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Heterogeneous Cell Density and Genetic Structure of Bacterial Pools Associated with Various Soil Microenvironments as Determined by Enumeration and DNA Fingerprinting Approach (RISA)

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ABSTRACT

The cell density and the genetic structure of bacterial subcommunities (further named pools) present in the various microenvironments of a silt loam soil were investigated. The microenvironments were isolated first using a procedure of soil washes that separated bacteria located outside aggregates (outer part) from those located inside aggregates (inner part). A nondestructive physical fractionation was then applied to the inner part in order to separate bacteria located inside stable aggregates of different size (size fractions, i.e., two macroaggregate fractions, two microaggregate fractions, and the dispersible day fraction). Bacterial densities measured by acridine orange direct counts (AODC) and viable heterotrophic (VH) cell enumerations showed the heterogeneous quantitative distribution of cells in soil. Bacteria were preferentially located in the inner part with 87.6% and 95.4% of the whole AODC and VH bacteria, respectively, and in the microaggregate and dispersible clay fractions of this part with more than 70% and 80% of the whole AODC and VH bacteria, respectively. The rRNA intergenic spacer analysis (RISA) was used to study the genetic structure of the bacterial pools. Different fingerprints and consequently different genetic structures were observed between the unfractionated soil and the microenvironments, and also among the various microenvironments, giving evidence that some populations were specific to a given location in addition to the common populations of all the microenvironments. Cluster and multivariate analysis of RISA profiles showed the weak contribution of the pools located in the macroaggregate fractions to the whole soil community structure, as well as the clear distinction between the pool associated to the macroaggregate fractions and the pools associated to the microaggregate ones.

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Furthermore, these statistical analyses allowed us to ascertain the influence of the clay and organic matter content of microenvironments on the genetic structure relatedness between pools.

Introduction

Bacteria represent abundant inhabitants of terrestrial ecosystems and are involved in the key processes of their functioning, i.e., decomposition of organic carbon and plant residues, nitrogen transformation, and soil aggregation. Sustained efforts have been devoted to investigating the functioning and the structure of soil bacterial communities. Up to now, studies on the diversity and structure of terrestrial bacterial communities have been performed at a large geographical or spatial scale. A recent study of Felske and Akkermans [8] on the presence and activity of prominent bacteria in a grassland soil community demonstrated the homogeneity of the spatial distribution at the field scale (several hundred square meters). However, at a size scale several orders of magnitude lower, the mineral and organic soil compounds form aggregates that define microenvironments differing in size, stability, and physical and chemical properties [10, 13, 18]. Various studies such as whole cell counting [12, 25] or biomass measurements [15, 32], as well as specific bacterial population enumerations [16, 20], showed that such intrinsic heterogeneity led to a heterogeneous quantitative distribution of bacteria. Similarly, activity hot spots were observed in certain microenvironments [3, 19, 21], demonstrating the presence of more favorable habitats [5]. Whether or not the heterogeneity of bacterial distribution in soil can be related to differences in community structure within the different microenvironments has not been investigated yet.

This study aims at comparing the cell density and the genetic structure of bacterial subcommunities (further called pools) associated with various soil microenvironments. In the present paper we considered the term *community* as the total bacterial cells present in the whole soil sample, whether or not species are interacting, and the term *pools* as the total bacterial cells present in the considered microenvironment. Microenvironments were obtained by a combination of soil washes [25] and a nondestructive physical fractionation adapted from Kabir et al. [16], to separate bacteria located outside aggregates (outer part) from those located inside stable aggregates (inner part) of different size (size fractions). Direct cell counts and viable heterotrophic cell enumerations were performed to determine the quantitative distribution of bacteria in the whole soil and in each microenvironment. The genetic structure of bacterial pools was investigated using a culture independent technique, the RISA (rRNA intergenic spacer analysis), that relies on the length polymorphism of the intergenic spacer between the small (16S) and large (23S) subunit rRNA genes. Fingerprinting methods based on DNA or rRNA sequence analysis such as the sequence-dependent separation of fragments in a denaturing or temperature-gradient gel electrophoresis (DGGE and TGGE), the amplified ribosomal DNA restriction analysis (ARDRA), and the rRNA intergenic spacer analysis (RISA) were demonstrated to be relevant for the studies of complex bacterial communities such as those indigenous to soil [4, 7, 23, 29]. Among all these methods, the RISA fingerprinting was used because it is easy to perform, allows the rapid examination of the composition of complex bacterial communities, and can also be performed without the use of specific and expensive equipment [1, 4, 28]. Cluster analysis and multivariate analysis of the RISA fingerprints allowed us to estimate genetic relatedness between the bacterial pools associated with the various microenvironments and to identify some of the environmental factors involved in these relationships.

Materials and Methods

Soil Sampling and Fractionation

Five samples of 10 kg were randomly collected from a field planted with corn in October 1996 at La Côte Saint André (LCSAc), France. Samples were collected from the upper layer (0–20 cm) between corn plants, pooled, sieved (mesh size, 2 mm) and homogenized to obtain several subsamples of 1 kg. This soil is a silt-loam and contained 1.45% organic carbon, 21.2% clay, 47.7% sand, and 35.3% silt. The soil water pH was 7.0.

The microenvironments were separated by two successive soil fractionation procedures: a soil washing and a physical fractionation procedure (Fig. 1). The entire procedure of fractionation was performed twice on 30 g soil (dry weight) taken from one subsample of 1 kg. The former was performed according to Ranjard et al. [25]. This step allows us to separate bacteria located outside aggregates, i.e., easily washed out from the surface of aggregates (outer part), from those located inside aggregates, i.e., retained in soil aggregates after washings (inner part). In brief, the method involved 15 successive soil washes in sterile 0.8% NaCl solution with gentle shaking on a gyratory shaker (100 rotations min^{-1}) for 1 min. The supernatants were then pooled and centrifuged at 9,800 $\times$ *g* for 20 min. The pellet (outer part) was resuspended in 50 ml

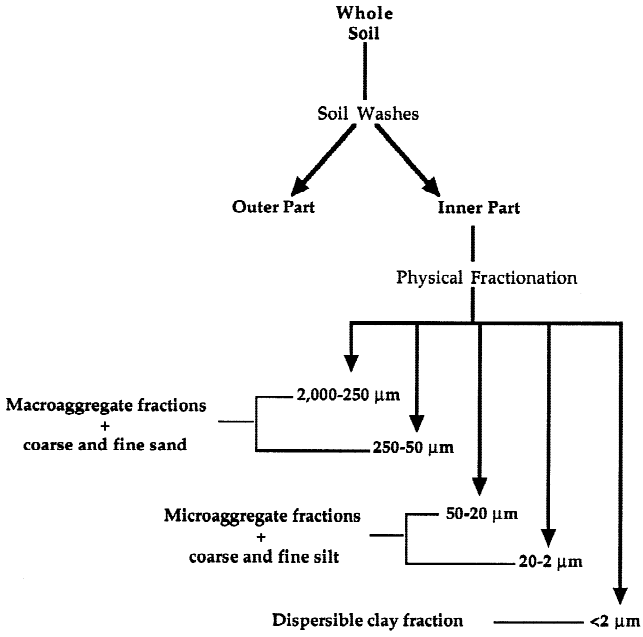

Fig. 1. Schematic representation of the soil fractionation to obtain the different soil microenvironments (parts and size fractions).

0.8% NaCl solution. An aliquot of the outer part suspension was dried (105°C, 24 h) to determine the weight representativity of the outer part corresponding to particles desorbed at the aggregate surfaces during soil washes. The remaining washed soil (inner part) was further fractionated to separate stable aggregates according to their size in order to obtain various soil size fractions, as developed by Kabir et al. [16]. Sand-size fractions (fractions 2,000 to 250 µm and 250 to 50 µm) containing stable aggregates plus coarse and fine sand, are further referred to as the macroaggregate fractions. They were obtained by wet sieving using sterile cooled (below 10°C) water to prevent quantitative modifications. Soil suspension containing aggregates and particles below 50 µm was aseptically transfered into a sedimentation flask. The silt-size fractions, 50–20 µm and 20–2 µm, contained microaggregates plus coarse and fine silt, respectively. The 50–20 µm fraction was obtained by sedimentation under gravity. Microaggregates and particles below 20 µm were removed by siphonation. The sedimentation step was repeated three times by resuspending the sedimented particles in cool sterile water. Microaggregates and fine silts (20–2 µm fraction) were pelleted from the supernatant by centrifugation at 90 × *g* at 10°C in a swinging bucket rotor in 250 ml centrifuge tubes. The dispersible clay fraction (<2 µm) was obtained by an overnight flocculation of the supernatants at 4°C after addition of $CaCl_2$ (50 mM).

Moist fractions 2,000–250, 250–50, and 50–20 µm were weighted in tared beakers. Subsamples were taken for direct bacterial counts, DNA extraction, and carbon and clay content estimation by sulfochromic oxydation and by textural analysis, respectively, and to determine dry weight of the fractions after a 24 h drying at 105°C. The 20–2 µm fraction and the dispersible clay fraction were resuspended in 200 ml of sterile cool water. Subsamples of 20 ml were used for the analysis mentioned above. Weight distribution, carbon and clay contents of soil, parts, and size fractions are listed in Table 1.

Bacterial cell counts were performed by acridine orange direct counts (AODC) as described by Richaume et al. [26]. Enumeration of colony forming units (CFU) corresponding to viable heterotrophic (VH) bacteria were performed on plate count agar media (PCA media) as previously described [25].

Extraction, Purification, and Quantification of Total DNA from Soil Samples

DNA was extracted from unfractionated soil and soil microenvironments using a direct lysis method according to the method described by Ranjard et al. [24]. This procedure involved sample homogenization and cell disruption by grinding in liquid nitrogen followed by enzymatic lysis (lysozyme and proteinase K). This method was shown to allow the recovery of (i) at least 60% of the DNA from the various samples and subsamples and (ii) DNA from diverse native bacterial groups.

rRNA Intergenic Spacer Analysis (RISA)

The intergenic spacers between the small- and large-subunit rRNA genes were amplified using the primers S-D-Bact-1522-b-S-20 (eubacterial rRNA small subunit, 5'-TGCGGCTGGATCCCCTCCTT-3') and L-D-Bact-132-a-A-18 (eubacterial rRNA large subunit, 5'-CCGGGTTTCCCCATTCGG-3') [22]. Amplified sequences contained the IGS *plus* 150 bp corresponding to the 20 nucleotides of the S-D-Bact-1522-b-S-20 primer and about 130 bp in the 23S rRNA gene. Reaction mixtures (50 µl) for PCR contained 5 µl of 10× dilution buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween 20 (vol/vol), 0.5% Nonidet P40 (vol/vol), 50% glycerol (vol/vol)), 1 µg T4 gene 32 protein (Boehringer Mannheim), 0.5 µM of primers, 200 µM of each dNTP, 2.0 U of Expand High Fidelity DNA polymerase (Boehringer Mannheim), and 100 ng of purified soil DNA. Amplification was performed in a Perkin Elmer Cetus 2400 after a hot start at 94°C for 3 min, followed by 25 cycles consisting of 94°C for 1 min, 55°C for 30s, and 72°C for 1 min, extension of incomplete products for 5 min at 72°C. The volume of PCR reactions loaded on gel was calibrated in order to get a similar intensity per profile. The influence of the number of PCR cycles on profiles was previously evaluated by performing 20, 25, or 30 PCR cycles. No changes were detected and 25 cycles were further performed in all PCR reactions.

Fragments ranging in size from approximately 300 to 1100 bp were resolved on 5% (w/vol) nondenaturing polyacrylamide–Tris–borate–EDTA gels (1.5 mm thick, 30 cm long). Gels were run at constant temperature (20°C) for 12 h at 60 V and 5 mA (DSG200-02, C.B.S. Scientific, Del Mar, Ca) and further stained with SYBR Green I (FMC Bioproducts, Le Perray en Yvelines, France) according to the manufacturer's instructions. The banding patterns were then photographed with Ilford FP4 film with a 302 nm UV source.

Table 1. Weight distribution, organic carbon and clay contents, total (AODC) and viable heterotrophic (VH) bacteria numbers, and distribution in LCSA-c soil

Soil sample	Weight distribution (%)	Organic carbon (%)*	Clay (%)	AODC (10^8 cells · g^{-1} soil sample)**	AODC distribution (%)	VH 10^7 cfu · g^{-1} soil sample**	VH distribution (%)‡
Unfractionated soil	100	1.45 ± 0.20	21.20	32.5 ± 2.8[a]	—	3.30 ± 0.7[d,e]	—
Outer part	7.9	2.05 ± 0.26	N.D	26.2 ± 5[a]	12.4	1.00 ± 030[e]	4.6
Inner part	92.1	1.03 ± 0.17	23.0	30.2 ± 0.2[a]	(87.6)§	3.71 ± 0.57[d]	(95.4)§
Fractions							
Macroaggregate fractions:							
+ Coarse sands: 2,000–250 μm	29	0.44 ± 0.14	9.1	4.2 ± 0.7[b]	7.2	0.26 ± 0.04[e]	4.7
+ Fine sands: 250–50 μm	20.7	0.51 ± 0.05	10.0	6 ± 1[b]	7.4	0.27 ± 0.11[e]	3.5
Microaggregate fractions:							
+ Coarse silt: 50–20 μm	19.5	0.58 ± 0.08	11.2	3.5 ± 2[b]	4.1	0.55 ± 0.15[e]	6.6
+ Fine silt: 20–2 μm	14.7	2.26 ± 0.22	34.4	35.9 ± 3.7[a]	31.6	5.38 ± 0.75[d]	49.4
Dispersible clay fraction <2 μm	8.2	3.41 ± 0.18	N.D†	73.4 ± 1.4[c]	37.2	6.07 ± 0.52[d]	31

* Mean ± standard deviation for duplicate samples.
** Mean counts ± standard deviation for duplicate samples.
† Size fraction <2 μm contains only clay minerals and organic carbon.
‡ AODC and VH distribution are expressed in percent of recovered bacteria.
§ Bacterial proportion in the inner part was the resultant of the sum of the proportion of the different size fraction.
a to e: Similar letters in superscript indicate no statistical pairwise differences at the 5% level (*t*-test) between associated mean counts, whereas different letters indicate significant differences.
N.D: Not determined.

Statistical Analysis of Data

Significant differences ($p < 0.05$) in AODC and VH bacterial numbers between soil samples and correlation factors were determined using Statview-SE with Student's *t*-test.

Pairwise comparisons of RISA profiles were manually performed using the negatives and matrices (presence–absence and relative intensity of each band) constructed. Bands were encoded by a number ranging from 0 (absence) to 4 (maximum intensity). In this analysis, whether two bands are present or absent as well as changes in band intensity are equally informative. Euclidian distances between pools were computed and subjected to hierarchical cluster analysis using the agglomerative second-order moment algorithm known as Ward's method [37] to produce the dendrograms.

Principal component analysis (PCA) on covariance matrix was performed on the data matrix (bacterial pools as rows and bands as columns). This method provided an ordination of bacterial pools and of encoded bands that were plotted in two dimensions based on scores on the first two principal components.

Cluster analysis and PCA were performed using the ADE-4 software [31].

Results

Bacterial Enumerations

Statistically ($p < 0.05$) different numbers of bacterial cells (AODC and VH) were found in the various soil microenvironments (Table 1). A slightly higher number of VH bacteria were found in the inner part than in the outer one but similar AODC numbers were found associated with both parts. However, the particles desorbed at the aggregate surfaces during soil washes and considered as the weight of the outer part represented only 7.9% of the total soil weight compared with 92.1% for the inner part, resulting in the presence of about 90% of the cells in the inner part (Table 1). AODC and VH numbers and proportions were highest in the dispersible clay and the 20–2 μm fractions. As shown in Table 2, AODC and VH numbers were found to be correlated with clay content ($r = 0.94$ and 0.79, respectively) and organic carbon content ($r = 0.96$ and 0.92).

RISA Profiles

RISA profiles differed in their complexity based on the number of bands as well as in the relative intensity of the bands (Fig. 2). The total number of bands detected per profile on the negatives and the number of discriminating bands (i.e., specific band to a profile or common band between profiles while relative intensity was different) between the profile of the unfractionated soil and the profiles of the fractions are listed in Table 2. The unfractionated soil and the inner part yielded identical profiles. The profiles of the size fractions

Table 2. Comparison of RISA profiles of bacterial pools associated with unfractionated soil and soil fractions

	Number of bands on RISA profile							
	Unfract. soil	Outer fraction	Inner fraction	2,000–250 μm	250–50 μm	50–20 μm	20–2 μm	<2 μm
Total number of bands:	40	32	40	46	45	47	44	32
Discriminative bands	—	18	0	23	26	21	22	28

were different from the profile of the inner part and were different from each other. All bands observed in the inner part profiles were found at least once in a microenvironment profile.

Nineteen bands were detected in the profiles of the various microenvironments that were not present in the profile of the unfractionated soil. Similarly, profiles of the various microenvironments exhibited a high number of bands that had different intensities compared to the equivalent size bands of the unfractionated soil profile.

Regarding fingerprint reproducibility, no difference could be detected between replicates of RISA profiles obtained from amplifications performed on a similar DNA extract (data not shown). Regarding profiles obtained from the DNA extracts from the two independent fractionation experiments, some differences were sometimes observed, i.e., repetitions of 250–50 μm and <2 μm fractions (Figs. 2 and 3). However, such differences were not as important as differences between different fractions could be.

Cluster and Principal Component Analyses (PCA)

A cluster analysis was performed to determine the relatedness of the bacterial community of the whole soil and the bacterial pools associated with the microenvironments (Fig. 3A). The resulting dendrogram revealed two main groupings: one with the unfractionated soil, the inner part, the outer part and the dispersible clay fraction, and the other with the macroaggregate fractions (2,000–250 μm and 250–50 μm) and the microaggregate ones (50–20 μm and 20–2 μm). Within the latter grouping a clear distinction was observed between the two macroaggregate fractions and the two microaggregate ones.

The principal component analysis (PCA) allowed us to ordinate bacterial pools and whole soil community (Fig. 3B) as well as bands (Fig. 4). The first principal component (PC1) explained 43% of the variance in the data and the second component (PC2) explained 20% of this variance. The factorial map (Fig. 3B) showed that ordination on PC1

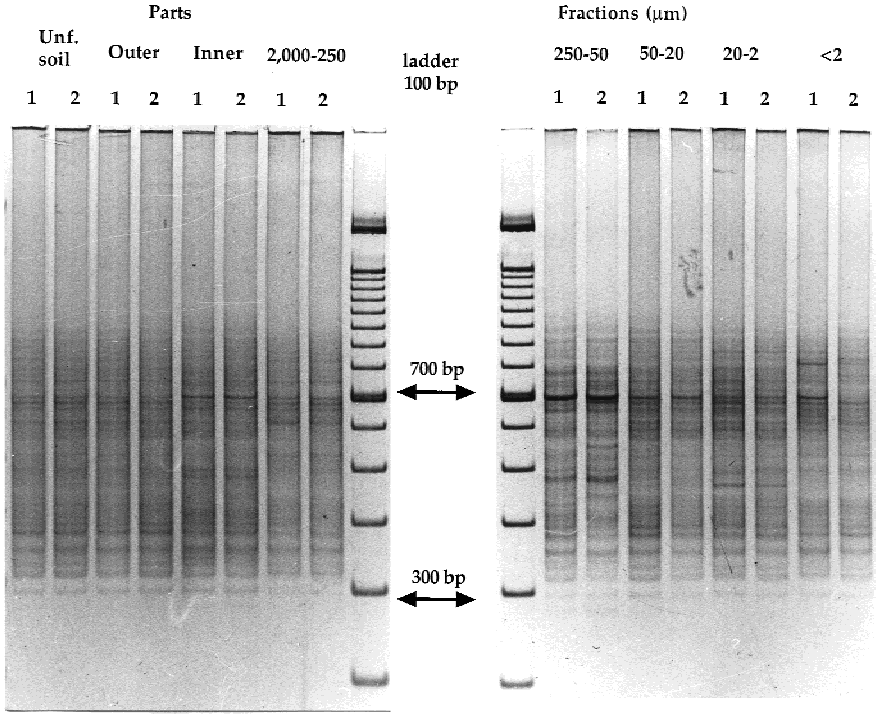

Fig. 2. RISA profiles from unfractionated soil and soil microenvironments resolved on 5% polyacrylamide. 1 and 2 represent the two independent repetitions.

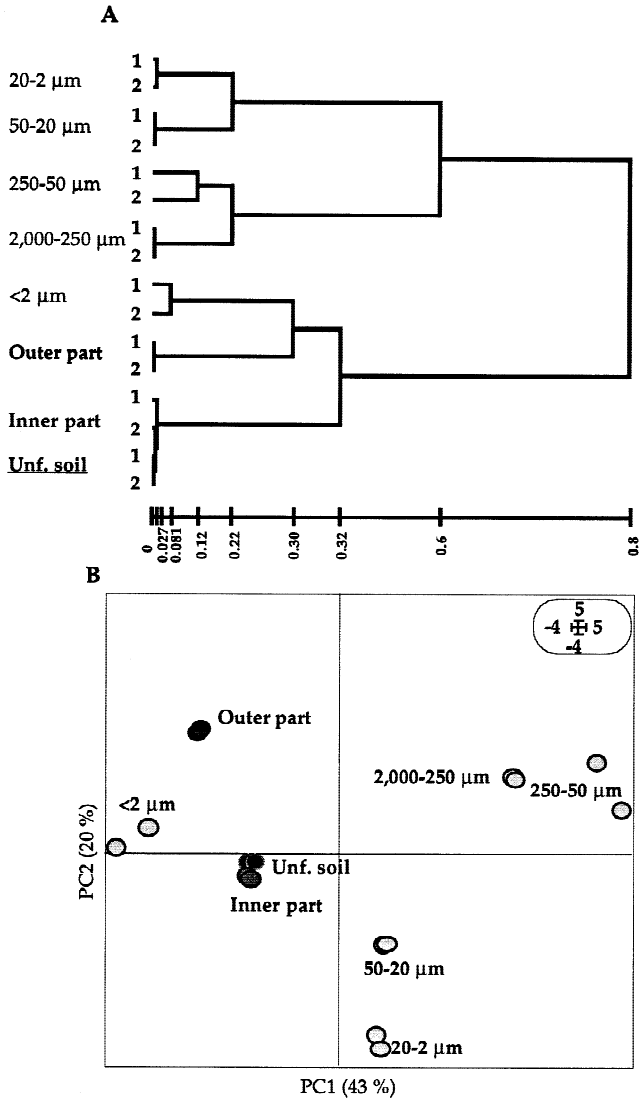

Fig. 3. Dendrogram (A) and principal component (PC1xPC2) plot (B) generated from RISA profiles of the bacterial pools from unfractionated soil and soil microenvironments. 1 and 2 represent the two independent repetitions.

mostly corresponds to aggregate size of the microenvironments. PC2 allowed us to differentiate the pools of the unfractionated soil and the inner part pools from that of the outer part. It also permitted us to differentiate the pools located in the microaggregate fractions (50–20 µm and 20–2 µm) from all the other ones. Correlation between soil sample characteristics and PC1 scores demonstrated a significant correlation with organic carbon and clay contents (Table 3). No significant correlation between these characteristics and ordination on the second component was observed.

PCA ordination of the bands allowed us to point out the contribution of the bands to the ordination of the bacterial

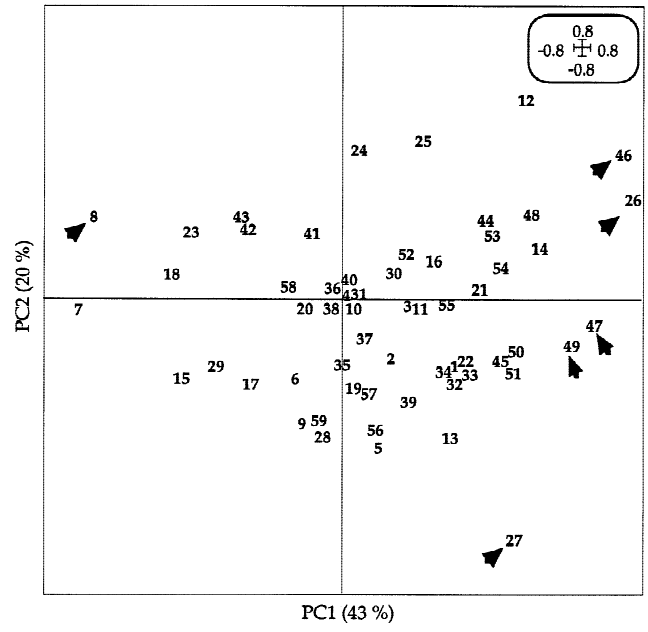

Fig. 4. Significance of the bands in explaining the variance between the RISA profiles of the bacterial pools from unfractionated soil and soil microenvironments.

pools and whole soil community (Fig. 4). The superimposition of bacterial pool distribution and band distribution allowed us to highlight the role of some bands to discriminate the different bacterial pools. The bands near the origin were common to all microenvironments. On the other hand, the more distant the bands were from the origin, the higher their environment specificity. For example, bands 46 (about 870 bp), 26 (about 480 bp), 47 (about 780 pb), and 49 (about 450 bp) characterized the macroaggregate fractions, whereas band 27 (about 470 bp) characterized the microaggregate ones and band 8 (about 820 bp) characterized the unfractionated soil, the inner part, the outer part, and the dispersible clay fraction.

Table 3. Correlation matrix for soil characteristics, bacterial cell abundance, and principal component analysis coordinates of bacterial pools

	Clay (%)	Organic carbon (%)	AODC	VH
Clay (%)	1			
Organic carbon (%)	0.93*	1		
AODC	0.94*	0.96*	1	
VH	0.79*	0.92*	0.92*	1
PC1 RISA	−0.81*	−0.80*	−0.83*	−0.78*
PC2RISA	−0.06	−0.2	−0.15	−0.2

* Indicates significant correlation at the 5% level.

Discussion

Cell densities and genetic structure of bacterial pools associated with various soil microenvironments of a cultivated silty-loam soil based on enumeration of total (AODC) and VH cells and on genetic fingerprints are reported. These microenvironments differed with regards to the size of the constituting particles, the size and stability of the aggregates, and the organic carbon and clay contents.

Heterogeneous Quantitative Distribution of Bacteria

Bacterial counts in the outer and inner parts showed that most bacteria were located in the inner part (Table 1). Quantitative differences between these two microenvironments had been previously reported a higher abundance of total bacteria (AODC), heterotrophic bacteria and nitrifying bacteria in the inner part than in the outer one [12, 25]. Within the inner part, the majority of bacteria (about 72% of total bacteria and 87% of VH bacteria) were found to be located in the microaggregate fractions and in the dispersible clay fraction. Several studies had previously reported the higher abundance of total [16, 26] and culturable [17] bacteria in these fractions. In the studied soil, the heterogeneous distribution of bacteria was found to be correlated to the level of organic carbon and clay contents (Table 2). Van Gestel et al. [33] demonstrated that both clay and organic carbon distribution mainly contributed to quantitative microbial biomass distribution in a silty loam soil. These two types of colloids may have beneficial functions with respect to microbial survival due to their capacity to provide and buffer nutrient supplies to microorganisms closely adhering to their surfaces, to modify cell metabolism due to charged surfaces, or to protect them from protozoan predation and desiccation [6, 27, 30, 33–36].

Genetic Structure of Bacterial Pools and Role of Soil Characteristics in the Relationships between Pools

Complex RISA profiles in terms of the number of bands as well as in the relative intensity, were observed in the unfractionated soil (Fig. 2) as reported by Borneman and Triplett [4] in forest soils. To various extents, such a complexity was also observed among the studied microenvironment profiles suggesting that a large diversity also exists at a microenvironmental scale. However, the fingerprinting approach used in this study may not be appropriate for an estimation of diversity in terms of richness and eveness since (i) only the most abundant populations were represented in a community profile, (ii) several sequences are certainly contained in one RISA band [9], and (iii) a single organism could be represented by several RISA bands [14]. Such observations led us to limit our study to a comparative analysis of the genetic structure of the whole soil community and the microenvironment pools.

Differences in the presence versus absence of bands as well as differences in band intensity between profiles (Fig. 2) might result from differences in population composition and/or their rank abundance. Comparison of the microenvironment profiles to the unfractionated soil one allowed us to detect new bands as well as higher intensity of some bands in the former ones (Fig. 2), suggesting the preferential location of some populations. Previous works on culturable bacteria reported similar observations by comparing the proportion of particular functional or taxonomic groups in the outer or inner part [12], or in different size fractions of a soil [16, 17, 19]. Studies on the distribution of *Azospirillum* populations, identified by colony hybridization, not only showed that some species were found in particular microenvironments but also that some species could not be detected in unfractionated soil [Jocteur Monrozier, personal communication]. These observations suggest that fractionation could contribute to detect minor populations undetectable by treating the unfractionated soil or without any previous enrichment step. Consequently, it could provide a more accurate view of the bacterial composition of indigenous soil communities than the current view deduced by considering only the whole soil.

Cluster analysis and multivariate analysis were adapted to such fingerprint profiles to highlight the genetic relationships between different pools and to assess their contribution to the observed genetic structure of the whole soil community. The dendrograms from cluster analysis and the results from the principal component analysis (Fig. 3) revealed a close genetic structure between the inner part and the unfractionated soil that can be explained by the important contribution of the inner part to the bacterial densities (87.6% of the AODC and 95.4% of the VH bacteria) (Table 1). Differences were observed in the genetic structure of the bacterial pools associated with both soil parts (ordination on PC2). Previous works of Hattori [12] and more recently Ranjard et al. [25] reported preferential location of culturable bacterial populations with a higher proportion of Gram-positive bacteria in the outer part than in the inner one. The genetic structure of the bacterial pool in the inner part was found closer to the pool of the two microaggregate

(50–20 μm and 20–2 μm) and the dispersible clay fractions than to the pool of the two macroaggregate (2,000–250 μm and 250–50 μm) fractions (ordination on PC1). This can be explained by the important proportion of bacteria located in the former ones (about 80% of the recovered inner part bacteria). As observed for the quantitative bacterial distribution (Table 1), PCA ordination showed that bacterial pools inhabiting macroaggregate fractions (2,000–250 μm and 250–50 μm) exhibit the most different genetic structure compared to the whole soil one (Fig. 3). Consequently, our results highlighted the strongest contribution of the pools associated with the microaggregate (50–20 μm and 20–2 μm) and dispersible clay fractions and the outer part to the observed genetic structure of the whole soil community. Genetic relatedness between pools has also been investigated with an ARDRA fingerprinting approach and showed a similar clustering (data not shown) emphasizing the validity of our interpretations.

Statistical analysis of profiles can be informative about the possible influence of soil physicochemical characteristics on the genetic relationships of pools located in the different microenvironments. Cluster and PCA ordination on PC1 grouped bacterial pools located in microenvironments exhibiting similar structural and granulometric characteristics, i.e., the two macroaggregate fractions (2,000–250 μm and 250–50 μm) are closely related as are the two microaggregate fractions (50–20 μm and 20–2 μm) and the <2 μm fraction with the outer part. In addition, correlation between the scores plotted on PC1 and clay and organic carbon contents has been found (Table 3). Therefore, aggregate and particle size but also clay and carbon content are involved in the relatedness between bacterial pools. Bacterial activities such as organic carbon mineralization, respiration, and denitrifying activities were also reported to be influenced by these soil parameters considering a similar microscale level [3, 6, 19, 20]. However, the influence of other soil characteristics would require further investigation, since PC2, which accounted for 20% of the variability, was unexplained by the studied characteristics.

Whether or not these findings could be extrapolated to other times and space samplings from the studied soil or to different soil types would need investigation. We may hypothesize that in microaggregate fractions (50–20 μm and 20–2 μm), our observations are likely to persist over space and time since these microenvironments were often referred as confined sites, whatever the soil type, in which bacterial cells are protected against environmental stress [5, 36]. On the contrary, the genetic structure of pools associated with macroaggregate fractions (2,000–250 μm and 250–50 μm) could be modified over time and space because of higher fluctuation of environmental conditions (water, nutrient and aeration status, toxicant input . . .) [12, 26]. The comparison of bacterial distribution between microenvironments in different soil types could be of great interest to confirm the influence of clay and organic matter, but also to investigate the influence of different types of clay and soil organic matter.

Band Implications for Relationships between Bacterial Pools

PCA analysis highlighted the involvement of each band in the discrimination of bacterial pools (Fig. 4). Such correspondence between bands and microenvironments would be difficult to achieve without statistical analysis because of the complexity of the fingerprint profiles. However, the RISA fingerprinting approach is limited by the lack of relationships between the size of the band and a precise species or genus identification (different-sized bands can derived from phylogenetically and/or functionally similar organisms and the opposite) [9, 14]. Consequently, RISA profiles provide a facile means to determine differences in microbial community structure, but fail to provide insight into the ecological significance of these differences. The RISA approach would need to be associated with further analyses aiming to establish correspondence between discriminant bands and bacterial taxa in order to elucidate the relationship between the occurrence of particular bacterial populations and specific surrounding conditions in a microenvironment. Various strategies such as profile hybridization with group- or species-specific probes or band isolation, sequencing, and comparison to known sequences could be adapted for such determination [1, 27]. Up to now, the strategies mentioned above are limited because of the incomplete sequence database corresponding to these ribosomal regions. An alternative would be to coamplify a part of the *rrs* sequence in order to use previously validated group- or species-specific probes or to identify sequences by comparing with a robust database. However, efforts must focus on the sequencing of the intergenic region because of its high potential in discriminating closely related species [2, 11, 22].

The results of this study showed that the RISA approach combined to cluster analysis and PCA can be a valuable tool to compare the structure of bacterial pools at a microscale and to assess their contribution to the whole soil community. Differences in cell density and genetic structure between microenvironments demonstrated a heterogeneous

quantitative and qualitative distribution of bacteria that might be related to structural organization and colloid contents. This microscale bacterial heterogeneity raises the question of interaction between bacterial populations inhabiting the different microenvironments. Then, the next challenge for microbial ecologists will be to detect the level of interactions between bacterial populations at the microscale. Knowledge of the scale of interactions would contribute to better use the term "community" according to the ecological definition: "A group of organisms that live alongside one another, and in which the different species and individuals interact with one another" [32].

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