African Journal of Microbiology Research Vol. 7(15), pp. 1443-1450, 9 April, 2013
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1950
ISSN 1996-0808 ©2013 Academic Journals

Full Length Research Paper

Distribution of microbial communities in Guiyu soils and sediments investigated by 16S rRNA gene library and denaturing gradient gel electrophoresis (DGGE) fingerprinting

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Accepted 8 January, 2013

Guiyu, a town in south China, seriously polluted by heavy metals and toxic organic compounds, takes an important part in the cycle of the Waste Electrical and Electronic Equipment. Here, culture-independent approaches were used to analyze the bacterial community structure of 7 soil and sediment samples. The objective of this paper was to examine the distribution of microbial communities in the e-waste contaminated areas of Guiyu. High concentrations of metals (Cu, Pb, Zn and Al) were detected in the soil and sediment samples collected from the e-waste contaminated areas at Guiyu. Denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene library technology were used to investigate the microbial communities. The 16S rRNA gene library could detect more bacteria than polymerase chain reaction (PCR)-DGGE. The main bacteria in the soil and sediment samples were *Deltaproteobacteria* (36%), *Firmicutes* (16%), *Alphaproteobacteria* (9%), *Bacteroidetes* (7%), *Verrucomicrobia* (7%) and *Acidobacteria* (5%); and 20% of the sequences in the 16S rRNA gene library had sequence identities less than 92% with known sequences.

Key words: Guiyu, microbial diversity, DGGE, 16S rRNA gene library.

INTRODUCTION

Guiyu, a town in south China, takes an important part in the cycle of the Waste Electrical and Electronic Equipment (WEEE), and gets involved in dismantling, sorting and recovering precious metals etc (Yang et al., 2008). Due to the adoption of backward technology, as well as the lack of pollution prevention, Guiyu regions are seriously polluted by heavy metals and toxic organic compounds. The physical health of human beings thereof faces the hazard (Hicks et al., 2005).

In 2001, Basel Action Network and Greenpeace for the first time reported the pollution condition in Guiyu, and their testing showed that the river in Guiyu region contained high concentrations of lead and cadmium, 1.9-24 mg/l [World Health Organization (WHO) limit: 0.01 mg/l] and 0.01-0.033mg/l [WHO limit: 0.003 mg/l] (Puckett et al., 2002), respectively. In soil, the heavy metals Cu, Pb, and Zn were the most abundant ones among the environmental samples. Cu concentrations at the printer roller dump site (712 mg/kg) and the burnt plastic dump site (496 mg/kg) exceeded the new Dutch list action value of 190 mg/kg (Leung et al., 2006). The pollution in the reservoir (RS) area, rice field (RF), areas near an open-burning site (NOBS) and at the open-burning site (OBS) was detected. The resulting total

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dioxins/furans (PCDD/F) concentrations ranged from 466 to 599,156 pg.g^{-1}, in the descending order: OBS > NOBS > RF > RS. And the concentrations of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) showed the same order (Wang et al., 2005; Yu et al., 2006). In OBS, the toxic organic compounds were higher than other cities in the southeast China, such as Hong Kong (Zhang et al., 2007) and Guangzhou (Chen et al., 2005). The Cd, Ni, Pb and Zn in the sediments were collected from acid leaching sites by approximately 5.8, 1.6, 2.5 and 2.5 times, respectively, and Cu exceeded the New Dutch List Action Value by three times (VROM, 2001).

Serious pollution situation had impacted the people's health in the region. The survey displayed that 165 children's blood lead levels (BLLs) ranged from 4.40 to 32.67 mg/dl, with a mean value of 15.3 mg/dl, while in Chen Zhen, the adjacent town, the BLLs of 61 children from 4.09 to 23.10 mg/dl, with a mean value of 9.94 mg/dl (Huo et al., 2007). And the blood lead and cadmium levels were related with the e-waste pollution (Zheng et al., 2008). The e-waste pollution had also impacted the PCB levels in fish, atmosphere and human milk (Xing et al., 2009) and it is harmful to the people in the town, especially the children and mothers (Yu et al., 2010; Liu et al., 2011). The pollution might also have negative effects on the birth outcome (Wu et al., 2012; Xu et al., 2012).

In the face of the special situation in Guiyu, it is important to know the status of microbial communities. The results from Zhang et al. (2010) revealed that β-*Proteobacteria* and *Firmicutes* were abundant bacterial lineages in PAH-polluted soils and confirmed that different levels of PAHs might affect the bacterial community by suppressing or favoring certain groups of bacteria. Zhao et al. (2011) compared the bacterial diversity of polluted and unpolluted sediment by brominated flame retardant, the *Epsilonproteobacteria* and *Chloroflexi* proved to be the predominant bacteria (44.9% of total clones) in the polluted sediment. Wu et al. (2012) studied the bacterial community structures of the sediments from a contaminated river in Guiyu and found that the predominant bacteria were *Betaproteobacteria* (34.1% of total clones). Here, culture-independent approaches were used to analyze the bacterial community structure of seven soil and sediment samples. The objective of this paper was to examine the distribution of microbial communities in the e-waste contaminated areas of Guiyu.

MATERIALS AND METHODS

Site description and sampling

Guiyu town is located in Chaoyang District, Shantou City, Guangdong Province, the southeast China, with a total area of 52 km^2 and a population of 150,000. Guiyu belongs to the sub-tropical climate, with an annual average temperature of 21.5°C and a mean annual rainfall of 1721 mm. A total of 7 samples were collected in November, 2007. The sediment samples were collected at four different sites: Sediments under the bridge of Fuchao (FS), in the Nanyang river (NS1, NS2) and in a pond near an open burning site (PS). In addition, three soil samples were collected, at the same time, respectively near the Guiyu government building (GD), near a leaching pool (LD), and beside the Nanyang river (ND). There were some dismantling workshops near the three soil sampling sites (Figure 1). Samples were then immediately kept on ice for transport to the laboratory and processed for analysis.

Sample analyses

The soil and sediment samples were freeze dried, shivered and mixed well. 0.50 g of each sample was used for the determination of heavy metal (Cd, Cr, Cu, Sn, Ni, Pb, and Zn) concentrations by wet digestion (USEPA 3050B). The elements were measured using inductively coupled plasma-atomic emission spectrometry (ICP-AES; Shimadzu ICPS-1000Ⅲ). As measures of quality control and assurance, standard solutions, reagent blanks were used. There were no indications of contamination. The recovery rates of the heavy metals of the standard reference materials were within the certificate values.

DNA extraction

DNA was extracted using the method described by Zhou et al. (1996) with some slight changes. Briefly, after the wash of about 0.6 g sample twice by PBS buffer (pH 7.4), 3 ml of DNA extraction buffer (100 mmol/L Tris-HCl, 100 mmol/L Na_2EDTA, 100 mmol/L phosphate buffer, 1.5 mol/L NaCl, 1% CTAB, pH 8.0) was added, and then added proteinase K (final concentration of 0.1 mg/ml). After 30 min of water bath at 37°C, 0.5 ml 10% SDS was added, and incubated at 65°C for two hours. The supernatant was used for DNA extraction by once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The nucleic acids in the supernatant were precipitated with two volumes of isopropanal, washed with 70% ethanol, dried and then dissolved with 100 µl TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

DNA quality was checked in 0.8% agarose gel electrophoresis stained with ethidium bromide solution. The DNA bands were recovered according to the gel extraction kit OMEGA manual operation, and used in the further PCR amplification.

PCR amplification

Nested PCR technique was applied to increase the sensitivity (da Silva et al., 2003). In the first round, the bacterial primers 27F and 1492R were used. PCR was performed in 50 µl reaction mixtures (2.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of each primer, 2.5 U of Taq DNA polymerase, and PCR buffer), for approximately 100 ng of extracted environmental genomic DNA for each sample. During the second PCR round, the universal bacterial primers 338F with an attached GC-clamp and 518R were used (Ovreas et al., 1997), and 2 µl PCR product obtained from the first round of PCR was added in a 50 µl PCR reaction mixtures as a template.

To increase the sensitivity in the second round, PCR amplification began with a high annealing temperature 61°C, and reduced 0.5°C every cycle until the annealing temperature was changed to 51°C. The program of PCR was designed with 5 min denaturing step at 94°C, followed by 33 cycles of 94°C for 1 min, annealed for 1 min, and 72°C for 0.5 min. The final cycle had an extension at 72°C for 9 min.

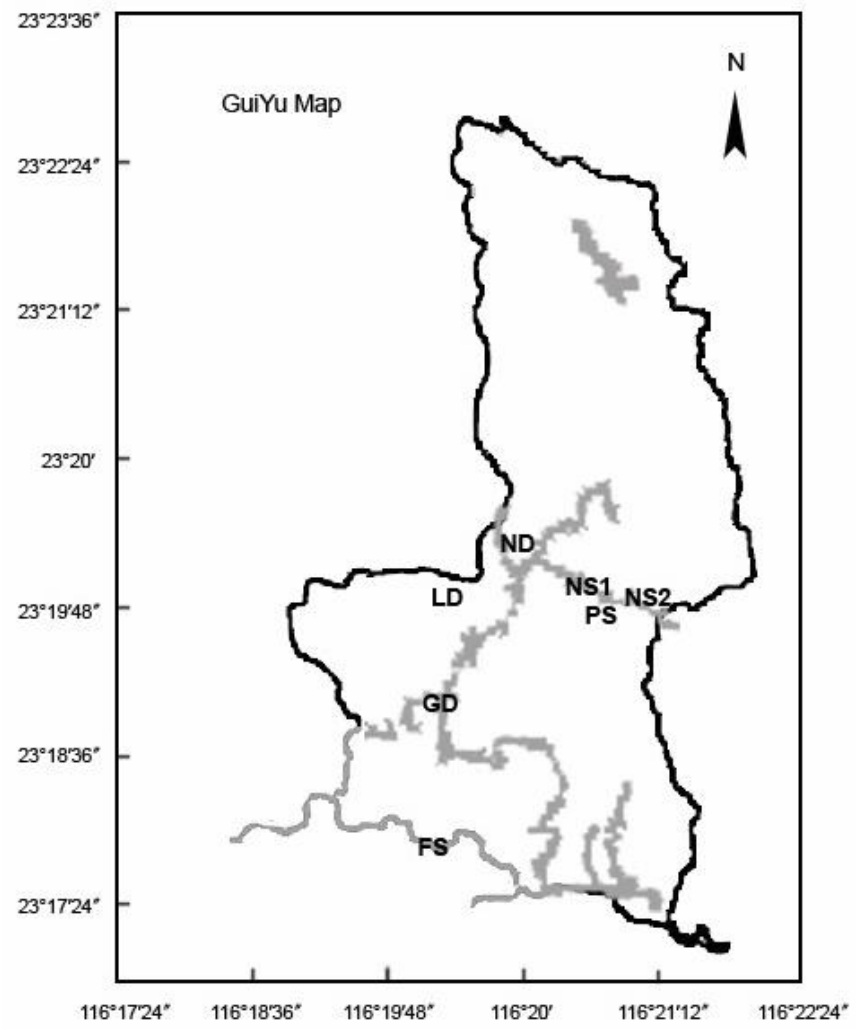

Figure 1. Sediment and soil collection sites of Guiyu. Sediments samples: sediments under the bridge of Fuchao (FS); in the Nanyang river (NS1, NS2); in a pond near an open burning site (PS). Soil samples: soil near the Guiyu government building (GD); near a leaching pool (LD) which was used to contain the waste water of acid pickling; beside the Nanyang river (ND).

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was performed using a DGGE-2100 system (C.B.S) with 10% polyacrylamide gels. A denaturing gradient of 40-70% was used, and the 100% denaturing solution contained 40% (v/v) formamide and 7 M urea. Electrophoresis was performed with a constant voltage of 130 V at 60°C for 7 h. Gels were stained in a 50 mg/ml ethidium bromide solution, rinsed with distilled water and photographed.

The bands in the DGGE gel were selected and dissolved in TE overnight. The supernatant was used as the template in the re-amplification using the primers F338 and 518R. The PCR products were cloned into plasmid vector pUCm-T and transformed into *Escherichia coli* DH5α cells. The positive clones were identified and sent to Invitrogen Biotechnology Co. for sequencing. The sequence identities were determined by BLAST in the GenBank database. The partial 16S rRNA gene sequences reported in this paper were submitted to GenBank database under the accession numbers FJ859702-FJ859718.

16S rRNA gene library analysis

The GD site's 16S rRNA gene products in the first round PCR were directly cloned into the pUCm-T vector. The ligation product was transformed into *Escherichia coli* DH5α cells. One hundred (100) positive clones, picked on a random basis, were identified and sequenced. The sequences were compared with those in GenBank by BLAST search tool and identified by Ribosomal Database Project II (RDP II). The partial 16S rRNA gene sequences were deposited in the GenBank database and assigned accession numbers FJ859751 -FJ859850.

RESULTS

Metal content of the samples

The Cu content of all sites was in excess of the New

Table 1. Metal content of the samples.

Sampling site	Element (mg/kg)						
	Cr	Ni	Cu	Zn	Cd	Sn	Pb
Sediment							
FS	5	10	1,890	430	-	165	1,762
NS1	5	9	834	339	-	70	1,389
NS2	11	16	767	485	-	103	1,232
PS	-	64	814	261	-	21	125
Soil							
GD	-	31	385	518	-	19	186
LD	-	21	1,533	365	-	64	2,806
ND	-	26	629	327	-	30	177
Soil quality standards							
Dutch							
Optimum value	100	35	36	140	0.8		85
Action value	380	210	190	720	12		530
China							
Grade Ⅰ	90	40	35	100	0.2		35
Grade Ⅱ	250	50	100	200	0.3		250
GradeⅢ	400	200	400	500	1.0		500

Sediments samples: sediments under the bridge of Fuchao (FS); in the Nanyang river (NS1, NS2); in a pond near an open burning site (PS). Soil samples: soil near the Guiyu government building (GD); near a leaching pool (LD) which was used to contain the waste water of acid pickling; beside the Nanyang river (ND).

Dutch list limit of United Kingdom (190 mg/kg) and the Pb content of sites FS, NS1, NS2 and LD had exceeded the action value (530 mg/kg) (VROM, 2001). The metal Cd had not been detected in all the sites, which might be mainly due to Cd's high mobility in the acidic environment (Table 1).

In the sediment samples, the Ni content of site PS was significantly higher than the FS, NS1, NS2 sites, the Sn and Pb contents were lower than the other sediment samples, and the Cu and Zn were in the same level. In the LD, the soil samples which were collected near the electronic dismantling dumps also had significant differences from other soil samples, with Al, Cu, Pb contents significantly higher.

DGGE patterns of bacterial communities

The DGGE band patterns are shown in Figure 2. Totally 26 different band types were detected on the DGGE gel using Quantity One software. The cluster analysis of the DGGE band patterns showed that the sampling sites could be separated into three small groups (Figure 3). The result, though similar to Dice Coefficient analysis (Table 2), is not significant.

Seventeen (17) bands were successfully sequenced and the sequences showed 90-100% similarities in the comparative analysis with the non-redundant nucleic acid database (Table 3). The microbial communities included: *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Spirochaetes* and *Verrucomicrobia*. *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* in the *Proteobacteria* had been detected.

16S rRNA gene clone library analyses

Through sequence alignment analysis, statistical results are shown in Table 4. The sequences revealed a wealth of microbial communities and 100 clones of the sequences mainly belonged to *Proteobacteria* (52%), *Firmicutes* (16%), *Bacteroidetes* (7%), *Verrucomicrobia* (7%), *Acidobacteria* (5%) and *Nitrospira* (4%). 20% of the sequences had sequence identities less than 92% with known sequences. 16S rRNA gene library had detected all the dominant groups of bacteria in the DGGE analyses and more species of bacteria.

DISCUSSION

Compared with previous research, the Pb content in the sites sampled in our work was close to those of open burning sites (856-7038 mg/kg) and the sites nearby (97.8-123 mg/kg), and the Cu content was close to those of open burning points (1374-14253 mg/kg) (Yu et al., 2006). It revealed that the samples collected in this paper were in serious contaminated areas of Guiyu.

The PCR-DGGE and 16S rRNA gene library technology were used to detect the distribution of microbial

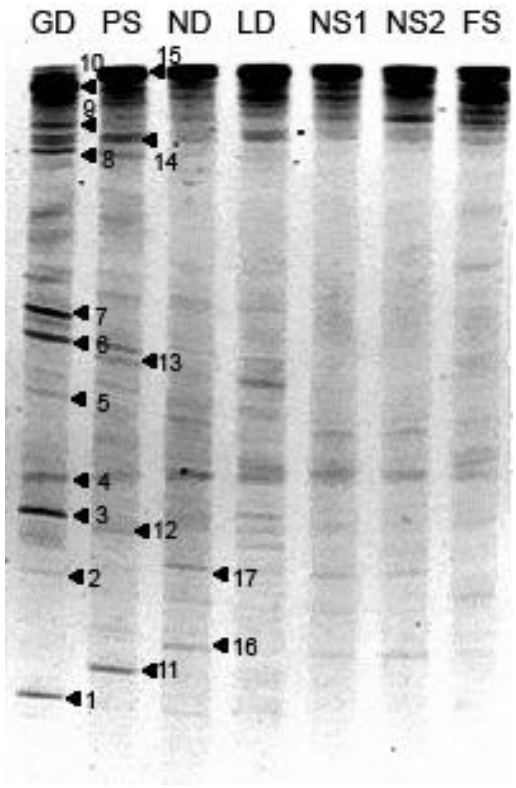

Figure 2. DGGE electrophoresis fingerprinting of 16S rRNA gene.

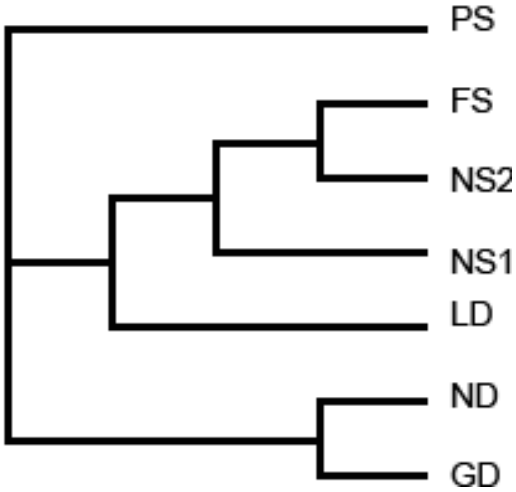

Figure 3. Cluster analysis of the samples using Neighbor Joining.

communities in e-waste polluted area of Guiyu. The two methods, as proved, could detect the dominant bacteria: *Proteobacteria*, *Firmicutes*. 16S rRNA gene library provided more information about the bacteria in non-dominant positions such as *Actinobacteria*, *Chloroflexi* and *Spirochaetes*. 16S rRNA gene library information displayed that 20% of the sequences had less than 92% sequence identity with known sequences. It showed that many microorganisms had not been well classified in the environment which failed to be separated and purified under the present experimental conditions.

The e-waste industry was widely distributed in Guiyu region, and the heavy-metal, PBDE, PAH and PCB contaminations caused by e-waste were severe (Wang et al., 2005; Yu et al., 2006). Studies had shown that long-term heavy metal pollution could significantly decrease the number of microorganisms and microbial activity in polluted region (Massieux et al., 2004; Muller et al., 2002). Heavy metals and toxic organic pollution provided a selection pressure on the microbial community, which might increase the number of tolerant microorganisms (Diaz-Ravina, 1996). Compared with Gram-positive bacteria, Gram-negative bacteria had more lipopolysaccharides in cell wall and induced pump systems, showing a greater advantage in the pollution resistance (Fernandes et al., 2003; Hubert et al., 2005). All of the *Proteobacteria* were Gram-negative, and they were the largest bacteria in the environment. The *Proteobacteria* occupied a dominant position in the PCR-DGGE and 16S rRNA gene library detection. Many strains of *Proteobacteria* showed a resistance to metals and toxic organics, the α-, β-, γ -, δ-*Proteobacteria* and high/low GC content of Gram-positive bacteria were found in the environment with different concentrations of lead, chromium and toxic organics (Joynt et al., 2006). In the marine sediments polluted by lead, copper, cadmium and zinc for more than 80 years, the γ-, δ-*Proteobacteria* and Cytophaga-Flexibacter-Bacteroides (CFB) were in a dominant position (Gillan et al., 2005).

Many strains of *Acidobacteria* were tolerant to heavy metals (Barns et al., 2007). The 16S rRNA gene library revealed that the *Acidobacteria* which could cumulate heavy metals occupied 60% of bacteria in the plant rhizosphere microbial communities and the other bacteria were *Firmicutes*, CFB (Gremion et al., 2003). A lot of *Firmicutes* bacteria could produce endospores, which could resist dehydration and survive in extreme conditions (Dib et al., 2008). In this paper, the CB01, CB02 and CB03 in the DGGE were detected to be *Bacilli* of *Firmicutes*. In the 16S rRNA gene library, 16% of bacteria clones were also detected to be *Firmicutes*, revealing their dominant position in extreme environment.

In wastewater treatment biofilm, the researchers discovered bacteria including *Firmicutes* (43.9%), *Proteobacteria* (28.6%) and *Bacteroidetes* (17.6%). The *Bacteroidetes* were in a dominant position, but they failed to be purified (Park et al., 2007). In this study, the *Bacteroidetes* bacteria clones occupied 7% in the 16S rRNA gene library, but were not detected in the DGGE analyses. The *Verrucomicrobia* which occupied 7% in the library were also detected in the DGGE analysis. The *Verrucomicrobia* were found in microorganisms related to *Thlaspi* which could accumulate high concentrations of Ni

Table 2. Similarity analysis of DGGE electrophoresis fingerprinting.

Lanes compared	NS1	NS2	FS	GD	PS	ND	LD
NS1	100.0						
NS2	70.4	100.0					
FS	63.5	62.1	100.0				
GD	56.7	57.9	56.0	100.0			
PS	56.7	55.0	49.2	48.7	100.0		
ND	62.3	66.9	61.4	60.7	60.3	100.0	
LD	65.4	60.4	56.8	52.6	58.3	65.1	100.0

Table 3. Alignment of sequences recovered from DGGE.

DGGE Band	Class	Accession No. of matched sequence	Similarity to matched sequence
CB01	*Bacilli*	EF528290	100%
CB02	*Bacilli*	AB241601	96%
CB03	*Bacilli*	EU784658	100%
CB04	*Alphaproteobacteria*	FJ764495	100%
CB05	*Deltaproteobacteria*	AY712423	97%
CB06	*Betaproteobacteria*	AM990021	96%
CB07	*Gammaproteobacteria*	EU863591	93%
CB08	*Acidobacteria*	EU299479	95%
CB09	*Acidobacteria*	AF392718	96%
CB10	*Alphaproteobacteria*	EU802247	97%
CB11	*Spirochaetes*	AB476706	98%
CB12	*Gammaproteobacteria*	DQ900624	90%
CB13	*Gammaproteobacteria*	DQ829256	98%
CB14	*Deltaproteobacteria*	AY294222	95%
CB15	*Deltaproteobacteria*	AY398688	97%
CB16	*Verrucomicrobia*	DQ996969	99%
CB17	*Deltaproteobacteria*	EF667662	98%

(Idris et al., 2004).

Squartini (2011) had analyzed the distributions of bacterial phyla in different environment in the Genbank database. In Squartini's report, there were 45.19% of *Proteobacteria* in the average bacterial community of soil including *Gammaproteobacteria* (14.64%), *Alphaproteobacteria* (13.76%), *Betaproteobacteria* (12.41%) and *Deltaproteobacteria* (4.38%). When it came to the heavy metal polluted bacterial community *Proteobacteria* had a slight increase to 47.49% including *Gammaproteobacteria* (19.32%), *Alphaproteobacteria* (13.1%), *Betaproteobacteria* (9.5%) and *Deltaproteobacteria* (5.57%). *Gammaproteobacteria* and *Deltaproteobacteria* were over-represented compared to the average soil community, while *Betaproteobacteria* were under-represented. In this study, total *Proteobacteria* had an increase to 52% in the Guiyu soil samples and *Deltaproteobacteria* were highly over-represented with a percentage of 36% while *Betaproteobacteria* were under-represented with 4%. However, *Gammaproteobacteria* dropped sharply to only 3%. Under the situation of "reducing" *Deltaproteobacteria* domains the bacterial community by 63% according to Squartini's report (2011). In this study the domain bacteria of heavy metal-polluted Guiyu samples proved to be *Deltaproteobacteria* (36%) indicating the abundance of metal-reducing bacteria. Another obvious under-represented example was for *Actinobacteria*. *Actinobacteria* had a percentage of 20.81 and 10.2% in the average soil and the heavy metal community, respectively, and in this study only 3% were *Actinobacteria*. The percentage of *Firmicutes* in the average soil increased from 12.28 to 26.7% in the heavy metal-polluted community while it had a slight increase to16% in this study. Therefore, the Guiyu soil had somewhat similarities of the bacterial community profile with the heavy metal-polluted community compared to the average soil in Squartini's report.

Table 4. Sequence alignment of 16S rRNA gene.

Phylum	Class	No. of class	Sum
Proteobacteria			52
	Deltaproteobacteria	36	
	Alphaproteobacteria	9	
	Betaproteobacteria	4	
	Gammaproteobacteria	3	
Firmicutes	*Clostridia*	16	16
Bacteroidetes			7
	Bacteroidia	5	
	Flavobacteria	2	
Verrucomicrobia	*Verrucomicrobiae*	7	7
Acidobacteria	*Acidobacteria*	5	5
Nitrospira	*Nitrospirae*	4	4
Actinobacteria	*Actinobacteria*	3	3
Spirochaetes	*Spirochaetes*	2	2
Chloroflexi			2
	Anaerolineae	1	
	Dehalococcoidetes	1	
Tenericutes	*Mollicutes*	1	1
Chlorobi	*Ignavibacteria*	1	1

Since the DGGE detection technology required the length of DNA sequence within 500 bp, the test results of the taxonomic status of the bacteria were generally only located at the levels of phylum or class. Using 16S rRNA gene library method might get more taxonomic status information about bacteria. If combined with amplified ribosomal DNA restriction analysis (ARDRA), restriction fragment length polymorphism (RFLP) and other technology, it would achieve better results (Jawad et al., 1998; Krizova et al., 2006).

Conclusion

The soil and sediment of the sampling sites in Guiyu were seriously contaminated by heavy metals. 16S rRNA gene library could provide more taxonomic status information about bacteria than the method of PCR-DGGE. The soil and sediment samples of Guiyu mainly contained *Deltaproteobacteria* (36%) and *Firmicutes* (16%). And 20% of the sequences in the 16S rRNA gene library had sequence identities less than 92% with known sequences.

ACKNOWLEDGEMENTS

This research was supported by National Natural Science Foundation of China (No. 30970106, 41076073) and Science and Technology Project of Shantou City of China (2011-160).

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