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Detection, isolation, and identification of cadmium-resistant bacteria based on PCR-DGGE

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Abstract

This study focused on the screening of cadmium-resistant bacterial strains from Pb-Zn tailing. We investigated the diversity of microbial community inhabiting Dong-san-cha Pb-Zn tailing in Beijing, China, by polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene of bacterial strain, and found two dominant strains in the DGGE profile. Using special culture media, we isolated two strong cadmium-resistant bacterial strains. On the basis of morphological, physiological, and biochemical characteristics, BIOLOG, and 16S rDNA sequencing, the two strains were identified as *Bacillus cereus* and *Enterobacter cloacae*. Minimal inhibitory concentrations (MICs) of heavy metals for the bacteria were determined. *E. cloacae* showed higher MIC values for heavy metals and a larger range of antibiotic resistance than *B. cereus*.

Key words: *Bacillus cereus*; *Enterobacter cloacae*; PCR-DGGE; cadmium resistance; antibiotic resistance

Introduction

Among heavy metals, cadmium (Cd) is widespread and one of the most toxic pollutants of the surface soil layer, released into the environment by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels (Tang *et al*., 2006). Cadmium is nonessential but poisonous for plants, animals, and humans (Gupta and Gupta, 1998). In plants, Cd inhibits root and shoot growth, affects nutrient uptake and homeostasis, and frequently is accumulated by agriculturally important crops. Cd is consumed by animals and humans in their diet and can cause diseases (Belimov *et al*., 2005). Human exposure to low levels of Cd can result in renal damage, osteomalacia, and lung cancer, as well as damage the cardiovascular system, liver, and reproductive system (Hrudey *et al*., 1995; USEPA, 1992). Cadmium has an extremely long biological half-life (>20 years) and is listed as one of the 126 priority contaminants by the US-EPA and as a human carcinogen by the International Agency for Research on Cancer (IARC, 1994). Thus, cadmium pollution attracts the most attention of environmentalists throughout the world.

Contamination of soil with heavy metals affects the qualitative and quantitative structure of microbial communities, resulting in decreased metabolic activity and diversity (Giller *et al*., 1998). Although many soil bacteria are tolerant to heavy metals and play important roles in mobilization of heavy metals (Gadd, 1990; Idris *et al*., 2004), microbial response to soil contamination varies because of variations in bioavailability and exposure to harmful metals. Soil bacteria can resist toxicity by transforming metals into less toxic forms, immobilizing metals on the cell surface or in intracellular polymers, and precipitation or biomethylation (Elena *et al*., 2005). Microorganisms inhabiting cadmium-contaminated sites are important material for both study and applications of bioremediation for differential targets.

Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 16S rDNA sequences obtained from community DNA extractions, which was described by Muyzer *et al*. (1993) and Muzyer *et al*. (1998), as a new culture-independent genetic fingerprinting technique, is a faster method for describing microbial community structure and diversity in environmental samples, either at a gross taxonomic level or at a more refined, e.g. genus level (Valria *et al*., 2006). The major advantage of this technique is that it allows the direct determination of bacterial genetic diversity, making it superior to cloning and subsequent sequencing. However, this does not exclude the latter techniques from identifying the key microorganisms (Rombaut *et al*., 2001).

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The aim of this study was to isolate and identify cadmium-resistant bacteria, which demonstrate greater resistance towards cadmium. It provides a foundation for on-line detection and bioremediation of cadmium contamination in the future. First, we detected the predominant bacterial community structure and diversity in Pb-Zn tailing in a suburb of Beijing using PCR-DGGE; and then, according to the main bands of the DGGE profile that had been sequenced, we selected and identify cadmium-resistant bacteria using the selective medium. Finally, we compared the antibiotic and heavy-metal resistance of isolated cadmium-resistance strains.

1 Materials and methods

1.1 Pb-Zn tailing and sample collection

Cadmium occurs mostly as an impurity of zinc ores (Phillips *et al*., 2005), due to similar geochemical behaviors of these metals. Contaminated sites can be a preferential source of microorganisms, which represent important material for both study and applications of bioremediation. So we selected Pb-Zn tailing as our sampling sites.

Dong-san-cha Pb-Zn tailing is located in a suburb of Beijing City. Soil samples contaminated by cadmium from five points were collected aseptically: plot (1) hill side, 14.6 g/kg; (2) slagheap, 168.6 g/kg; (3) woods, 2.4 g/kg; (4) ore of the mine, 115.5 g/kg; (5) inside of the mine, 226.9 g/kg. Each soil sample (about 1 kg) was taken from the plough layer (0–20 cm) and passed through a 60-mesh sieve. Total concentration of cadmium in all samples analyzed using atomic absorption spectrophotometer ranged from 2.4 μg/g to 226.9 μg/g, and the lowest and the highest concentrations of Cd were about 2.6 times and 241.4 times higher than the average (0.94 μg/g; Phillips *et al*., 2005) in the control paddy soils, respectively.

1.2 Extraction of total DNA

For PCR amplification of 16S rDNA, total DNA was extracted as described by Zhou *et al*. (1996) with minimal modification. Purification of total DNA using silver bead DNA gel extraction kit (shanghai Sangon: SK111) according to the manufacturer's instructions and followed by suspension in 30 μl of TE. DNA solution was estimated by agarose gel electrophoresis (1.0%, containing ethidium bromide).

1.3 PCR amplification and DGGE conditions

Total DNA from the five samples was used as a template to amplify the V3 variable region of bacterial 16S rRNA gene by PCR using the universal primers F357 (5′-TACGGGAGGCAGCAG-3′) and R518 (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al*., 1993). A GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGG-3′) was linked to the first primer to obtain F357-GC (Ana and Baltasar, 2006).

PCR reaction system (50 μl) included 0.25 μl of Taq polymerase (5 U), 1 μl of primers F357 and R518 (5 pmol), 1 μl of DNA dilution (approximately 1 ng), 5 μl of ten-fold PCR buffer, 5 μl of $MgCl_2$ (25 mmol/L), and 37.75 μl of UV-sterile water and the system was programmed with 35 cycles at 94°C for 30 s, 56°C for 20 s, and 68°C for 40 s. The results were analyzed by 1.2% agarose gel electrophoresis.

The expected size of the amplified fragment was 240 bp. DGGE analysis was performed in a DGGE apparatus (Bio-Rad, Richmond, CA, USA) at 60°C on 8% polyacrylamide gels with denaturing ranges from 30% to 50%. The electrophoresis condition was 120 V for 5 h. Bands were visualized using a UV transilluminator after staining the gel with ethidium bromide and photographed.

1.4 Sequencing of selected DGGE bands

In order to select cadmium-resistant strains in cadmium-contaminated soil samples, the bands that were dominant and distinct in DGGE profiles in higher concentration of cadmium-contaminated samples were identified.

The selective bands on the EB-stained DGGE gel were assigned to different species after their isolation, reamplification by PCR, and sequencing. The selective bands were incised and then placed in 1.5 ml tube to reclaim the DNA. DNA was reclaimed using DNA reclaim kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The reclaimed DNA was used as a template to reamplify the bands with the same pair of primers (not containing the GC clamp) and the same PCR conditions as described earlier. Amplicons were then purified by EZ-10 DNA Gel Extraction Kit (BBI.) according to the manufacturer's protocol and sequenced with one of the amplification primers. These sequences were finally compared with similar sequences in the Genbank DNA database using BLAST analysis (Basic logical alignment search tool, BLAST at NCBI) (Li *et al*., 2006).

1.5 Isolation of cadmium-resistant bacteria

According to the result of sequencing selective DGGE bands, we knew there were two dominant species of cadmium-resistant bacteria in Dong-san-cha Cd-Zn tailing: *Bacillus* sp. and *Enterobacter* sp. Thus, selective enrichment cultures were used to obtain these strains that were capable of being cadmium resistant.

Bacillus sp. was isolated with *Bacillus cereus* selective agar (Chen, 1995.) (polymyxin-mannitol-egg yolk-phenol red agar, PMYPA), 1.0% peptone, 1.0% beef extract, 1.0% mannitol, 1.0% sodium chloride, 1.5% agar, 0.003% phenol red solution, supplemented with 100 mg/L of polymyxin B sulfate, and 100 ml/L of egg yolk sterile, pH 7.0–7.2, and this medium was designed by Mossel *et al*. (1967).

Enterobacter sp. was isolated with MacConkey agar (No. 3): peptone 2.0%, lactose 1.0%, bile salt 0.15%, sodium chloride 0.5%, neutral red 0.003%, crystal violet 0.0001%, agar 1.5%, pH 7.0–7.2 (Chen, 1995).

The bacterial isolation was carried out following the conventional procedure: 1 g of mixed soils from Cd-Zn tailing samples was added to 25 ml Peptone water medium with 100 mg/L $CdCl_2$ (analytical-grade, filter sterilized) as

selective stress. The enrichment of bacteria was incubated overnight in 100 ml flasks at 30°C and 180 r/min in a rotary shaker. The suspension of enrichment cultures were plated onto their own selective media with 100 mg/L $CdCl_2$, respectively, and cultivated for 5 d at 30°C. Morphologically distinct colonies were picked and routinely maintained at 30°C, and at the same time stored at 4°C for further analysis. Monthly transfer was performed under aseptic condition with 100 mg/L $CdCl_2$, respectively.

1.6 Identification of bacteria

Preliminary characterizations of the isolates were based on observation of cell growth on agar plates, the Gram-staining reaction, microscopic observation of cell dimensions, and some standard biochemical techniques. Isolates were further identified by both BIOLOG system and analyzing 16s rDNA sequences

BIOLOG system uses a 96-well microplate with a different carbon source in each well (Biolog, 2002). The microplates are specific for Gram positive and Gram negative isolates. Lawns of each isolate were streaked on TSA plates and incubated at 28°C for 24 h. Cultures were then suspended in phosphate buffer solution (PBS), and the absorbance was measured using a BIOLOG spectrophotometer at the wavelength of 600 nm. Gram-negative bacteria were suspended to a transmittance of 50%, and Gram-positive bacteria were suspended to a transmittance of 20%–28%. The suspension was then poured into a sterile container, and 10 μl was dispensed into the wells of the BIOLOG plate using an 8-tip micropipette. The plates were then incubated at 28°C for 24 h. Individual wells containing media that was purple indicated positive growth and wells that remained colorless indicated negative growth. The pattern of positive and negative results was entered into the Microlog computer program (BIOLOG), and a database search was conducted for the closest match.

16S rDNA genes were amplified by PCR using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) (Wilson *et al*., 1990). PCR products were quantified by electrophoresis on an agarose gel (1.2%) and purified using an EZ-10 DNA Gel Extration KIT (BBI.). A more complete 16S rDNA sequence of approximately 1400 bp were compared to those in the Genbank database and those of the Ribosomal Database Project using the BLAST program. Sequences with a percentage identity of 97% or higher were considered to represent the same species (Ana and Baltasar, 2006).

1.7 Cadmium and other heavy metal resistance

Tolerance to Cd was estimated visually by minimal inhibitory concentration (MIC) containing Cd at concentrations from 0 to 2000 mg/L Cd^{2+}. A MIC of Cd was determined for each strain. Tolerance of bacteria to other heavy metals was also monitored in the same manner. The standard strain of *B. cereus* ATCC 14579 and *E. coli* ATCC 25922 (source: China Center for Type Culture Collection) were used as control. All the tests were conducted twice with three replicates for each strain. The lowest concentration of heavy metals at which no growth occurred, when compared with the control plate was considered as MIC.

All metal salts were added to the medium after autoclaving and cooling at 45 to 50°C from the filter-sterilized stock solutions. The metal salts used for the study included Co $(CH_3COO)_2{\cdot}4H_2O$, $Pb(NO_3)_2$, $CdCl_2{\cdot}2.5H_2O$, $ZnCl_2$ and $CuSO_4{\cdot}5H_2O$.

1.8 Antibiotic profile

Antibiotic susceptibility of each isolate was determined by the disk diffusion method (Bauer *et al*., 1966). Disks of ampacillin (10 μg), erythromycin (15 μg), tetracycline (30 μg), kanamycin (30 μg), rifampicin (5 μg), chloramphenicol (30 μg), and streptomycin(300 μg) were used. The sensitivity and resistance of each isolate were determined by the criteria of the National Committee for Clinical Laboratory Standards (1997). The standard strain of *B. cereus* ATCC 14579 and *E. coli* ATCC 25922 (source: China Center for Type Culture Collection) were used as control.

2 Results and discussion

2.1 PCR-DGGE analysis and identification of selected DGGE bands

The diversity of bacterial community in each sample was studied with PCR-DGGE analysis of amplified V3 region 16S rDNA genes. The DGGE analysis was performed on the total DNA extracted directly from five soil samples of Pb-Zn tailing.

The DGGE banding patterns showed that the number and intensity of migrating bands of the DNA profiles of all samples were changeable (Fig.1). Each sample showed a specific profile. Higher numbers of bands were obtained

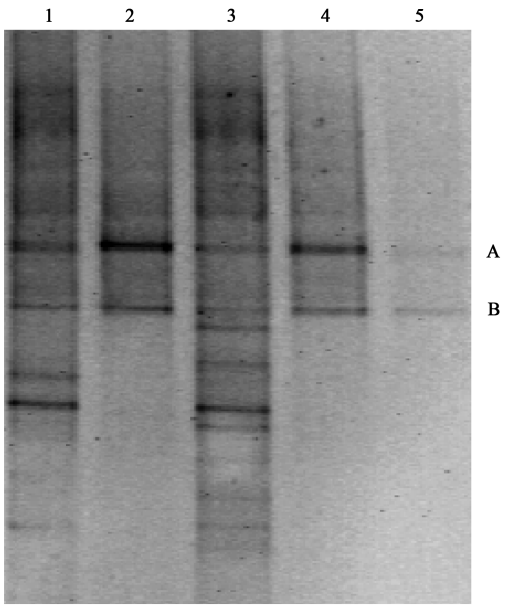

Fig. 1 DGGE profiles of the 240-bp PCR fragment of 16S rDNA genes (V3 region) amplified from different soil samples: Lanes 1–5 correspond to 1–5 soil samples, respectively.

with 1 and 3 soils. This decrease in band numbers was found with the 2, 4 and 5 soils, respectively. This DGGE gel showed that the numbers of bands were dependent on the level of contamination with cadmium: more cadmium contamination, less bands.

Two common bands (band A and B, Fig.1) were shared by all profiles, and it appeared more clearly in soils 1 and 3, whereas other bands were faint in several lanes and more varied across profiles. Although the two dominant bands in soil 5 were faint, maybe because of nutrition shortage for bacteria to live, but existent. The DGGE analysis showed that the community structure and genetic diversity were badly affected by heavy metals, and the increase of heavy metals led to the decrease of diversity. In the most contaminated soil there are only two evident bands indicating the most predominant bacteria in the Pb-Zn tailing. In order to isolate greater cadmium-resistant bacteria, the two prominent bands (bands A and B) in the soil profiles from the five soil samples were selected for sequence analysis and showed maximum homology to *Bacillus* sp. (A: 100% identity to *B. cereus*) and *Enterobacter* sp. (B: 99% identity to *E. cloacae*) in the sequence of the V3 region analyzed, respectively.

The bands on DGGE gel represented microbial species (Ercolini, 2004), so the two dominant bands were considered to be the king strains (*B. cereus* and *E. cloacae*) in the Pb-Zn tailing. The DGGE experiments suggested that potential strains with higher cadmium resistance in the highly contaminated Pb-Zn tailing were *Bacillus* sp. and *Enterobacter* sp.

2.2 Isolation and identification of strains

According to the result of DGGE analysis, we knew that the dominant bacteria in the highly contaminated Pb-Zn tailing were *Bacillus* sp. and *Enterobacter* sp., and we used selective media, which were beneficial for the growth of these two strains, to isolate the cadmium-resistant bacteria. Finally, two Cd-resistant bacterial strains were isolated from the soil samples of the Pb-Zn tailing designated as BG and BS. The results for strains BG and BS are shown in Table 1. According to its morphology, cultural appearance, physiological and biochemical characteristics, together with the BIOLOG identification system and analysis of 16S rRNA gene sequences, BG was identified as *Bacillus cereus*, and BS was identified as *Enterobacter cloacae*.

Photographs of Gram-stained isolated strains (*B. cereus* and *E. cloacae*) from solid medium in Fig.2 were distinguished as Gram-positive bacterium (*B. cereus*), Gram-negative bacterium (*E. cloacae*), and rod-shape strains according to the micrographs. SEM micrographs of isolated strains are shown in Fig.3. From SEM micrographs it was possible to determine cellular dimensions: (0.9–1.2) × (3.0–5.0) μm (*B. cereus*) and (0.6–0.8) × (1.2–3.0) μm (*E. cloacae*).

2.3 Heavy metal resistance and antibiotic tolerance of isolate strains

2.3.1 Heavy metal resistance

A MIC (minimal inhibitory concentration) is the lowest concentration of the heavy metals that completely inhibited strain growth (Froidevaux *et al*., 2001). MIC of cadmium

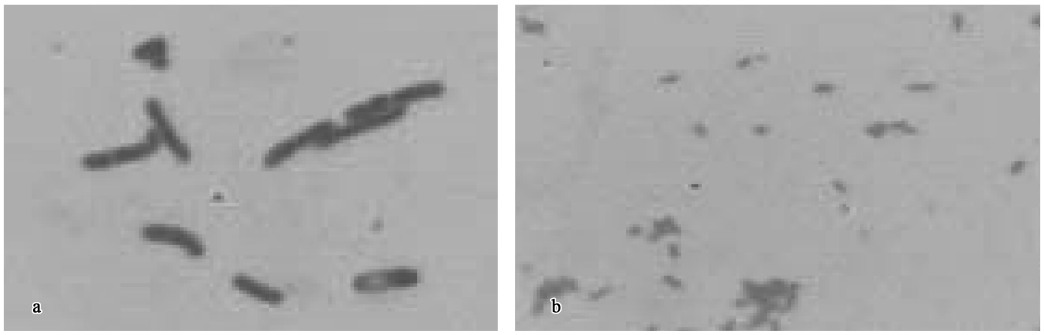

Fig. 2 Photographs of Gram-stained isolated strains. (a) *Bacillus cereus* (BG); (b) *Enterobacter cloacae* (BS).

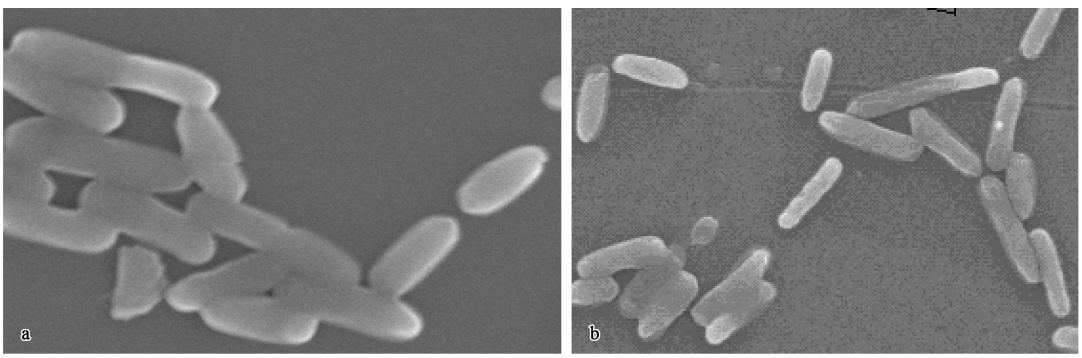

Fig. 3 SEM images of isolated strains. (a) *Bacillus cereus* (BG); (b) *Enterobacter cloacae* (BS).

Table 1 Summary of characteristics of strains *Bacillus cereus* (BG) and *Enterobacter cloacae* (BS)

Content of identification	BG	BS
Form size (μm)	Rod (0.9–l.2) × (3.0–5.0)	Rod (0.6–0.8) × (1.2–3.0)
Gram stain	Positive	Negative
Gemma	+	–
Flagellum	+	+
Motility	+	+
Yellow pigment formed	–	–
V-P reaction	+	+
Contactwise	+	N
Indole production	–	–
d-glucose	+	+
l-Arabinose	–	+
Raffinose	+	+
Cellobiose	+	+
Gas from glucose	+	+
Phenylalanine deaminase	–	–
Hydrolysis of gelatin	+	+
Use of propionate	–	+
SIM value of BIOLOG	0.96	0.78
Homology of 16s rDNA sequences	100%	99%

–: 0–10% positive; +: 90%–100% positive; N: not identify. *d*-Glucose, *l*-arabinose, raffinose and cellobiose belong to reaction of acid produce. SIM value of BIOLOG >0.5, considered to be the same species. 16s rDNA sequences with a percentage identity of 97% or higher were considered to represent the same species.

Table 2 Minimal inhibitory concentration (MIC) of various heavy metals to the isolates

Metal (mg/L)	BG	*B. cereus* (ATCC14579)	BS	*E. coli* (ATCC25922)
Cadmium	1200	50	2000	50
Cobalt	200	5	250	10
Copper	600	25	550	20
Lead	690	25	700	85
Zinc	450	20	500	65

and other heavy metals are shown in Table 2. As can be seen in Table 2, these two strains showed significant resistance to high concentrations of cadmium, and also have great tolerance to copper, lead, zinc and cobalt. MICs were almost identical in both strains, which implied that these strains presented relatively good resistance against Cu^{2+}, Pb^{2+} and Zn^{2+}. The order of toxicity of the metals to the two strains was found to be Co>Zn>Cu>Pb>Cd. In contrast, the standard strain ATCC14579 and ATCC25922 showed very low heavy metals resistance in the test.

Some heavy metals, such as nickel, cobalt, and zinc are essential to microorganisms as trace nutrients, in contrast to others such as cadmium and lead, which have no known beneficial roles (Nies, 1992). However, all heavy metals are toxic in micromolar or millimolar concentrations, yet certain bacteria are capable of growing in metal contaminated areas. In this study, we found the same conclusion: The growth of two strains was stimulated at low concentrations of lead and copper and then was inhibited as the concentrations increase. It is well known that Cd^{2+} is one of the most powerful biological inhibitors, so the growth of two strains were inhibited with cadmium, even at low concentrations.

In general, BS had a higher tolerance than BG to Cd and other heavy metals tested such as lead, cobalt, and zinc. Gram-positive bacteria are less tolerant to heavy metals than Gram-negative bacteria, so gram-negative bacilli, BS were selected for further studies.

2.3.2 Antibiotic profile

The ability of microorganisms to resist antibiotics and tolerate metals seem to be the result of exposure to metal-contaminated environments that cause coincidental selection of resistance factors for heavy metals and antibiotics (Foster, 1983; Ramteke, 1997), so antibiotic resistance of isolate strains were also studied.

Susceptibilities to the antibiotics differed among each strain (Table 3). BS showed resistance to Ampicillin, Erythromycin, kanamycin, rifampicin. BG showed resistance to Ampicillin (Table 3). In contrast, the standard strain ATCC14579 and ATCC25922 showed no resistance in the test. The antibiotic resistance and susceptibility profile matched the published profile for these organisms.

Table 3 Antibiotic profile results for the two isolates

Antibiotic/ strains	BS	*E. coli* (ATCC25922)	BG	*B. cereus* (ATCC14579)
Ampicillin	+	–	+	–
Tetracycline	–	–	–	–
Erythromycin	+	–	–	–
Kanamycin	+	–	–	–
Rifampicin	+	–	–	–
Streptomycin	–	–	–	–
Chloramphenicol	–	–	–	–

+: resistant, –: susceptible.

3 Conclusions

In this study, according to the PCR-DGGE technique, we found distinct differences in microbial community structure, with a lower diversity in soil more contaminated with heavy metals compared with that of lower contaminated soil, the same as Smit *et al*. (1997). Furthermore, we isolated two new cadmium-tolerant strains by DGGE profile from the Pb-Zn tailing. The two highly resilient cadmium-resistant strains were identified as BG and BS, respectively, according to their morphological characteristics, physiological and biochemical characteristics, and analysis of 16S rRNA gene sequences.

We concluded that the Gram-negative strain BS has higher resistance to cadmium and other heavy metals, and wider antibiotic range than the Gram-positive strain BG. The ability of microorganisms to resist antibiotics and tolerate metals appears to be the result of exposure to metal-contaminated environments that cause coincidental selection of resistance factors for heavy metals and antibiotics. Elucidation of the exact resistance mechanisms needs further investigation.

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