Bacterial diversity in soils around a lead and zinc mine

HU Qing, QI Hong-yan*, ZENG Jing-hai, ZHANG Hong-xun

Department of Environmental Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: huboqing2003@yahoo.com.cn

Received 12 January 2006; revised 7 March 2006; accepted 17 March 2006

Abstract

Five samples of soil collected from a lead and zinc mine were used to assess the effect of combined contamination of heavy metals on soil bacterial communities using a polyphasic approach including characterization of isolates by culture method, community level catabolic profiling in BIOLOG GN microplates, and genetic community fingerprinting by denaturing gradient gel electrophoresis of 16S rDNA fragments amplified by PCR from community DNA (PCR-DGGE). The structure of the bacterial community was affected to a certain extent by heavy metals. The PCR-DGGE analysis of 16S rRNA genes showed that there were significant differences in the structure of the microbial community among the soil samples, which were related to the contamination levels. The number of bacteria and the number of denaturing gradient gel electrophoresis (DGGE) bands in the soils increased with increasing distance from the lead and zinc mine tailing, whereas the concentration of lead (Pb) and cadmium (Cd) was decreased. Heavily polluted soils could be characterized by a community that differs from those of lightly polluted soils in richness and structure of dominating bacterial populations. The clustering analysis of the DGGE profiles showed that the bacteria in all the five samples of soil belonged to three clusters. The data from the BIOLOG analysis also showed the same result. This study showed that heavy metal contamination decreased both the biomass and diversity of the bacterial community in soil.

Key words: BIOLOG system; DGGE; heavy metal; microbial diversity; soils

Introduction

Heavy metal pollution is a serious environmental problem. Heavy metals have an obvious effect on or are potentially harmful to biota. Among heavy metals, cadmium (Cd), commonly associated with soil pollution, is considered to be particularly toxic and responsible for significant decreases in biological activities in soils (Smith et al., 1997).

Discharge of heavy metal has detrimental effects on human health and the environment. Lead and zinc mines are major sources that release significant amounts of Cd, lead (Pb), and chromium (Cr) into the natural environment.

Recently, the effect of metal toxicity on microorganisms has received special attention because microorganisms are key components for recycling of nutrients. The effects of heavy metals on the soils around lead and zinc mine are complex and very difficult to study because of the diversity of the microorganisms that are present in the soils. Some microorganisms can tolerate toxicity of heavy metals, whereas others cannot. However, microorganisms that have the ability to resist the toxicity are not yet well known. Previous studies merely show that some microorganisms have the ability to remove heavy metals from polluted environments. However, at a given concentration, a metal may be toxic to one species, whereas it may serve as a growth stimulant to others (Filiz et al., 1996). To date, the influence of metals on the microbial communities is not precisely known.

In soil, heavy metals are present in various forms as a result of their interactions with various soil components; therefore, the total concentration of heavy metals in soils cannot provide a precise index for evaluating their influence on soil microorganisms (Welp and Brummer, 1997; Kunito et al., 1999). To date, few attempts have been made to study the relationship between heavy metals in soils and their influence on microbial properties (Kunito et al., 1999), and very little information is currently available in the published literature on this subject.

In this study, five samples of soil from around a lead and zinc mine located in the suburbs of Beijing City in North China were collected and a polyphasic approach was used, including cultivation-based methods and molecular techniques, to analyze the soil bacterial communities in the samples. The microbial communities were characterized using the BIOLOG system and the denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments that were amplified from the total bacterial DNA extracted from the soil (Miller et al., 1999; Picard et al., 1992; Torsvik et al., 1990; Tsai and Olson, 1992; Zhou et al., 1996). The main objective of this study was to obtain information about the microbial diversity of soils that were polluted by mixed heavy metals from the lead and zinc.
mine. Furthermore, a study on the patterns of microbial distribution in the soil samples was also attempted. This provided a valuable basis for further investigation on heavy metal-resistant bacteria.

1 Materials and methods

1.1 Soil samples

Soil sample was collected from the surface layer (0–10 cm) from a lead and zinc mine located in the suburbs of Beijing City in North China (40°07′N; 116°38′E). This mine had been continuously exploited for 20 years. Five individual samples of soil were collected from the interior of the mine (S1), a stacking site of cinder at a distance of 20 m from the mine (S2), an orchard at a distance of 60 m from the mine (S3), a brook at a distance of 40 m from the mine (S4), and at the entrance of the mine (S5). The sampling depth was 0–20 cm. The samples were collected in the plastic bags and transported on ice to the laboratory, where they were stored at 4°C.

1.2 Determination of metal concentration

A total of 0.4 g of dried soil sample was used for the determination of lead, cadmium, and chromium. The sample was wet-digested with 5 ml of concentrated nitric acid in a closed polytetrafluoroethylene (PTFE) vessel in a microwave oven. The digest was diluted to 25 ml with redistilled water and filtered. A Spectra AA640 apparatus (Varian Techtron, Australia) was used for atomic absorption spectrometry measurements.

1.3 Enumeration and morphological examination of colony-forming units (CFU)

100 µl of appropriate soil (S1–S5) dilutions were spread on LB agar plates that were supplemented with fungicide (25 µg Nystatin/ml) and incubated at 25°C for 14 d. The visible colonies were enumerated and marked daily throughout the incubation period. For typing of colony morphology on LB plates, the 30–100 colonies that had developed on each plate after 4–5 d of incubation were grouped into morphotypes on the basis of visual characteristics such as colony color, diameter, edge, surface, and other special characteristics. All morphological examinations were carried out on three replicates of each soil. While analyzing cultivable heterotrophic and genetic diversity, Richness (S), Shannon-Wiener indices (H), and Evenness (EH) were used according to the following equations:

\[ H = - \sum_{i=1}^{S} p_i \ln p_i = - \sum_{i=1}^{S} (N_i/N) \ln(N_i/N) \]  

(1)

\[ E_H = H/H_{\text{max}} = H/ \ln S \]  

(2)

Where \( p_i \) is the ratio between the number in a specific group (\( N_i \)) and the total number (\( N \)), \( S \) is the total number of morphotypes in cultivable heterotrophic diversity.

1.4 DNA extraction methods

The cetyltrimethylammonium bromide (CTAB) method was used to extract the DNA from the bacterial biomass that was grown in the plates (Mette and Heils, 2002). This method was adapted to extract DNA from soils. Soil sample (250 mg) was added to 250 mg of acid-washed glass beads (0.4–0.52 mm in diameter) in a 2-ml microcentrifuge tube. Buffer A (1 ml of 100 mmol/L Tris-HCl, 100 mmol/L EDTA (pH 8.0), 100 mmol/L phosphate buffer (pH 8.0), 1.5 mol/L NaCl, 1% CTAB) was added to a mixture of 175 µg of proteinase K and 1 mg of lysozyme. It was then agitated on a platform shaker at maximum speed for 20 min. Subsequently, 120 µl of 20% sodium dodecyl sulfate was added, and the samples were incubated at 65°C for 30 min. The samples were then centrifuged at 2800 g for 2 min. The supernatant was transferred to a fresh tube, and the pellet was reextracted with 300 µl of buffer A and was centrifuged again. The combined supernatants were extracted with an equal volume of chloroform-isooamyl alcohol (24-1, v/v). The aqueous phase was precipitated with 0.6 volume of isopropanol at –20°C for 30 min. The pellet was washed in 300 ml of 70% (v/v) ethanol and air-dried before resuspending in 100 µl of 10 mmol/L trithylhydroxymethyl aminomethane (Tris) (pH 8.5). The DNA preparations were purified by gel electrophoresis, which served to separate humic acids from the DNA. Aliquots (50 µl) were run on a 0.7% agarose gel at 60 V for 2 h. DNA bands of approximately 10–30 kb were excised from the gel, and the DNA was extracted using the Silver Bead DNA Gel Recovery Kit (manufactured by Shanghai Sangon Co., Ltd.). Essentially, the gel slice was solubilized and bound to the glass particles in solution at pH 7.5. It was then washed and eluted in 10 mmol/L Tris (pH 8.5). This was then used directly for subsequent PCR amplification.

1.5 PCR amplification of 16S rRNA genes from the extracted DNA

The partial bacterial 16S rRNA genes (16S rDNA fragments that are about 230 bp) were amplified with the forward primer F357 (5′-CCT ACG GGA GGC AGG-3′) and the reverse primer R518 (5′-ATT ACC GGG GCT GCT GG-3′) (Zhou et al., 1996). As forward primer, a 40-base GC clamp (5′-CGC CCG CGC CGC GGC GGC GGC GGC GGG GCA CGG GGG G-3′) was added to the 5′ end to stabilize the melting behavior of the DNA fragments. PCR was performed with Applied Biosystem Gene Amp PCR system 2700. The following cycle conditions were used for the primer pair: 95°C for 15 min (for enzyme activation and target denaturation), followed by 20 cycles of 95°C for 1 min, 65°C (reduced by 0.5°C each cycle) for 45 s, and 72°C for 1 min; 10 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 5 min.

1.6 DGGE analysis

The Dcode System for DGGE (Bio-Rad Laboratories Ltd., Hertfordshire, United Kingdom) was used. Samples were loaded on 10% polyacrylamide-bisacrylamide
(37.5:1) gels having denaturation gradients from 35% to 60% (where 100% is 7 mol/L urea and 40% (v/v) deionized formamide) in 1×TAE electrophoresis buffer. Electrophoresis was carried out at 180 V at a temperature of 60°C for 4.5 h. Gels were then stained with EB in 1×TAE for 30 min at room temperature and observed under UV illumination. Bands of interest were excised, and DNA was eluted with an equal volume of diffusion buffer (0.5 mol/L ammonium acetate, 10 mmol/L magnesium acetate, 1 mmol/L EDTA (pH 8.0), and 0.1% (wt/v) sodium dodecyl sulfate) at 50°C for 30 min. The resulting solution (2 µl) was used as target DNA for subsequent PCR amplification with primers F357 and R518. The purity and correct running position of each fragment was confirmed by further DGGE analysis.

### 1.7 BIOLOG analysis

The metabolic diversity patterns were analyzed using BIOLOG (BIOLOG, Hayward, CA). Soil samples (20 g) were shaken for 15 min along with sterile phosphate buffer. Soil particles were removed by centrifugation for 10 min at 2600 g. The GN microplates were prepared and inoculated according to the BIOLOG manufacturer’s directions. Microplates were covered and incubated at 30°C. The plates were read with a BIOLOG microplate reader (590 nm) at 4 h intervals, beginning when the color became visible on the microplate (18±2.4 h after inoculation) and ending when the wells no longer changed color (36±5.2 h after inoculation). The average well color development (AWCD) was calculated for each microplate. The AWCD for each microplate was calculated by subtracting the optical density (OD) of control well from the OD of substrate well (blank substrate wells), setting any resultant blanked substrate wells with negative values to zero and taking the mean of the 95 blanked substrate wells. The mean of the AWCD for each set of triplicate plates was calculated. A reference point of 0.25 AWCD was used to compare BIOLOG patterns. The readings obtained from the selected BIOLOG microplates with 0.25 AWCD were further analyzed and compared using the principal component analysis (PCA).

### 2 Results

#### 2.1 Atomic absorption spectroscopic analysis

Three major toxic heavy metals (Pb, Cd, and Cr) in the five samples of soil were estimated by the atomic absorption spectrophotometer (AAS). The results are shown in Table 1.

On the basis of the data given in Table 1, it can be seen that the amount of Pb and Cd in the soils was extremely high and was distributed such that the contents decreased with increase in distance from the mine. In the lead and zinc mine, the Pb content of the soil was 204 µg/g. Outside the mine, the Pb content of soil distributed averagely. This shows that Pb had already penetrated into the soils around the lead and zinc mine tailing, including the soil at the brook, which was at a distance of 40 m from the mine, and the soil in the orchard, which was at a distance of 60 m from the mine. The distribution of Cd was coincident with the distribution of the mineral, with concentrations in soil being high in the mine and around the cinder and low in the soil at the brook, which was at a distance of 40 m from the mine, and the soil in the orchard, which was at a distance of 60 m from the mine. The concentration of Cr gradually increased with the increase in the distance from the mine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pb (µg/g)</th>
<th>Cd (µg/g)</th>
<th>Cr (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>204</td>
<td>226.9</td>
<td>5</td>
</tr>
<tr>
<td>S2</td>
<td>96</td>
<td>115.5</td>
<td>8</td>
</tr>
<tr>
<td>S3</td>
<td>67</td>
<td>168.6</td>
<td>2</td>
</tr>
<tr>
<td>S4</td>
<td>63</td>
<td>14.6</td>
<td>27</td>
</tr>
<tr>
<td>S5</td>
<td>57</td>
<td>2.4</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 1 Concentration of toxic heavy metals (Pb, Cd, and Cr) in the five samples of soil

S1: from the interior of the mine; S2: from the mine 20 m; S3: at a distance of 60 m from the mine; S4: at a distance of 40 m from the mine; S5: at the entrance of the mine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shannon-Wiener index (H)</th>
<th>Richness (S)</th>
<th>Evenness (Ei)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1*</td>
<td>1.506</td>
<td>6</td>
<td>0.84</td>
</tr>
<tr>
<td>S2*</td>
<td>0.954</td>
<td>4</td>
<td>0.69</td>
</tr>
<tr>
<td>S3*</td>
<td>1.686</td>
<td>10</td>
<td>0.73</td>
</tr>
<tr>
<td>S4*</td>
<td>2.317</td>
<td>12</td>
<td>0.93</td>
</tr>
<tr>
<td>S5*</td>
<td>1.827</td>
<td>8</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Table 2 Diversity analysis based on colony morphology

*SDetails of samples are similar to those given in Table 1.

Fig. 1 Number of heterotrophic bacteria capable of forming colonies in five samples of soil.
2.3 DGGE band analysis

The method of PCR-DGGE based on 16S rDNA and denaturing gradient gel electrophoresis fingerprinting technology has been increasingly used to assess changes in soil microbial communities (Friedrich et al., 1997). The strength of DGGE as a screening method for diversity lies in its ability to monitor spatial and temporal changes in community structure in response to changes in environmental parameters (Mette and Neils, 2002). Here the method of PCR-DGGE was used to investigate the changes in the structure of microbial community in all the five field sites contaminated by heavy metals (Cd, Pb, and Cr), which were at varying distances from the lead and zinc mine tailing. Bacterial DGGE profiles generated from the universal bacterial primers (F\textit{357} and R\textit{518}) showed the structural composition of communities in soil samples (Fig.2). Each of the distinguishable bands in the separation pattern represents an individual bacterial species (Luca et al., 2002). Soil sample S4 showed the most complex DGGE pattern with 12 visible bands, indicating the presence of a high number of different bacterial species. The visible band numbers of DGGE patterns of all other samples were 4–10, with a decrease of 16.6%–33% compared with sample S4. Soil sample S2 showed the simplest DGGE pattern with four visible bands. Two special crisp bands (labeled band 1 and band 2 in Fig.2) were found in the soil samples S1, S2, and S5, indicating that special heavy metal-resistant bacteria may have colonized during the long-term deposition of heavy metals in the soil. Clustering of the profiles showed that there were very large differences among the profiles of the soil samples (Fig.3).

The greatest difference was found between the profile of S5 and all the other samples. The profile of S5 belongs to a single cluster. The profiles of the other four samples were separated into two major clusters: profiles of S1 and Fig. 3 Similarity of the five samples of soil based on NEIGHBOR JOINING method.

S2 into one and profiles of S3 and S4 into another. Profiles of S1 and S2 showed approximately 77.8% similarity with respect to the clustering. Profiles of S3 and S4, however, showed a similarity of approximately 89.6%. These data indicate that the bacterial communities in the soil near the lead and zinc mine tailing had changed substantially during the long-term period of pollution.

2.4 Bacterial diversity analysis

Bacterial diversity was analyzed by DGGE. Based on the intensity of each band, S-W indices were calculated by computerized image analysis. Diversity indices are useful as a first approach to estimate the diversity of microbial communities, i.e., the higher \(H\) is, the greater is the diversity of the microbial community. A diversity index consists of two components: (1) the total numbers of species present or species richness and (2) the distribution of the number of individuals among those different species, called species evenness, or species equability. The results in Table 3 showed the bacterial diversity of all the soils sampled.

Clustering analysis of the DGGE profiles showed that bacteria in the five samples of soil belonged to three clusters. The bacterial communities in soils sampled in the mine and at the cinder stacking site and those in soil sampled at the entrance of the mine belonged to a single cluster. Bacterial community in soils sampled at a brook that was at a distance of 40 m from the mine tailing and an orchard that was at a distance of 60 m from the mine tailing belonged to another cluster.

2.5 Result of BIOLOG analysis

Principal component analysis (PCA) of the color response data of the soil samples showed the existence of different microbial communities. PCA was carried out to characterize the associations between samples, taking into account the absorbance values for all 96-response wells at Table 3 Shannon-Wiener index \((H)\), Richness \((S)\), and Evenness \((E_H)\) of each sample

<table>
<thead>
<tr>
<th>Samples</th>
<th>(H)</th>
<th>(S)</th>
<th>(E_H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1*</td>
<td>1.506</td>
<td>6</td>
<td>0.84</td>
</tr>
<tr>
<td>S2*</td>
<td>0.954</td>
<td>4</td>
<td>0.69</td>
</tr>
<tr>
<td>S3*</td>
<td>1.686</td>
<td>10</td>
<td>0.73</td>
</tr>
<tr>
<td>S4*</td>
<td>2.317</td>
<td>12</td>
<td>0.93</td>
</tr>
<tr>
<td>S5*</td>
<td>1.827</td>
<td>8</td>
<td>0.88</td>
</tr>
</tbody>
</table>

*Details of samples are similar to those given in Table 1.
different times of incubation. Two principal factors were found from the patterns through S1 and S5 that explained 68% of the variation: factor 1 was the absorbance values for the wells, whereas factor 2 was the incubation time.

The relationship between the microbial community patterns was further analyzed using hierarchical clustering. In Fig.4, the results of cluster analysis also showed that the metabolic activities of S1 were more “closely” related to those of S2 compared with S3 and S4. The metabolic activities of S5 were a single cluster that was distantly related to the S1–S2 cluster and the S3–S4 cluster. The heavily contaminated and lightly contaminated soils differed in this respect. The PCA illustrated that the microbial communities in different soils were distinctive in relation to the contamination level of the heavy metals.

![Component plot](image)

**Fig. 4** Principal component analysis of the five samples of soil based on SPSS software.

### 3 Discussion

The contamination of soil with heavy metals can have a detrimental effect on microbial activity and function. Heavy metal contamination decreases soil respiration and microbial biomass (Bååth, 1989) and hampers the decomposition of litter in soils (Berg et al., 1991). In this study, the contaminated soils sampled around the lead and zinc mine had been subject to pollution over a long time period. The changes in structure of microbial community around the lead and zinc mine tailing could illustrate real changes in theses communities of microbial organisms in soils contaminated with Cd, Pb, and Cr. Soils around the lead and zinc mine had received high amounts of heavy metals such as Cd and Pb, which were considered as dominant pollutants in this study. In this study, a decrease in microbial community diversity was found. Similar results were also found using the method of BIOLOG.

Many studies have shown that even small amounts of heavy metals in the environment have detrimental effects on all living organisms and decrease litter decomposition and subsequently nutrient cycling in the whole ecosystem. Heavy metal pollution inhibits microbial processes such as N mineralization in soil (Chander et al., 1995) and litter decomposition (Fritze et al., 1989). Therefore, the normal functioning of a microbial community in soils around the lead and zinc mine tailing might have been weakened. Among all the profiles of DGGE in soils, that of S1 was the simplest. However, in this study, cluster analysis of DGGE profiles showed that the bacteria in S1 and S3 belonged to the same cluster. This confirmed the result that was obtained using the method of BIOLOG. S1 was the soil sampled in the mine and S3 was the soil sampled near the cinder stacking site, which was 20 m away from the lead and zinc mine tailing. It showed that discharge the cinder into the soil environment would destroy the structure of the microbial community.

In the present study, the generations of heavy metal-resistant bacteria were found in the profile of DGGE in S1. The increased heavy metal tolerance of the microbial community could be due to an acquired tolerance by adaptation, a genetically altered tolerance, or a shift in species composition. It is possible to acquire heavy metal tolerance in the environment because the genes in most organisms controlling metal resistance are presented in plasmids.

The results also indicate that some heavy metal-resistant bacteria can survive in the soils with high amount of Pb (204 µg/g soil) and Cd (226.9 µg/g soil). The appropriate level of heavy metals should be given when domesticking new bacteria resistant to combined heavy metals.

Compared with the traditional method of plate counts, PCR-DGGE and BIOLOG methods can provide more detailed information about the shift and diversity in structure of microbial community in the soil environment.

**Acknowledgements:** The authors thank Mr. Li J Z for providing the PCR Cycle instrument and Mr. Liu S J for help with the Dcode™ mutation Detection System.

### References


Luca C, Daniele A, Marisa M et al., 2002. An application of