obtained only from the soil surrounding the *S. citrinum* mycorrhizae. According to the Biolog method results, these organisms were able to use very few carbon sources, including trehalose, a fungus disaccharide that accumulated in *S. citrinum* mycelium (data not shown). This strongly suggests that *Collimonas* isolates are fungus dependent. In the *Scleroderma*-oak ectomycorrhizosphere, the *Collimonas* isolates could use trehalose exuded by the fungus or, because of their chitin-degrading ability (13), could hydrolyze the hyphae to release trehalose.

According to the phylogenetic analysis of the *rrs* gene, the 61 bacterial isolates were clustered in six groups. *Burkholderia* isolates, representing the most abundant genus, were clustered in three groups with variable mineral-weathering efficacies. The isolates in clusters C and D were obtained from all of the soil compartments and had mean mineral-weathering efficacies of 0.17 and 0.39 mg/liter Fe, respectively. In contrast, isolates in cluster E had the greatest mineral-weathering efficacy (1.13 mg/liter Fe). These isolates exhibited high levels of sequence homology with *B. glathei* and were obtained from the vicinity of the fungi. Interestingly, one of these strains, strain PML1(12), was recently described as an organism that was able to significantly increase biotite weathering and pine growth (11). The abilities of various *Burkholderia* strains to solubilize phosphate have been described previously (30, 46), but to our knowledge this is only the second report for *B. glathei* (23).

Multivariate analysis of the metabolic fingerprints confirmed that bacterial isolates belonging to the same genera grouped together and allowed us to distinguish the bulk soil isolates from the isolates from the mycorrhizosphere and the symbiotic mantle (Fig. 4). Such phenotypic (i.e., Biolog) grouping of isolates from the mycorrhizosphere was also observed in the mycorrhizosphere of Douglas fir-*L. bicolor* in a forest nursery soil (18). The Biolog fingerprints were used to identify carbon substrates preferentially metabolized by each group of isolates. Interestingly, 23% of the bacterial isolates were trehalose users; these isolates included only two isolates from the bulk soil and 13 isolates obtained from the vicinity of *Scleroderma*. Thus, the high proportion of trehalose users associated with *Scleroderma* suggests that this symbiont could exert trehalose-mediated selection on bacterial communities, as previously shown by Frey et al. (18) for fluorescent *Pseudomonas* communities.

As the aims of this study were to compare the mineral-weathering efficacies of 61 isolates having different origins and to assess the impact of the ectomycorrhizosphere on the structure of the bacterial community, two substrates were tested in the mineral-weathering bioassay: glucose and mannitol. Indeed, it has been demonstrated previously that silicate weathering is sometimes driven by the nutrient requirements of the microbial consortium (8) and that carbon sources determine the activity of various P-solubilizing bacteria (35). In our study, glucose and mannitol were chosen because (i) glucose utilization is a classical bacterial trait and (ii) mannitol is a common polyol found in fungal exudates (31, 32, 33, 40). Interestingly, whereas most of our isolates from all soil compartments were able to use mannitol, only a single *Burkholderia* isolate, obtained from the ectomycorrhizosphere, was significantly more efficient for weathering biotite in the presence of mannitol than in the presence of glucose. The contrasting results obtained using glucose in this bioassay validate the use of this assay for screening the bacterial collection.

Microorganisms are known to influence the formation or the dissolution of soil minerals by different mechanisms, playing a central role in soil fertility (4, 16). Two main mechanisms are suspected to be involved in mineral weathering in aerobic conditions: acidification and complexation. One hypothesis is that these mechanisms are linked to the production of geochemically reactive by-products of bacterial metabolism. Indeed, acidification is generally linked to the production of microbial organic acids and protons, and complexation is generally linked to the production of microbial chelating molecules, like siderophores or organic acids. In our study, comparison of the mineral-weathering efficacies of the 61 bacterial isolates to the two reference curves for acidification (HCl) and complexation (citrate) suggested that both mechanisms were involved. Most of the bacterial isolates that effectively weathered the biotite were able to strongly acidify the culture media. This acidification is likely linked to the production of organic acids from glucose metabolism. Several *Burkholderia*, *Pseudomonas*, and *Serratia* strains were described as organisms that

were able to dissolve inorganic phosphate by producing gluconic acid (23, 24, 30, 42). This acid results from glucose oxidation by the quinoprotein dehydrogenase (3). In contrast, some isolates in the collection able to efficiently weather biotite did not acidify the medium, suggesting that there was production of chelating molecules. The role of bacterial chelators or siderophores in the dissolution of minerals has been assessed previously. It was presumed that the catechol derivative produced by a strain of *Streptomyces* sp. could influence hornblende dissolution (22, 29).

Mycorrhizal fungi are well-known plant growth promoters (17). This characteristic results from the ability of these fungi to efficiently remove organic and inorganic nutrients from soil organic matter and minerals (10, 26). In nature, mycorrhizal fungi live in close association with bacterial communities, forming multitrophic complexes. Here, we demonstrated that the mineral-weathering potential of the bacterial isolates living in the vicinity of *Scleroderma* mycelium was greater than the mineral-weathering potential of the isolates living in the surrounding bulk soil. Therefore, we suspect that some of the functional activities, like mineral weathering, that have classically been attributed to ectomycorrhizal fungi could result at least partially from the activity of the associated exo- or even endobacterial communities (9) that have been selected by the ectomycorrhizal symbiosis. In this context, workers should also address the question of possible functional complementation between the ectomycorrhizal fungi and the associated bacterial communities, which would improve the mineral-weathering efficacy of the ectomycorrhizal complexes.

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