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Microbial diversity of soil bacteria in agricultural field contaminated with heavy metals

CHIEN Chihching[1,*], KUO Yumei[2], CHEN Changchieh[1], HUNG Chunwei[1], YEH Chihwei[1], YEH Weijen[1]

1. Graduate School of Biotechnology and Bioengineering, Yuan Ze University, Chung-Li, Taiwan 320, China. E-mail: ccchien@saturn.yzu.edu.tw
2. Department of Occupational Safety and Health, Chung Hwa University of Medical Technology, Tainan, Taiwan, China

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Abstract

In this study we evaluated the bacterial diversity in a soil sample from a site next to a chemical industrial factory previously contaminated with heavy metals. Analysis of 16S rDNA sequences amplified from DNA directly extracted from the soil revealed 17 different bacterial types (genera and/or species). They included *Polyangium* spp., *Sphingomonas* spp., *Variovorax* spp., *Hafina* spp., *Clostridia*, *Acidobacteria*, the enterics and some uncultured strains. Microbes able to tolerate high concentrations of cadmium (500 μmol/L and above) were also isolated from the soil. These isolates included strains of *Acinetobacter* (strain CD06), *Enterobacter* sp. (strains CD01, CD03, CD04 and CD08) (similar strains also identified in culture-independent approach) and a strain of *Stenotrophomonas* sp. The results indicated that the species identified from direct analysis of 16S rDNA of the soil can be quite different from those strains obtained from enrichment cultures and the microbial activities for heavy metal resistance might be more appropriately addressed by the actual isolates.

Key words: cadmium; heavy metal; heavy metal resistant; microbial diversity; soil bacteria

Introduction

Heavy metal contamination is a common environmental problem which presents a significant health hazard and requires expensive cleanup costs. The input of these pollutants into the environment has significantly increased due to the irresponsible disposal of wastes by various industries. Agricultural fields contaminated with the heavy metal cadmium have been a particular concern since it results in the contamination of the agricultural products such as rice. Additionally, heavy metals can accumulate in biological systems and ultimately be introduced into food web via different mechanisms (Giller *et al*., 1998). Metals are a significant toxic factor to biota in the environment in their free ionic forms. However, studies on bacterial diversity in heavy metal contaminated sites still demonstrated a high diversity of microorganisms (Dean-Ross and Mills, 1989; Hattori 1992; Gelmi *et al*., 1994; Roane and Kellogg, 1996; Filali *et al*., 2000; Ellis *et al*., 2003; Konstantinidis *et al*., 2003). They are indigenous organisms that have not only adapted to the new environments but have also flourished under them (Haq and Shakoori, 2000; Roane and Pepper, 2000). Microorganisms possess a variety of mechanisms to deal with high concentrations of heavy metals and often are specific to one or a few metals (Silver and Mistra, 1988; Silver and Phung, 1996; Mejare and Bulow, 2001; Nies, 2003; Piddock, 2006). Therefore, specific heavy metal resistant determinants can be used as parameters for environmental forensic as biosensors (Haq and Shakoori, 2000; Zhou, 2003; Verma and Singh, 2005).

The required methods to most reliably determine the bacterial diversity of an environment is often hotly debated. It is widely accepted that the enrichment culture approach only identifies a small proportion of microorganisms in any given sample. And these are usually the organisms that grow best under the enrichment conditions provided. On the other hand, the use of nucleic acid-based methods including biochips is believed to be more appropriate for the assessment of microbial community than those based on culturing techniques (Ovreas and Torsvik, 1998). Unfortunately, there are drawbacks of these culture-independent approaches such as the relevance of the microbial activities to the metal pollutants can not be totally elucidated. Study on the relationship between the diversity of the culturable portion of the microbial community and the microbial diversity obtained by direct amplification of 16S rDNA genes from soil have been discussed (Ellis *et al*., 2003). Also from the application of bioremediation point of view, the isolation of the microorganisms and the bacterial effects in the removal of heavy metals is essential.

In this study, we studied the bacterial diversity of a soil

* Corresponding author. E-mail: ccchien@saturn.yzu.edu.tw.

sample from an agricultural field previously contaminated with heavy metals. Examination of the microbiota was carried out both by direct amplification of 16S rDNA genes from the sample as well as cultivation of the microorganisms. As expected, our result indicated that the species identified from direct analysis of 16S rDNA genes of the soil was quite different from those strains obtained from enrichment cultures. All the isolates showed robust-growth in the medium containing relative high concentration of heavy metals. This suggests that from the application point of view, only the "actual" isolation of microorganisms may provide the ability to study the relationship between *in situ* microbial activities and those isolates able to be used for bioremediation applications.

1 Materials and methods

1.1 Experimental soil

The experiment was conducted with soil from an agricultural field contaminated with heavy metals in Lu-Chu, Taoyuan, Taiwan, due to the wastewater discharged to irrigation channel from a chemical industrial factory nearby in 1980s. Although the field was under remediation process in late 1990s, the farmland is still not available for agriculture until recently. The sample was collected from the top 5–20 cm layer of the field. The pH of the collected sample was 4.6 and the organic carbon content was 3.7%. The composition of the sample was 28% sand, 42% silt and 30% clay. The concentration of heavy metals was 3 mg/kg for cadmium and 115 mg/kg for chromium. The analysis of the soil was conducted by soil survey and testing center, "National" Chung-Hsing University, Taichung, Taiwan, China.

1.2 Isolation of cadmium resistant bacteria

Bacteria were isolated from soil described above using Luria Bertani (LB) broth medium supplemented with 500 μmol/L $CdCl_2$ (Lennox, 1955). Soil (0.5 g) was inoculated into 250-ml Erlenmeyer flask containing 50 ml medium and incubated at 30°C with shaking (150 r/min) to maintain aerobic conditions. Pure cultures were obtained on the same medium solidified by addition of Bacto agar (BD Diagnostics, Sparks, MD, USA).

1.3 DNA extraction from the soil and bacterial isolates

Bacterial DNA was extracted directly from soil using the SoilMaster™ DNA extraction kit (Epicentre® Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. DNA was also extracted directly from soil using bead beater and hot detergent treatment (Kuske *et al*., 1998). For pure cultures, cells were harvested and washed twice with 1 ml of sterile phosphate-buffered saline and the DNA was extracted from cell pellets by Triton-Prep method.

1.4 Amplification of 16S rDNA genes by polymerase chain reaction (PCR) and analysis of the PCR products

The 16S rDNA genes were amplified with bacterial universal primers F24 (Forward AGTTTGATYMTGGCTCAG) and F25 (Reverse, AAGGAGGTGWTCCARCC) as described (Dewhirst *et al*., 1999). The PCR (20 μl) contained 1 μl of the DNA preparation, 1X Taq polymerase buffer (provided by the manufacturer), 1 mmol/L of each forward and reverse primer, 200 mmol/L of each deoxynucleotide (ddATP, ddGTP, ddCTP and ddTTP) and Taq polymerase (Yeastern Biotech Co., Ltd., Taipei, Taiwan) 2.5 U. PCR conditions were as follows: 95°C for 5 min followed by amplification in which the following conditions were used: denaturation at 95°C for 45 s, annealing at 55°C for 60 s and elongation for 90 s at 72°C with an additional 10 min at 72°C for each cycle for the 30 cycles. The PCR products obtained from DNA extracted from soil were first analyzed by electrophoresis in 1% agarose gel and was stained with ethidium bromide and visualized under short-wavelength UV light. Purified PCR products (about 1.5 kb) were then inserted into a commercial plasmid vector yT&A® (Yeastern Biotech. Co., Ltd., Taiwan) and then transformed into competent *Escherichia coli* DH5α. Random selected clones were sequenced using a LICOR IR^2 4200 DNA sequencer (LICOR, Lincoln, NE, USA) according to manufacturer's instructions. Purified PCR products obtained from pure cultures of the isolates were also subjected for sequencing as described. Sequences obtained were compared to the non-redundant nucleotide database at the National Center for Biotechnology Information by using their World Wide Website, and the BLAST (Basic Local Alignment Search Tool) algorithm.

1.5 Heavy metal tolerance of isolated strains

To examine the ability of the isolate strains to resist heavy metals, cells of strains CD01, CD06 and CD08 were grown in 250-ml Erlenmeyer flasks containing 50 ml of LB medium supplemented with 500 mol/L of heavy metals (Cd in $CdCl_2$, Cr in $K_2Cr_2O_7$, Ni in $NiSO_4$, Zn in $ZnSO_4$, Pb in $Pb(NO_3)_2$, and Cu in $CuSO_4$). Cultures were incubated at 30°C with shaking to maintain aerobic conditions. Cell growth was determined as measurements of optical density at 600 nm ($OD_{600\,nm}$). Another isolate (a strain of *Stenotrophomonas*) possess a peculiar ability for heavy metal resistance and was published elsewhere (Chien *et al*., 2007).

2 Results and discussion

2.1 Bacterial diversity determined by culture-independent 16S rDNA analysis

From the selected clones with insertion of direct amplification of 16S rDNA from soil, seventeen different microbial types (genera and/or species) were identified from 40 random selected clones. They include bacteria belong to genera *Polyangium*, *Sphingomonas*, *Variovorax*, *Hafina*, *Clostridia*, *Acidobacteria*, bacteria belonging to the members of *Enteribacteriaceae* and some uncultured strains. The identities were determined by partial 16S rDNA sequence analysis of these representative clones and sequence accession numbers are shown in Table 1. The genera that are closely related to the clones as determined by the similarity the alignments of partial 16S rDNA sequences in this study are also shown in Table 1.

2.2 Identification of cultured cadmium resistant microorganisms

Six strains of bacteria able to grow in the presence of cadmium (at least 500 μmol/L) were isolated from the soil sample. The partial 16S rDNA sequences of these bacteria were compared with the 16S rDNA sequences available in the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) public database. The results showed that the six isolates belonged to three different groups of bacteria. One of the isolates was identified as a *Stenotrophomonas* sp. and was published elsewhere since it has peculiar characteristics for cadmium resistance and antibiotic resistance (Chien *et al*., 2007). The other strains were identified as *Enterobacter* sp. (designed as strain CD01, CD03, CD04 and CD08) and *Acinetobacter* sp. (designed as strain CD06), respectively. The nucleotide sequence of partial 16S rDNA of strains CD06 and CD08 have been deposited in the GenBank database under accession numbers EF198175 and EF198176, respectively.

2.3 Heavy metal resistance levels of strains CD01, CD06 and CD08

Strains CD01, CD06 and CD08 were able to resist to a variety of heavy metals. Strains CD01 and CD08 showed the same pattern of heavy metal resistance, which also indicated these isolates maybe the same strains (*Enterobacter* sp.). Table 2 shows the ability of strain CD08 grown in medium containing different heavy metals. Strain CD06 was identified as an *Acinetobacter* sp., and is most closely related to *Acinetobacter radioresistens* by 16S rDNA sequence alignment. *A. radioresistens* capable of degrading aromatic compounds has been reported to be sensitive to heavy metals regardless the bacterium possessed abundant plasmids (Pessione and Giunta, 1997). In contrast, strain CD06 is able to resist to a variety of heavy metals to rather high concentrations (Table 2). We also examined *A. radioresistence* ATCC15425 for its ability to resist cadmium and a much lower minimum inhibitory concentration was observed (500 μmol/L) compared to that of strain CD06 (1500 μmol/L). This has been also noticed in *Stenotrophomonas* sp. in which the strain isolated from heavy metal contaminated soil demonstrated much higher tolerance to heavy metals than those obtained from culture collection (Chien *et al*., 2007). The growth curves of strain CD06, CD08 and an *E. coli* in the medium without addition of heavy metals in comparison to the growth curve in the medium supplemented with selected concentrations of different heavy metals are shown in Fig.1. The results showed that compared to the heavy metal resistant strains, *E. coli* did not grow in the medium containing Cd and Cr, and also had remarkably reduced growth in the medium

Table 1 List of the selected clones and their closest genera identified by 16S rDNA sequences

Clone	Most closely related strain by 16S rDNA[a]	GenBank accession number
MB-01 (1)[b]	α-*Proteobacterium* (AB121772)[c] (96%)	EF133672
MB-02 (2)	*Hafnia alvei* (Z83203) (98%)	EF133673
MB-03 (4)	Uncultured bacterium CCM6b (AY221067) (97%)	EF133674
MB-04 (1)	*Variovorax paradoxus* (DQ256487) (98%)	EF133675
MB-06 (1)	Uncultured bacterium clone (DQ537533) (95%)	EF133676
MB 5-02 (1)	Uncultured bacterium clone (AF507681) (92%)	EF133677
A-04 (1)	*Polyangium cellulosum* (AF467674) (96%)	EF133678
A-05 (2)	Uncultured *Enterobacteriaceae* (AY635972) (98%)	EF133679
A-07 (2)	*Sphingomonas* sp. (AF324199) (99%)	EF133680
B-15 (3)	Uncultured *Acidobacteria* bacterium (DQ829252) (97%)	EF133681
MB 36 (3)	*Enterobacter* sp. (AB114268) (98%)	EF133682
MB 43 (1)	α-*Proteobacterium* (AB245349) (99%)	EF133683
MB 49 (12)	Uncultured *Clostridiales* (DQ069224) (97%)	EF133684
MB 52 (1)	Uncultured *Planctomyces* sp. (DQ084252) (92%)	EF133685
MB 56 (2)	Uncultured bacterium (AY457688) (98%)	EF133686
MB 57 (1)	Uncultured bacterium (AY921936) (94%)	EF133687
MB 67 (2)	Uncultured *Rhodospirillales* bacterium (AY755403) (98%)	EF133688

[a] Determined by the similarity of 16S rDNA sequence (Similarity value from 92% to 99% as shown in square brackets); [b] the number of clones obtained from the examined clones; [c] GenBank accession number of reference strains.

Table 2 Growth of *Enterobacter* sp. CD08 and *Acinetobacter* sp. CD06 on Luria Bertani supplemented with different heavy metals

Heavy metal concentration (μmol/L)	$OD_{600\ nm}$ for *Enterobacter* sp. CD08						$OD_{600\ nm}$ for *Acinetobacter* sp. CD06					
	500	1000	2000	3000	4000	5000	500	1000	2000	3000	4000	5000
Cd	2.4	1.6	0.3	–	–	–	4.7	2.4	–	–	–	–
Cr	3.8	–	–	–	–	–	3.5	1.7	–	–	–	–
Pb	4.9	4.9	4.3	4.1	3.9	3.8	7.8	7.7	7.6	6.3	5.1	4.7
Cu	6.0	5.6	5.5	4.0	4.4	4.2	9.0	8.4	9.0	6.4	6.1	5.8
Ni	4.9	5.3	4.4	3.5	0.3	–	6.9	6.6	5.6	3.7	3.5	–
Zn	5.0	4.9	3.2	1.7	0.4	0.4	5.7	6.9	2.6	2.1	2.4	0.6

Numerals indicate the growth as measured by $OD_{600\ nm}$ (measurement of optical density at 600 nm) within a growth period of 5 d (the highest growth measurement is listed). (–) indicates a measured value less than 0.1 after 5-d incubation. Growth after 24 h in the medium without heavy metal was 5.7 as measured by $OD_{600\ nm}$.

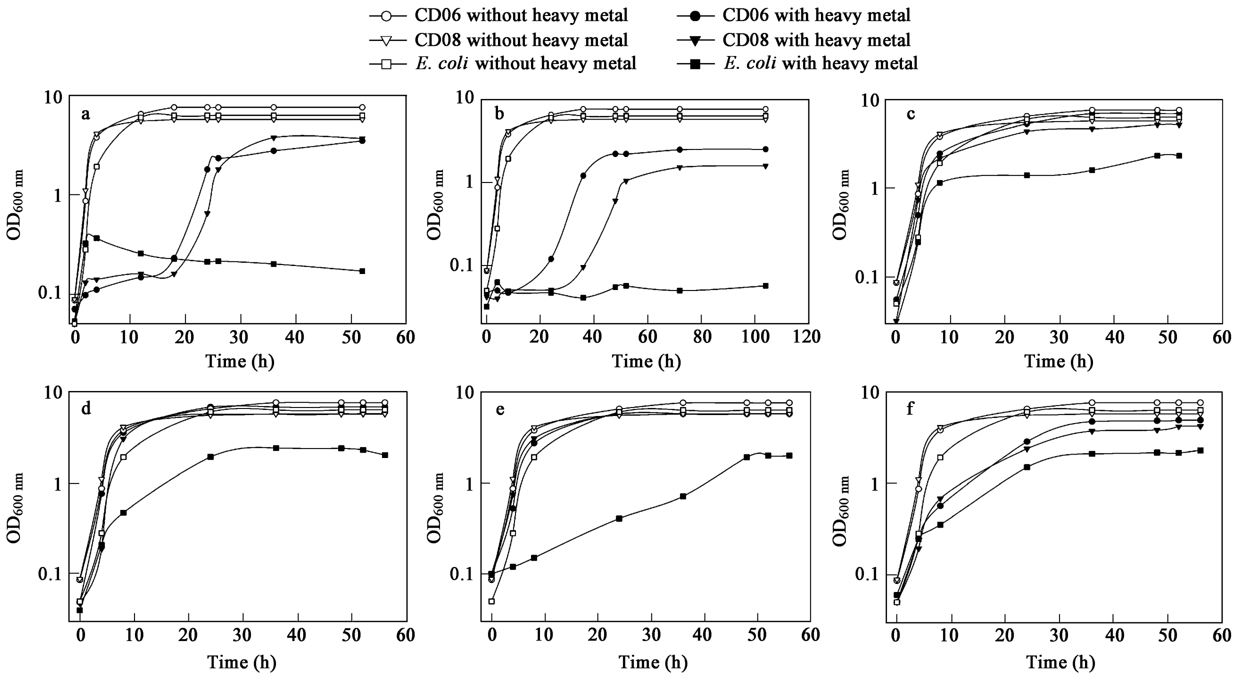

Fig. 1 Growth curves of strain CD06, CD08 and an *Escherichia coli* in the medium with and without the addition of heavy metals. The media were supplemented with heavy metals where applicable: (a) 500 μmol/L Cr; (b) 1000 μmol/L Cd; (c) 1000 μmol/L Zn; (d) 1000 μmol/L Ni; (e) 5000 μmol/L Cu; (f) 5000 μmol/L Pb.

containing other heavy metals examined.

From the enrichment culture, four out of six isolates were identified as *Enterobacter* sp. according to the 16S rDNA sequence similarity. Bacteria belong to *Enterobacteriaceae* were also identified in the culture-independent approach in higher proportion compared to many other type of microorganisms. The other microorganism that was identified from culture-independent approach with the highest proportion was bacteria belong to *Clostridiales*. We did not employ anaerobic culturing techniques for our enrichment culture isolation, therefore, no *Clostridium* and related microorganisms were isolated in this study. However, anaerobic microorganisms unquestionably are one of the most significant communities in such environment, and may also valuable for further bioremediation applications.

Heavy metals resistant traits are often carried by plasmids. *Enterobacter cloacae* and *Klebsiella* sp. isolated from industrial effluents capable to resist to heavy metals were demonstrated to harbor related plasmids (Haq *et al.*, 1999). An attempt to isolate plasmids was unsuccessful. Further investigation is required for the confirmation of the presence of the plasmids as well as the mechanisms of heavy metals resistance of these isolates.

3 Conclusions

The microbial diversity in a soil sample from a site next to a chemical industrial factory previously heavily contaminated with cadmium was examined by both culture-independent and culture-dependent approaches. Three different types of microorganisms that resist to high level of heavy metals were isolated in pure cultures. However, seventeen types of microbes were identified by culture-independent method. The different results between culture-independent and culture-dependent approaches were due to both enrichment culture techniques bias and the relevance of the microbial resistant to the metal pollutants may not be totally relevant in culture-independent results. However, from the application of bioremediation point of view, the isolation of the microorganisms and the bacterial effects on the removal of heavy metals are essential.

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