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Full Length Research Paper

The effects of aluminum stress on bacterial community diversity in acidic red soils by polymerase chain reaction (PCR)-amplified restriction fragment length polymorphism

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The soil bacterial communities in response to aluminum (Al) treatments were evaluated by polymerase chain reaction-amplified restriction fragment length polymorphism (PCR-RFLP) of 16S rRNA genes in acidic red soil samples in this study. A total of 6 bacterial communities were sampled from two representative soil types under different Al-treated concentrations. Bacterial genomic DNA was extracted and nested PCR-amplified to obtain 16S rDNA fragments which were cloned to construct 6 16S rDNA libraries. Clones of each library were selected randomly for PCR-RFLP analysis of rDNA fragments, and eventually 60 genotypes were identified by RFLP fingerprinting. These 60 genotypes were sequenced and their respective phylotype was identified through the Basic Local Alignment Search Tool (BLAST) of NCBI (similarity 93 to 100%) and phylogenetic analyzed. The phylotype richness, frequency distribution (evenness) and composition of the clone libraries were investigated by using a variety of diversity indices. Among these phylotypes, 96.7% belonged to the genera *α*-, *β*-, γ-, δ-Proteobacteria, Acidobacteria, Cytophaga-Flavobacteria-Bacteroides (CFB group), green nonsulfur (GNS) bacteria, Gemmatimonadetes, high seed sludge (GC) Gram-positive and *Nitrospira*. Sequence analyses revealed that 56.7% (34) of clone sequences were similar to those of uncultured soil bacteria in the environment. In addition, the bacterial diversities and compositions were clearly displayed in different soil samples. More genera were discovered in A_0 soil sample than any other, and some special species, such as *Nitrospira*, disappeared in Al-treated soils.

Key words: Acidic red soil, aluminum stress, soil bacteria, 16S rDNA, restriction fragment length polymorphism (RFLP).

INTRODUCTION

Acidic red soils, which contain low pH, high level of monomeric aluminum (Al) and low content of organic matter, are one of the agriculturally important soils in subtropical areas of China. Al appears in the 3^+ oxidation state and Al minerals are almost insoluble when soil is weakly acidic or neutral (Kochian et al., 2004). As the pH decreases, this element becomes more soluble in the soil solution and toxic to the living organisms (Amonette et al., 2003; Zheng et al., 2004). Adequate and continuous applications of lime and farm-yardmanure are known to have effects on reducing the toxicity of Al stress (Meng et al., 2004). However, Al toxicity in the subsoil restricts the development of crop root system. Due to acidic

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deposition and strong rain leach, the concentration of monomeric Al in the soil solution becomes higher and higher in recent years (Driscoll et al., 2003). Guida et al. (1991) found that the growth inhibition of *Escherichia coli* was markedly dependent on pH, recording its sensitivity to 0.9 and 2.25 mM Al^{3+} at pH 5.4 and 6.6, respectively. In addition, the continual enrichment of Al^{3+} directly resulted in the change of the soil microbial ecosystem and the decrease of soil quality (Wood, 1995). Numerous toxic effects of Al^{3+} had been demonstrated in a great number of investigations (Fischer et al., 2002; Amonette et al., 2003; Papathanasiou et al., 2011), while few studies on the effects of Al^{3+} on the soil microbial community composition were carried out.

Soil is a complex ecosystem in which microorganisms occur in heterogeneous communities. Long-term application of lime and fertilizer will change the original soil ecological environment in the agricultural soils (AGR soils) (Mota et al., 2008). In contrast, forest soils (FOR soils) were able to maintain a unique red soil ecosystem. Since the microbial community is an integral component of soil quality, it is essential to assess its different responses when Al^{3+} is added continually to soil. Analysis of bacterial communities and soil diversity was traditionally focused on how to cultivate microorganisms from the environment. The technique of such a traditional analysis is very limited because of the inability of a wide range of bacteria to be efficiently cultured in the laboratory (Torsvik et al., 2002). Otherwise, Given that the culture-independent methods for soil bacteria are time-consuming and expensive; they do not exactly reflect the construction of natural microbial bacteria compositions (Moter and Ulf, 2000), thus, various molecular techniques including restriction fragment length polymorphism (RFLP) (Lajbner and Kotlik, 2011), fluorescence *in situ* hybridization (FISH) (Pernthaler et al., 2002), denaturing gradient gel electrophoresis (DGGE) (Brons and Elsas, 2008), temporal temperature gradient gel electrophoresis (TTGE) (Alejandra et al., 2005), random amplification of polymorphic DNA (RAPD) (Giancarlo et al., 2005), terminal-RFLP (T-RFLP) (Schutte et al., 2008) and the immunological method (Schloter et al., 1995) have been applied to learn about more comprehensive and precise characterizations of bacteria in soil environments in recent years.

In this study, we applied 16S rDNA genes as an operational taxonomic unit (OTU) to bacterial community analysis, and used RFLP patterns generated by restriction enzyme digestion and sequences to perform bacterial clone library analysis. The fingerprints provided a graphical representation of the composition of the bacterial assemblages in order to allow for a rapid visual comparison of samples varying under different Al concentrations. The objective of this study was to analyze the microbial community diversity under different concentrations of Al stress, and compare the differences in bacterial diversity between AGR soil sample and FOR soil samples by RFLP in acidic red soils.

MATERIALS AND METHODS

Sample collection

The studied soils represent a typical red forest acidic soil (that is, FOR soil) collected from the middle section of the Lohsiao Mountain range and an adjacent agricultural field (that is, AGR soil) that was less than 200 m apart. Both sites are located at the foot of the Chingkang Mountains in Jiangxi Province, Eastern China (27°06′ N, 115°01′ E, 83 m altitude; a wettish area of continental subtropical monsoon with mild climate, abundant rainfall and four distinct seasons). The FOR soil had been isolated from anthropogenic intervention for more than 100 years. The site is occupied mainly by shrubs. The AGR soil was in the continuous cultivation of single-cropped rice under conventional tillage (one moldboard plowing and two diskings every year) for more than 10 years. Two experimental soils were sampled at a depth of 2 to 20 cm. After it was transported to the laboratory, the soil was air-dried, ground, passed through a 2 mm mesh and stored as the stock sample for test. Some important properties of the soils studied are shown in Table 1.

Experimental treatments

The soil samples were adjusted to 60% water-holding capacity and preincubated at 28°C for 7 days in 250 mL glass conical flask. Al was added to the soil as an aqueous mixture of salt $AlCl_3{\cdot}6H_2O$. The concentrations of Al^{3+} were 50 and 100 mg kg^{-1} of dry weight soil. The control received distilled water. All the tested soil samples were designated F_0 (FOR soil, 0 mg/kg Al^{3+}), F_1 (FOR soil, 50 mg/kg Al^{3+}), F_2 (FOR soil, 100 mg/kg Al^{3+}) and A_0 (AGR soil, 0 mg/kg Al^{3+}), A_1 (AGR soil, 50 mg/kg Al^{3+}), A_2 (AGR soil, 100 mg/kg Al^{3+}). After thorough mixing, the treated soils were incubated at 28°C in climate-control chamber, maintaining the same moisture content for 30 days. After 30 days, the monomeric Al concentrations in soil samples were tested by pyrocatechol violet (PCV) colorimetric method (Zheng et al., 2004) and showed in Table 2.

DNA extraction

DNA extraction was performed according to the procedure described by Zhou et al. (1996) with small modifications. About 10 g aliquots of each of the 6 soil samples was suspended in 10 ml DNA extraction buffer [100 mM Tris HCl, 100 mM Na_2 ethylenediaminetetraacetic acid (EDTA), 100 mM Na_3PO_4, 1.5 M NaCl and 1% cetyltrimethylammonium bromide (CTAB) at pH 8.0; containing 100 µL proteinase-K (10 mg mL^{-1}; Sangon) and 1 mL lysozyme (60 mg ml^{-1}, Sangon], and incubated at 37°C for 30 min. Then, 1.5 mL of 10% sodium dodecyl sulfate (SDS) was added to the mixture, and incubated at 65°C for 2 h. Three cycles of freezing at 80°C for 20 min and thawing in a 65°C water bath for 30 min were followed to release DNA from the microbial cells. 1.0 mL of the DNA solution was taken to a microtube, added with 1 mL of phenolchloroform- isoamylalcohol (25:24:1) mixture, shaken gently, and centrifuged at 12,000 g for 5 min. The supernatant phase was transferred to a new microtube, added with the same volume of chloroform-isoamylalcohol (24:1) mixture, and again centrifuged at 12,000 g for 5 min. The aqueous phase was transferred to a new tube containing same volume of isopropanol, and kept for 10 min statically at room temperature. The pellet was obtained by centrifugation at 12,000 g for 20 min. After the supernatant was removed, 1 mL of 70% cold ethanol (-20°C) was added and centrifuged at 12,000 g for 2 min. Then the aqueous phase was removed and the precipitate was dried up. The extracted DNA was dissolved in 50 mL TE (10 mM Tris HCl, 1 mM Na_2EDTA, pH 8.0).

Table 1. The basic physicochemical properties of studied soil.

Parameter[a]	FOR soil	AGR soil
pH	4.46	6.23
OM/(g·kg^{-1})	17.8	80.0
AN/(mg· kg^{-1})	127.5	1066
AP/(mg·kg-1)	4.05	58.7
AK/(mg·kg^{-1})	48.5	198.5
Fe_2O_3/(mg·kg^{-1})	21.4	10.3
SiO_2/(mg·kg^{-1})	40.6	39.8
Monomeric Al (μmol/L)	42.6	6.8
EC /(dS·m^{-1})	0.15	5.25

aOM, organic matter; AN, available N; AP, available P; AK, available K; EC, Electrical conductivity.

Table 2. The monomeric Al concentrations in the soil samples.

Parameter	F_0	F_1	F_2	A_0	A_1	A_2
Monomeric Al (μmol/L)	42.6	44.9	50.3	6.8	7.4	9.3
Monomeric Al (μmol/L, after 30 days)	38.3	40.6	49.1	5.2	6.6	8.7

PCR amplification of 16S rDNAs and cloning

Two rounds of PCR (nested-PCR) were performed. The first round was carried out in a final volume of 25 μL with 1 μL (1 ng) extracted DNA as template, 0.5 μL each of the primers (10 μM), 2 μl deoxyribonucleotide triphosphate (dNTP) mixture (2.5 mM), 1.5 μL $MgCl_2$ (25 mM), 2.5 μL 10 × PCR reaction buffer and 0.2 μL *Taq* DNA polymerase (5 units/μL, Takara). The primers in the first round were 27f (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492r (5’-TACCTTGTTACGACTT-3’). The thermocycler program was 5 min at 94°C for initial denaturation, 10 cycles of 30 s at 94°C, 40 s at 55°C and 1 min at 72°C, followed by a 10 min final elongation at 72°C. For second round, the first round PCR products were used as template and the primers were: 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1389r (5'-ACGGGCGGTGTGTACAAG-3'), which could specifically anneal to the PCR products of the first round. In this round, 30 cycles was set and other conditions were much the same as those of the first round. The nested-PCR products were visualized on 1% ethidium bromide (EB) agarose gel and the bands over 1300 bp should be the target products according to the experimental design.

Clone libraries were created from 16S rDNA sequences amplified from extracted DNA. PCR amplicons approximately 1.3 kb long were ligated into the pMD18-T plasmid vector (TaKaRa.) and transformed into *E. coli* DH5α competent cells. About 200 positive colonies of each sample were randomly selected and rDNA inserts were confirmed by colony-PCR with two vector primers M13F (5’-CGC CAG GGT TTT CCC AGT CAC GAC-3’) and M13R (5’- GAG CGG ATA ACA ATT TCA CAC AGG-3’). Six (6) 16S rDNA libraries corresponding to each soil sample were thus, constructed. Clones were designated F_0, F_1, F_2 and A_0, A_1, A_2, followed by the clone number (1 to 215).

RFLP and sequence analysis

The 63F-1389R PCR-screened products of positive colonies were digested with two restriction enzymes (*Hha*I and *Rsa*I). Digested RFLP fragments were resolved via 2% (w/v) agarose gel (H, sangon), visualized by EB-staining, and photographed under ultraviolet (UV) illumination. Clones with common RFLP patterns were regarded as one OTU. A representative clone of each OUT was sequenced at Sangon Biotech (Shanghai) Co., Ltd., China. Then, the sequences were phylotyped by the Basic Local Alignment Search Tool (BLAST) (www.ncb.nlm.gov/blast/blast.cgi) and Ribosomal Database Project (RDP) classifier (http://rdp.cme.msu.edu/).

Statistical analysis

Library total clone number and OUT number were used to express compute library coverage (*C*; Giovannoni et al., 1995):

$C= [1-(n_1/N)]*100\%$.

Annotated: n_1 is the number of OTUs which appeared only once in a library; N is the library size; If C is less than 70%, the library should be rebuilt. To analyze the distribution of abundant taxa within libraries, groups were constructed using Mothur at a distance of less than or equal to 0.02 (Schloss et al., 2009). A dendrogram according to the sequences was constructed from the distance matrix using softwares clustal X 2.0 (Larkin et al., 2007) and Mega 4.0 (Tamura et al., 2007).

RESULTS

DNA extraction and PCR amplification of 16S rDNA genes

DNAs were extracted from all the 6 samplings and amplified with the used ribosomal primers by nested-PCR and analyzed by electrophoresis on a 1.0% (w/v) agarose gel in Tris-Borate-EDTA (TBE) buffer. All the samples

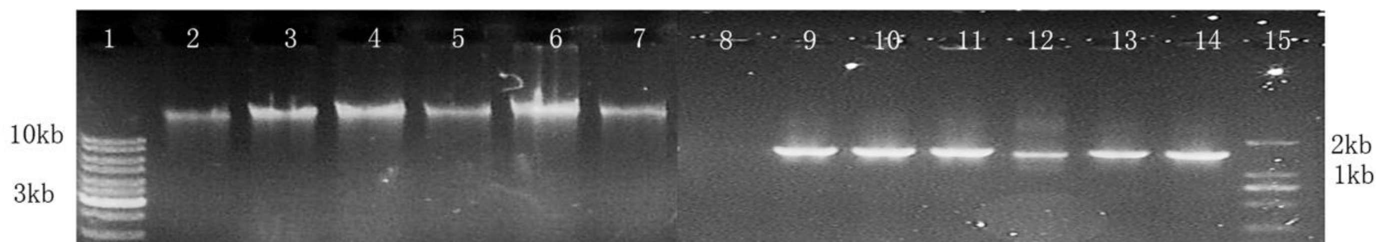

Figure 1. Soil DNA and PCR amplification of 16S rDNA fragments using primer pair 27f/1492r followed by nested primers 63f/1389r from template DNA. Lane 1 indicates 1 kb DNA ladder (Sangon); Lanes 2, 3, 4, 5, 6, 7 indicate soil DNA extracted from F_0, F_1, F_2, A_0, A_1 and A_2, respectively. Line 8 indicates PCR products amplified from control sample (ddH_2O); Lanes 9, 10, 11, 12, 13, 14 indicate PCR products amplified from template DNA F_0, F_1, F_2, A_0, A_1, A_2, respectively. Lane 15 indicates DL2000 DNA ladder (Takara).

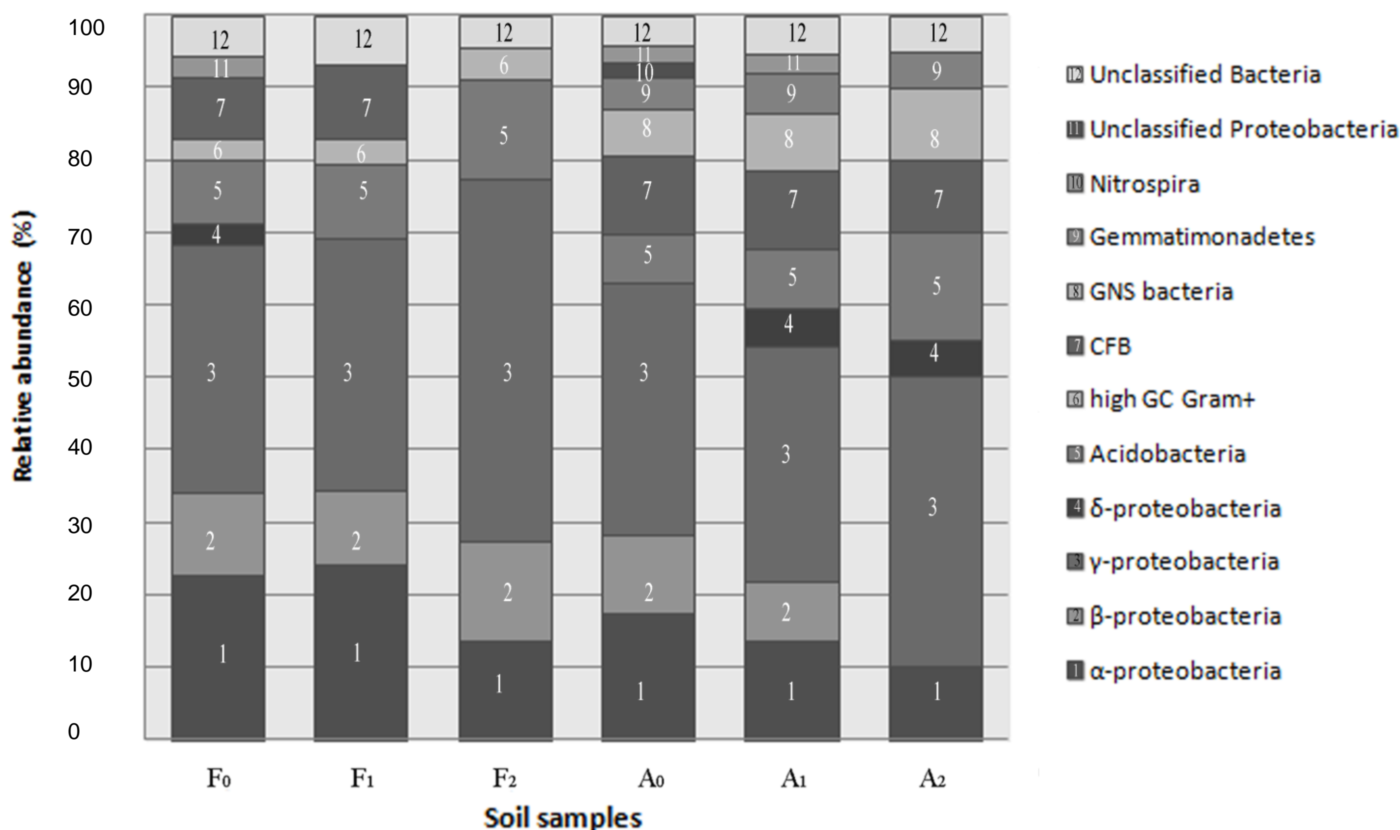

Figure 2. Relative abundances of dominant bacterial taxa in different Al-treated soil samples.

produced a single band corresponding to the expected size (Figure 1).

16S rDNA libraries construction and PCR–RFLP analysis

A total of 1241 16S rDNAs clones of 6 soil samples were analyzed by PCR-RFLP with restriction enzymes *Rsa*I and *Hal*I, whereby 60 unique RFLP fingerprints OTU were identified. Representative clones of each were sequenced. Sequences were submitted to DDBJ with the following accession numbers: AB679919-AB679978. Taxonomic classification with BLAST and RDP based on phylogenetic analysis of these sequences showed that 96.7% (58) of phylotypes belonged to 11 major clusters with similarity of 93 to 100% and 3.3% (2) of them to unclassified bacteria (Figure 2). The results showed that 24 phylotypes were closely matched to γ- Proteobacteria (Methylococcales, Pseudomonadales, Xanthomonadales and unclassified_γ- Proteobacteria), 11 sequences to α-Proteobacteria (Sphingomonadaceae, Caulobacterales,

Rhodospirillales and Rhizobiales), 5 sequence to Cytophaga-Flavobacteria-Bacteroides (CFB; Sphingobacteria), 5 sequence to β- proteobacteria (Burkholderia and unclassified_β-proteobacteria), 3 sequences to Acidobacteria (Acidobacteriales), 3 sequences to green nonsulfur bacteria (GNS; Chloroflexi), 2 sequences to δ- Proteobacteria (Myxococcales and unclassified_δ-Proteobacteria), 2 sequences to Gemmatimonadetes, 1 sequence to high seed sludge (GC) Gram-positive (Actinobacteria), 1 sequence to *Nitrospira and* 1 sequence to unclassified_Proteobacteria. As shown in Figure 2, γ-Proteobacteria was the most abundant group in all tested samples. Its relative abundances amounted to 34.3, 34.7 and 50.2% in FOR soils, and 34.9, 32.4 and 40.0% in AGR soils.

Across all the 6 samples, 34 (56.7%) sequences similar to the uncultured bacteria in all of the 60 sequences were obtained, which implied that a large part of potential species existed in the acidic red soils and a higher proportion was found in Al-treated soil samples. Their relative abundance ranged from 57.8 to 64.7% in FOR soils, and 51.6 to 67.6% in AGR soil. A dendogram was constructed according to the sequences of 16S *r*RNA genes of classified bacteria with a high similarity of 97 to 100% to visualize the phylogenetic relationships among them (Figure 3).

Changes of bacterial community diversity by Al stress in acidic soils

Coverages of 16S rDNA libraries ranged from 78.0 to 91.0% (Table 3 and Figure 4), indicating that the clone numbers in all samples were adequate for diversity analysis. Rarefaction analysis showed that 16S rDNA libraries could reflect the bacterial community diversity in all soil samples (Figure 4). Community bacterial composition and diversity affected obviously by Al stress (Tables 3 and 4). Diversity and evenness values generated from Simpson and Shannon indices indicated that Al addition lowered the soil microbial diversity and evenness (Table 3). Phylotype richness and diversity was the highest in A_0 soil sample. More genera were discovered in A_0 than any others, and some special species disappeared in Al-treated soil samples. δ- Proteobacteria appeared in F_0, and disappeared in F_1 and F_2. *Nitrospira* appeared in A_0, and disappeared in A_1 and A_2. CFB appeared in F_0 and F_1, and disappeared in F_2 (Table 4). Comparison on the bacteria composition between FOR and AGR communities was also performed. Members of the γ-Proteobacteria were the dominant groups in both FOR and AGR soils, accounting for more than 1/3 in FOR and AGR. Members of α-,γ- Proteobacteria and Acidobacteria were found in FOR and AGR communities in every Al-treated concentrations, while CFB was distributed in different Al-treated concentrations. High GC Gram-positive was the specific genus in FOR soil, whereas GNS bacteria, Gemmatimonadetes and *Nitrospira* were some specific genera in AGR soil (Table 4).

DISCUSSION

The toxicity effects of Al stress on soil organism have been studied in many plants and some "model" microbiologies (Kawai et al., 2000; Amonette et al., 2003; Kochian et al., 2004). However, its effect on soil microbial communities is little studied, especially in red soils. This was not the first report on bacteria diversity revealed by molecular method but the first report on the effect of Al stress on bacterial diversity in acidic red soils. According to some research, Acidobacteria and Proteobacteria have ubiquitous distribution in soils. A study in Western Amazonia soil indicated a predominance of Proteobacteria and Acidobacteria in 16S rRNA clone libraries, the latter comprising approximately 30% of the sequences (Kim et al., 2007). In another study on the bacterial diversity of the Brazilian Cerrado soil, the contribution of Proteobacteria and Acidobacteria was 30 and 22%, respectively (Quirino et al., 2009). Our phylogenetic analysis indicated that the sequenced clones fell into three major lineages within the domain bacteria. Among them, members of Proteobacteria were the typical and dominant groups (Figure 2), including α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, etc. In Proteobacteria, γ-Proteobacteria was found to be the preponderant genus in all soil samples. The results indicated that γ-Proteobacteria was widely distributed in acidic soil environments. However, the contribution of Acidobacteria was lower than the previous results (Pradhan et al., 2010). In addition, a large part of potential species existed in the acidic red soils and a higher proportion were found in Al-treated soil samples.

In the present study, two types of soil sample collected from the region of typical acidic soil in South China were treated by Al stress and analyzed for bacterial diversity. Our findings were that the composition of the bacterial communities in AGR soils differed from those of the FOR soils, and many of the abundant OTUs were different. Likewise, the diversity of bacterial communities from FOR was less than that from AGR. These results suggested that some of the bacterial communities in PRs in AGR soil consisted of bacterial members similar to those associated with organic matter decomposition in Paddy field soil and to soil inhabitants (Upchurch et al., 2008). Meanwhile, their clear differences could be found in different Al-treated soil samples. After 30 days of Al exposure, the composition of the bacterial soil community of the Al-treated soil samples (F_1 and F_2, A_1 and A_2) was changed into that of the control (F_0 and A_0). Furthermore, significant changes occurred in the dominant species and some special species. The results earlier mentioned showed that Al addition lowered the soil microbial

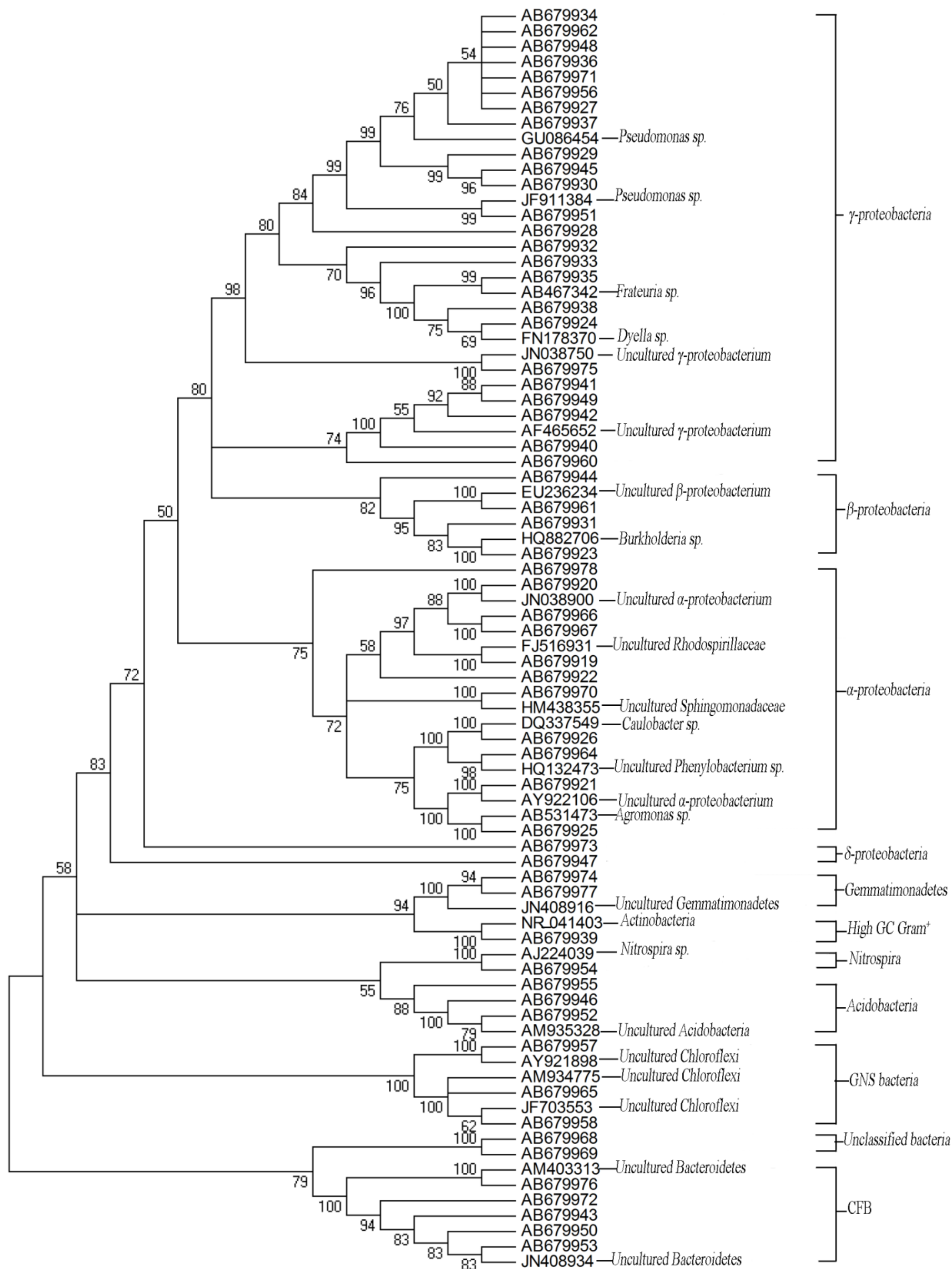

Figure 3. Dendrogram of genetic relationships among 16S rDNA genotypes of bacteria classified. The tree showed the relationship based on partial sequences of the 16S rRNA gene of selected clones, excluding those similarities less than 96%. The sequence alignment was performed by means of the Clustal X program, and the tree was generated by the neighbor-joining method in MEGA 4 software. The DDBJ accession numbers indicated bacteria obtained in this study, while the genus and accession numbers were the most closely related genera. The class and subclasss to which the strains belong were presented on the right.

Table 3. Bacterial diversity of OTUs in rDNA clone libraries from tested soils.

Soil sample	OUT number	Total clones	Coverage richness (%)	Chao1 index	ACE richness index	Simpson index	Shannon index
F_0	35	208	83.2	219.0	1040.7	0.019	3.29
F_1	29	203	85.7	139.5	635.0	0.027	3.04
F_2	22	197	88.8	28.8	63.9	0.056	2.54
A_0	46	215	78.6	143.6	381.1	0.014	3.53
A_1	37	214	82.7	78.6	258.7	0.029	3.17
A_2	20	204	90.2	69.5	93.9	0.021	2.76

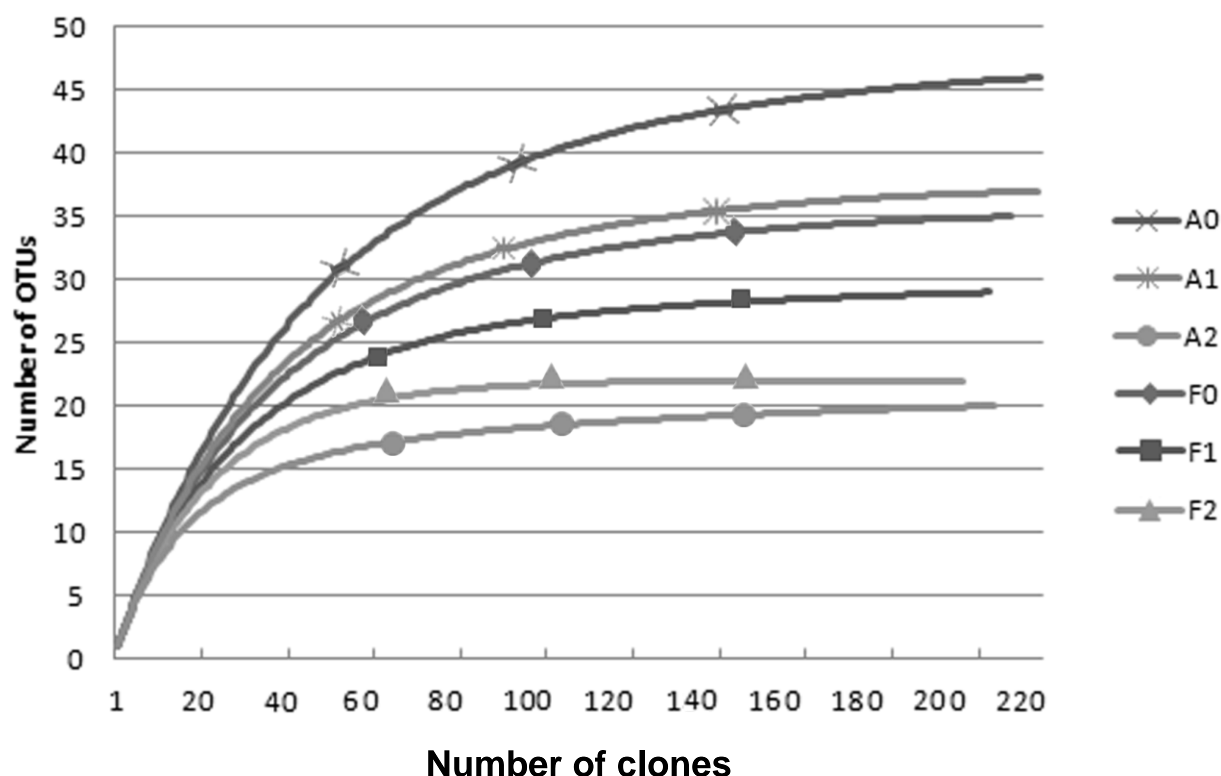

Figure 4. Rarefaction curves for acidic red soil samples OTUs of 16s rDNAs gene clones in different Al treatment.

richness and diversity, and changed the soil microbial contribution. All this information is critical for the determination of the most representative types of bacteria that inhabit the red soil, and can provide valuable information for the isolation and identification of Al-tolerant bacteria.

Conclusions

Our 16S rDNA sequence analyses detected, significantly, the decrease in microbial diversity of soil samples and the differences in bacterial diversity between AGR soil and FOR soil samples, which indicated the great effects of Al addition on the microbial community function of acidic red soil in China. Due to the complexity of the bacterial community, more studies on the red soil are needed to obtain more overall microbial community structures in order to further understand the relationships among the red soil environment, Al stress and microorganisms, and make a better exploitation of some special microbial resources in such areas.

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Table 4. Presence of all of the tested clones by PCR–RFLP assays.

Characteristic or group	Number of clones[a]					
	F_0	F_1	F_2	A_0	A_1	A_2
Total[b]	208	203	197	215	214	204
α-proteobacteria	37	49	42	41	35	22
Caulobacterales	9	11	0	7	8	0
Rhodospirillales	12	30	35	9	9	0
Rhizobiales	8	2	0	10	7	0
Sphingomonadales	3	0	0	7	5	10
Unclassified	5	6	7	8	6	12
β-proteobacteria	20	18	21	21	23	0
Burkholderiales	13	12	10	14	12	0
Unclassified	7	6	11	7	11	0
γ-proteobacteria	101	82	86	68	86	112
Xanthomonadales	14	11	06	12	0	0
Methylococcales	0	0	5	0	5	10
Pseudomonadales	62	51	57	46	61	79
Unclassified	25	20	18	10	20	23
δ-proteobacteria	2	0	0	8	6	1
Myxococcales	0	0	0	8	0	0
Unclassified	2	0	0	0	6	1
Acidobacteria	18	23	31	17	23	37
Acidobacteria Gp3	8	11	11	7	10	17
Acidobacteria Gp6	10	12	20	10	13	20
High GC Gram-positive	2	8	9	0	0	0
Actinobacteria	2	8	9	0	0	0
CFB	4	9	0	14	9	3
Bacteroidetes	4	7	0	8	5	0
Unclassified	0	2	0	6	4	3
GNS bacteria	0	0	0	19	9	6
Chloroflexi	0	0	0	8	4	3
Unclassified	0	0	0	11	5	3
Gemmatimonadetes	0	0	0	2	7	15
Gemmatimonadales	0	0	0	2	7	15
Nitrospira	0	0	0	4	0	0
Nitrospirales	0	0	0	4	0	0
Unclassified *Proteobacteria*	3	0	0	2	0	0
Unclassified bacteria	21	14	8	19	16	8

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