Applied and Environmental Microbiology, Jan. 2008, p. 526–530 Vol. 74, No. 2
0099-2240/08/$08.00+0 doi:10.1128/AEM.00843-07
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Actinobacterial Community Structure in Soils Receiving Long-Term Organic and Inorganic Amendments▽†

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Received 13 April 2007/Accepted 1 October 2007

The impact of long-term organic and inorganic amendments on the actinobacterial community in soils was studied. Denaturing gradient gel electrophoresis patterns based on the V3 region of 16S rRNA suggested that there was no significant difference between the communities occurring in the different amendments. However, analysis of the clone libraries of the actinobacterial communities by the use of multiple statistical approaches showed that these communities were significantly different from each other. Results showed that long-term organic and inorganic soil amendments did not significantly alter the overall phylogenetic diversity of the actinobacterial communities but did significantly change the community structure.

Actinobacteria, ubiquitously found in terrestrial (7, 9, 20), freshwater (1, 18), and marine (3, 15, 25, 29) ecosystems, are dominant soil bacterial taxa (9, 10) and probably play multiple roles in the environment (8, 14, 17, 19, 27). Applying organic amendments to soil is a common agronomic practice that likely has a significant impact on the diversity and community structure of actinobacteria. Extensive work has been directed toward understanding the effects of soil amendments on general rather than group-specific soil microbial communities. The purpose of the present study was to compare the community structure and diversity of actinobacteria in untreated soil with those of actinobacteria in soil treated with manure for 25 years.

The site of the long-term experiment, established in 1980, is in Suzhou (31°32′45″N; 120°41′57″E), Jiangsu Province, China. The site was initially selected on the basis of its relatively uniform crop growth, the flatness of the soil surface, and the relatively uniform soil fertility, as evidenced by the levels of total nitrogen, phosphorus, and potassium measured in 1980. Rice-wheat rotation, a typical cropping system used for more than 1,500 years in the area (4, 5), was adopted (with only six exceptional crops over 25 years) during the experimental period. The total experimental area was 1,600 m^2, which was split into 42 plots (14 treatments in triplicate), each with 20 m^2 of effective cropping area. The plots were separated by cement plates extending from 50 cm beneath to 15 cm above the soil surface to limit the mixing of water between plots. Separate ditches for irrigation or drainage were designed to ensure uniform control of the water regime. All field agronomic practices were performed manually. The soil is clay loam in texture. Soil fertility variables measured in 2004 (23) indicated that triplicate plots within each treatment were reasonably uniform.

A control and four treatments were included in the present study: (i) CK, in which the soil remained unamended (the control); (ii) NPK, in which the soil received N, P, and K fertilizers in combination; (iii) SN, in which the soil received straw plus N; (iv) M, in which the soil received pig manure only; and (v) MNPK, in which the soil received N, P, and K in addition to pig manure. Additional information on the field plots can be found elsewhere (23). Ten soil cores (15 cm in height by 8 cm in diameter) randomly distributed within each 20-m^2 plot were sampled, combined, sieved to remove the roots, and brought on ice to the laboratory, where the total DNA was extracted. The soils were sampled twice during the wheat growing season, on 8 April 2006, when wheat was in the early earing stage, and on 1 July 2006, after the wheat harvest (rice transplanting was delayed in this particular year).

For DNA extraction, 2 g of soil was repeatedly homogenized by vortexing it in 20 ml phosphate-buffered saline and centrifuged at 200 × *g* for 2 min. Microbial cells in the combined supernatant liquid were collected by centrifugation at 12,000 × *g* for 10 min, washed three times with TENP buffer (50 mM Tris, 20 mM EDTA, 100 mM NaCl, 1% polyvinylpyrrolidone, pH 10; 1-liter volume), and lysed by bead beating. Briefly, the tubes containing the cells, 0.3 g of 0.1-mm zirconium beads, and 150 μl redistilled phenol (pH 8.0) were agitated on a bead beater (Mini-Bead-Beater-8; Biospec Products) at the highest speed for 80 s and then allowed to sit on ice for 1 min. This was repeated twice more for a total of three runs per sample. Next, 110 μl of sodium dodecyl sulfate (10%) was added and gently mixed, and the sample was incubated on ice for 10 min. After this, 150 μl chloroform-isopropanol (25:1, vol/vol) was added, gently mixed, and then centrifuged at 15,000 × *g* for 10 min. The supernatant liquid was collected, and 1/10 volume of 3 M sodium acetate and 1 volume of phenol were added, followed by centrifugation at 15,000 × *g* for 10 min. The supernatant was then extracted twice with chloroform-isopropanol (24:1, vol/vol). Nucleic acids in the supernatant were precipitated with cold ethanol. The size of the extracted DNA, as deter-

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 12 October 2007.

mined by electrophoresis on a 0.5% agarose gel, was found to be ~10 to 15 kbp.

The V3 region of the 16S rRNA gene was amplified for denaturing gradient gel electrophoresis (DGGE) analysis using primers P2 and P3 with a 40-bp GC clamp at the 5′ end of P3 (16), as previously described (13). The amplified products were separated with a Dcode system (Bio-Rad, Hercules, CA) in an 8% (wt/vol) polyacrylamide gel containing a linear, 35 to 60% denaturant gradient. Electrophoresis was carried out using 1× Tris-acetate-EDTA buffer at 200 V and 60°C for 200 min. The DNA bands were stained with Sybr green (Amresco, Solon, OH) and photographed with a UVI gel documentation system (UVItec, Cambridge, United Kingdom). The images were analyzed with Quantity One software version 4.4 (Bio-Rad, Hercules, CA). A dendrogram of the bands was constructed based on the Dice similarity coefficient using the unweighted-pair group arithmetic average clustering algorithm.

For clone library construction, actinobacterial 16S rRNA genes were amplified with primers S-C-Act-0235-a-S-20 and S-C-Act-0878-a-A-19 by use of previously described PCR conditions (26). The amplified products were purified and concentrated with the UltraClean 15 purification kit (Mo Bio, Inc.), ligated with T4 DNA ligase into a pGEM-T Easy vector (Promega), and electrotransformed into competent *Escherichia coli* DH5α cells according to the manufacturer's instructions. The transformed cells were plated onto LB agar containing ampicillin (100 μg ml^{-1}; Amresco), isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 μg ml^{-1}). Positive colonies were screened by PCR using the amplifying primers. The plasmids containing the correct inserts were sequenced commercially with an ABI 3730 DNA analyzer (Applied Biosystems) using the S-C-Act-0235-a-S-20 primer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.). The sequences were double checked for chimeras by use of both the Chimera Detection program from the Ribosomal Database Project II (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su = SSU) and Mallard version 1.02 (2).

Actinobacterial 16S rRNA gene sequences were aligned using the CLUSTAL X interface (28). Operational taxonomic units (OTUs) were identified with DOTUR 1.53 (21) at a DNA distance cutoff of 0.01. The closest representative for each OTU sequence was identified using BLASTn. Phylogenetic neighbor-joining trees were constructed with MEGA version 3.1 (12) using a Jukes-Cantor model. Libraries were compared using the LIBSHUFF program (24) by treating each cloned sequence as a separate sample. Two nonparametric richness estimators (S_{ACE} and S_{Chao1}), calculated with web-based software (11), were used to estimate whether the libraries were large enough to yield stable phylotype richness estimates. Nonparametric estimators of the fraction and richness of OTUs shared between two communities were analyzed using SONS software (22). The phylogenetic diversity within each community was estimated using DOTUR 1.53, SPADE v2.1 (A. Chao and T.-J. Shen [http://chao.stat.nthu.edu.tw/softwareCE.html]), and Arlequin v3.01 (6).

The DGGE patterns of the 16S rRNA amplified from all of the treatments were generally similar (Fig. 1). The most notable differences were the band intensities rather than the band positions. The dendrogram generated from the bands showed

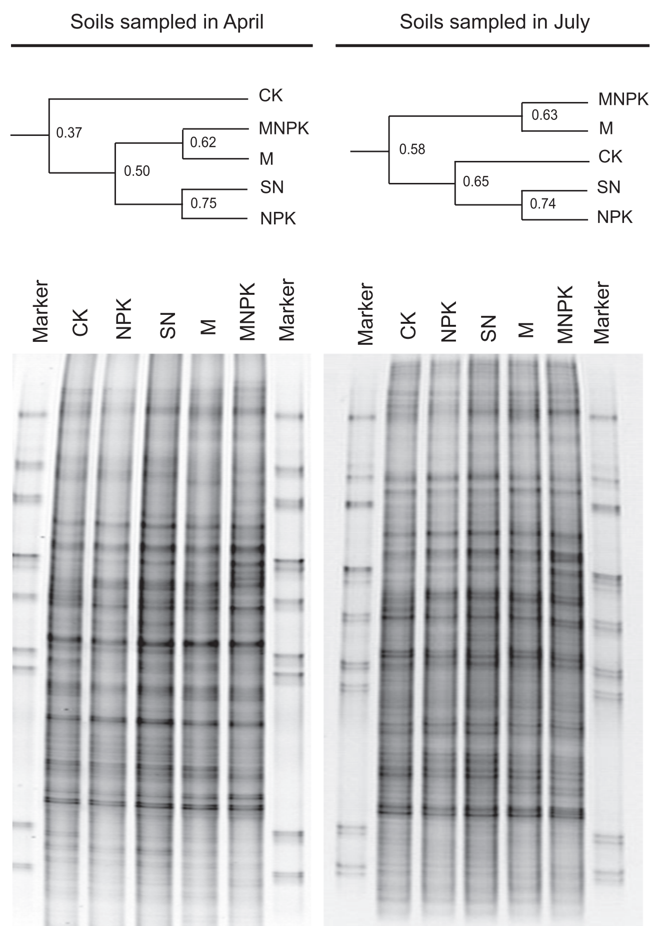

FIG. 1. DGGE patterns of soil samples treated with CK, NPK, M, NPK, and SN based on the sequence of the V3 region in the 16S rRNA gene. See the text for descriptions of treatments.

that the April soils could be placed into three clusters, representing CK, manure-treated, and non-manure-treated soils. Similarly to the April soils, the July soils also showed similar DGGE patterns among the treatments (Fig. 1). Note that communities from the April and July samples could not be compared directly by band positions because they were from two separate gels. However, analysis of both the April and July gels generated three clusters that represented the same treatments, indicating that there was no substantial community change over time. CK, NPK, and MNPK soils were, therefore, chosen as the representative soils for further clone library analysis. Clone libraries were constructed from the July samples only.

Of the 247 clones in total, one was detected as a putative chimera and was set aside for further analysis, one belonged to the genus *Gemmatimonas*, and the remaining 245 were actinobacterial in origin. The numbers of OTUs derived from the different samples are listed in Table 1. The library size estimated by the method described in reference 11 showed that the S_{Chao1} and S_{ACE} richness estimators did not reach an asymptotic maxima but tended to converge into a narrow range that exceeded the unstable estimator-producing stage when the subsample sizes approach the actual library sizes (see Fig. S1 in

TABLE 1. Comparison of ecological and molecular estimates of sequence diversity for actinobacterial communities in differently treated soils

Community in soil with indicated treatment[a]	No. of unique sequences	No. of OTUs	Value determined by DOTUR[b]			$H_{Jackknife}$ value determined by SPADE[c]	Value determined by Arlequin[d]		
			$H_{Shannon}$	S_{Chao1}	C_{ACE}		Gene diversity	Nucleotide diversity	$\theta_{(\pi)}$
CK	61	46	3.7 (~3.5 to 3.9)	77 (~59 to 123)	89 (~64 to 146)	4.29 (0.11)	1.0 (0.003)	0.12 (0.06)	76.1 (36.8)
NPK	84	53	3.8 (~3.6 to 3.9)	101 (~73 to 170)	106 (~77 to 172)	4.27 (0.11)	1.0 (0.002)	0.11 (0.05)	72.0 (34.7)
MNPK	75	49	3.7 (~3.5 to 3.9)	89 (~65 to 148)	96 (~70 to 154)	4.16 (0.11)	1.0 (0.002)	0.11 (0.05)	71.6 (34.5)
CK+NPK+MNPK	214	106	4.3 (~4.2 to 4.5)	190 (~148 to 274)	194 (~156 to 263)	4.68 (0.08)	1.0 (0.0004)	0.11 (0.05)	72.7 (34.7)

[a] A "+" means that the libraries from the different soils were pooled.
[b] The OTUs are defined at a DNA distance of 0.01. The numbers in parentheses are the ranges at the 95% confidence interval. $H_{Shannon}$, Shannon diversity index.
[c] The OTUs are defined at a DNA distance of 0.01. The numbers in parentheses are standard errors. $H_{Jackknife}$, Jackknife diversity index.
[d] All clone sequences, each treated as an individual haplotype, were uploaded to the program. The numbers in parentheses are standard errors. $\theta_{(\pi)}$, molecular diversity index calculated on the basis of the number of nucleotide differences between two randomly chosen sequences from a population.

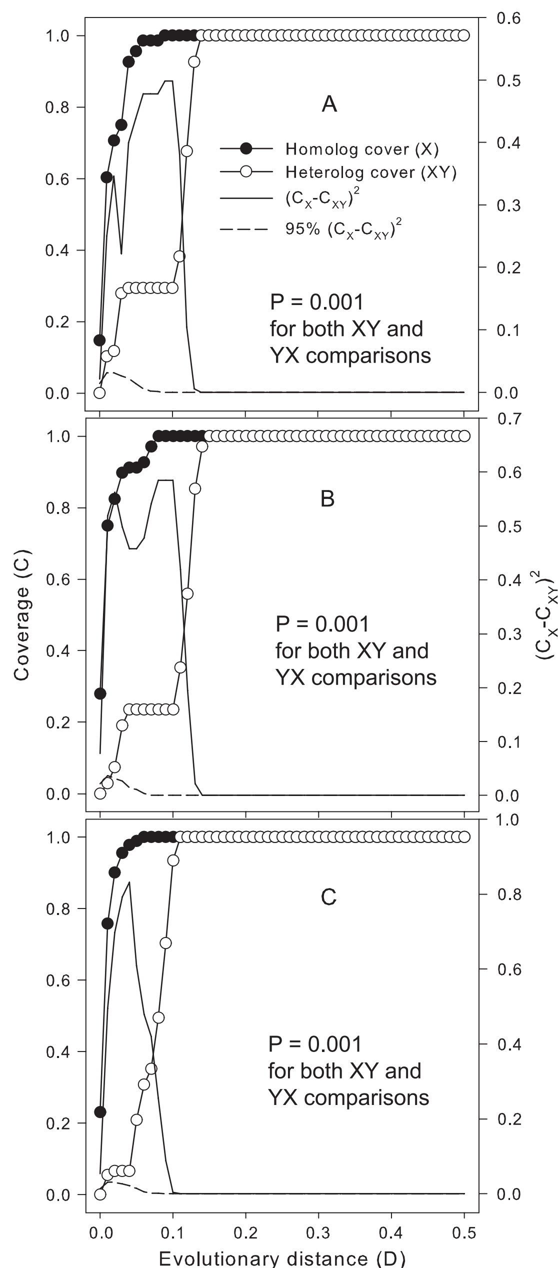

FIG. 2. LIBSHUFF comparison of the homologous and heterogenous coverages between the CK and NPK libraries (A), the CK and MNPK libraries (B), and the NPK and MNPK libraries (C). *X* represents the CK library in panels A and B and the NPK library in panel C. *Y* represents the NPK library in panel A and the MNPK library in panels B and C. The fact that the *P* values produced by both the *XY* and *YX* comparisons are smaller than the critical *P* value of 0.025 indicates that the two libraries being compared are different.

the supplemental material). S_{Chao1} and S_{ACE} richness estimator patterns were, in fact, very similar to the patterns that are generated from libraries not considered to have been exhaustively sampled but that were large enough to yield stable and unbiased estimates of phylotype richness (11). We therefore considered that the actinobacterial libraries were of sufficient size to allow valid comparisons.

LIBSHUFF (24) analysis of homologous and heterologous

TABLE 2. Nonparametric estimates of actinobacterial library comparisons using SONS at an OTU of 0.01

Libraries from soils with indicated treatment compared[a]		Estimated value[b]							
A	*B*	U_{est}	V_{est}	$S_{A,B\ Chao}$	J_{clas}	A_{shared}	B_{shared}	J_{abund}	θ_{YC}
CK	NPK	0.74 (0.16)	0.57 (0.11)	39	0.24	0.46	0.36	0.48 (0.10)	0.39 (0.09)
CK	MNPK	0.47 (0.15)	0.47 (0.12)	27	0.20	0.34	0.32	0.48 (0.09)	0.24 (0.07)
NPK	MNPK	0.50 (0.11)	0.85 (0.15)	46	0.25	0.38	0.42	0.48 (0.12)	0.38 (0.08)
CK+NPK	MNPK	0.50 (0.11)	0.84 (0.12)	49	0.25	0.33	0.54	0.45 (0.11)	0.36 (0.07)
CK+MNPK	NPK	0.81 (0.14)	0.74 (0.11)	63	0.29	0.38	0.55	0.63 (0.12)	0.49 (0.08)
CK	NPK+MNPK	0.77 (0.12)	0.54 (0.11)	42	0.25	0.55	0.31	0.46 (0.09)	0.36 (0.08)

[a] A "+" means that the libraries from different soils were pooled.

[b] The numbers in parentheses are standard errors. The detailed definitions and calculations of the estimates are described by Schloss and Handelsman (22). U_{est} and V_{est}, the fractions of sequences from libraries *A* and *B*, respectively, that belong to a shared OTU; J_{clas}, the classic Jaccard similarity index; A_{shared}, the ratio of shared OTUs to the total number of OTUs in library *A*; B_{shared}, the ratio of shared OTUs to the total number of OTUs in library *B*.

coverage (*C*) curves indicated that the three libraries were significantly different from each other, as the *P* values produced by both *XY* and *YX* comparisons (where *X* and *Y* represent two different libraries) were all 0.001, smaller than the critical *P* value of 0.025 (Fig. 2). These results suggested that most sequences in the libraries were different. Significant differences between $(C_X - C_{XY})^2$ and 95% of $(C_X - C_{XY})^2$ values were found only at a distance (*D*) of less than 0.10, indicating that the libraries differed greatly at a *D* of <0.10. At a *D* of >0.10, both $(C_X - C_{XY})^2$ and 95% of $(C_X - C_{XY})^2$ values were virtually zero, suggesting that deep phylogenetic groups were not found in the libraries (see Fig. S2 in the supplemental material).

The diversity indices generated for each individual community by DOTUR (21), SPADE, and Arlequin (6) were very similar (Table 1), suggesting that soil amendments did not significantly change the diversity levels of the actinobacterial communities. When the communities were pooled, most of the estimators did not change significantly. Species richness (S_{Chao1}) and coverage (C_{ACE}) values were the exceptions, since they were significantly higher for the pooled library than for the individual libraries. These results indicated that one library contained a fraction of the species that were not found in the other individual libraries, which is further supported by SONS analysis.

Nonparametric estimates obtained by SONS analysis (22) are listed in Table 2. The J_{abund} value, defined as the probability that a randomly selected OTU is found in the libraries under comparison, is a measure of community overlap. In our case, all J_{abund} values between any two individual communities under comparison were low and were significantly different from 1.0 (Table 2), suggesting that the communities were different from each other. This is further supported by low θ_{YC} values (Table 2), measures of community structure similarity, as well as by LIBSHUFF results (Fig. 2). When two libraries were pooled and compared with a third one, the resulting estimates of J_{abund} values were also low and significantly different from 1.0. When CK was compared with NPK, the J_{abund} value, for example, was 0.48. When CK was pooled with MNPK (CK+MNPK) and compared with NPK again, the J_{abund} value was 0.63, higher than 0.48 but still significantly different from 1.0. These results indicate that the pooled community did not result in a significant increase in overlap.

By combining the $S_{A,B\ Chao}$ values in Tables 1 and 2 (where $S_{A,B\ Chao}$ is the richness estimator of the number of shared OTUs between libraries *A* and *B*), a Venn diagram was generated (Fig. 3). The numbers in each section or intersection of the diagram are nonparametric $S_{A,B\ Chao}$ values. The fraction of $S_{A,B\ Chao}$ values in each intersection over the S_{Chao1} value of a specific library could be roughly viewed as the fraction of shared OTUs between the two libraries concerned. The CK library, for example, contained approximately 49% (38/77) unique OTU sequences, 16% (12/77) shared with the NPK library, 0% shared with MNPK, and 35% (27/77) shared with pooled NPK and MNPK libraries. The central intersection in Fig. 3 labeled "27" accounted for roughly 30% of the mean $S_{A,B\ Chao}$ value of the three libraries (27/[(77 + 101 + 89)/3]). The fraction of OTUs that were not shared with any other communities are probably the OTUs that could be changed easily in response to soil amendments. In contrast, the fraction of OTUs shared by all three communities are probably the OTUs that are unaffected by amendments.

In summary, our results showed that DGGE patterns based

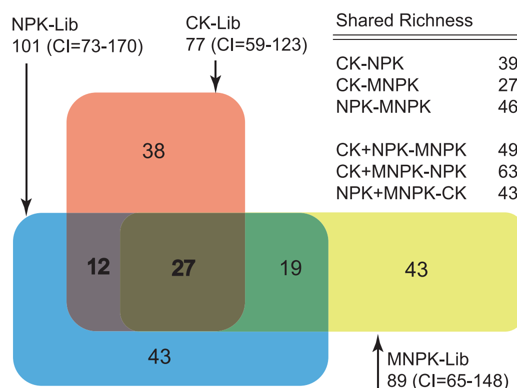

FIG. 3. A Venn diagram comparing the $OTU_{0.01}$ (OTU assigned at a DNA distance of 0.01) memberships found in the CK (n = 68), NPK (n = 91), and MNPK (n = 89) libraries. Below each library's name are the Chao1 richness estimate and the 95% confidence interval estimated by DOTUR for the respective community. The richness of the overlapping regions was estimated based on the pairwise $S_{A,B\ Chao}$ richness estimates shared by the three communities and by pooling two communities and estimating the fraction shared with a third community. The Chao1 richness estimate for the three libraries pooled together was 190 (confidence interval, 148 to 274), and the sum of the richness estimates for the individual sectors in the diagram was 182.

on the V3 region of 16S rRNA gene fingerprints were remarkably similar among the soils receiving long-term amendments. However, actinobacterium-specific clone libraries showed that the communities were significantly different from each other. With the aid of multiple statistical approaches, it was shown that long-term organic and inorganic soil amendments did not significantly alter the phylogenetic diversity of the actinobacterial communities but did significantly change the community structure. These results demonstrated the advantage of group-specific community analysis over general community analysis in revealing the impact of soil amendments on highly complex soil communities. Our results also demonstrated the usefulness of multiple statistical approaches in revealing community structure and diversity.

Nucleotide sequence accession numbers. The nucleotide sequences derived from this study have been deposited in the GenBank database under accession numbers EF134967 to EF135072.

We are grateful to M. X. Shen and his group for maintaining the long-term field plots and the sampling. S. Y. thanks James P. Shapleigh for his comments and help in revising the text. The comments from two anonymous reviewers are appreciated.

Financial support from the National Science Foundation of China (40471072), the National Key Basic Research and Development Program of China (2005CB121108), and the Knowledge Innovation Program of the Chinese Academy of Sciences (KZCX2-YW-407) is acknowledged.

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