

Isolation and Phylogenetic Analysis of Chromium(VI) Reducing Bacteria of a Magnetite Mine Drainage from Hebei China

Zhitang Lu(Corresponding author), Xiufang An & Weiwei Zhang

College of Life Science, Hebei University,

Key Lab of Microbial Biodiversity Research and Application of Hebei Province, Baoding 071002, China

Tel: 86-312-507-9696 Fax: 86-312-507-9364 E-mail: lzt325@sina.com

Received: January 7, 2011

Accepted: January 20, 2011

doi:10.5539/mas.v5n2p113

This research was funded by National Natural Science Foundation of China (No.30800003).

Abstract

A magnetite mine drainage sample collected from Han-Xing Iron mine area in Hebei Province was domesticated with potassium dichromate. Four kinds of media supplemented with 300mg/L chromium(VI) were used to isolate chromium(VI) tolerant bacteria. 10 Cr(VI) reducing strains which can tolerant at least 350 mg/L Cr(VI) and reduce the Cr(VI) efficiently are screened by observing the growth station and measuring the remaining chromium (VI) concentration in media. 16S rDNA sequencing and phylogenetic analysis showed that *Brevundimonas* spp. are the main group of chromium(VI) tolerant bacteria in the magnetite mine drainage water sample.

Keywords: Magnetite, Cr(VI) reducing bacteria, Isolation, Phylogenetic analysis, *Brevundimonas* spp

Hexavalent chromium has strong toxicity than trivalent chromium, it can cause mutation to organism, and it is one of the crucial sources for heavy metal environmental pollution. The chromium(VI) (Cr(VI)) contained waste water is produced in large quantities and discharged to the environment by the chromic salts production, chromium electroplating industry and leather industry etc., which caused considerable environmental problems and aroused widespread concern all over the world. Many microorganisms have been found that can tolerant to an extremely high concentration of heavy metal ions and relieve the toxicity to growth, and we can take the advantage of the characteristics and mechanism of the resistance to heavy metal of some microorganisms in the hazard-free treatment of various heavy metal polluted environments (Chen et al., 2002). "*Pseudomonas dechromaticans*" is the first bacteria which was found have the ability to reduce chromium(VI), it can utilize chromates and bichromates as oxygen donors during growth on organic substances in anaerobic conditions, and reduce the hexavalent chromium to trivalent (Romanenko et al., 1977). From then on, widely chromium (VI) reduction bacteria were found, such as *Pseudomonas fluorescens* strain LB300 (Lawrence et al., 1988), *Pseudomonas putida* (Ishibashi et al., 1990), *Escherichia coli* ATCC 33456 (Shen et al., 1993), and so on. Microbial treatment on Cr(VI) pollution has the advantages of economy, efficiency and no secondary pollution (Long et al., 2004). So, it is environmental friendliness and is hoped to be used to treat many kinds of chromium (VI) containing waste water in many countries (Cheng et al., 2008).

In our previous study, we found there are a lot of chromium (VI) tolerant bacteria in the magnetite mine drainage samples. Thus, we tried to isolate the Cr(VI) reducing bacteria from a magnetite mine drainage sample and determine the phylogenetic status of them .

1. Materials and methods

1.1 Source of the sample

The magnetite mine drainage used for bacteria isolation was collected from the Qianshan Mining of Han-Xing iron mine area in Shahe, Hebei Province, China, on November 4, 2009. The depth of the mine is about 300 meters; the pH value of the water is 6.5 by field testing with a portable acidometer. The mine drainage sample was collected by a sterilized plastic bottle and used for domestication immediately after taken back to the laboratory.

1.2 Domestication and isolation

The chromium tolerant bacteria in the sample were domesticated by the following procedure: the mine drainage

sample was inoculated by 5%(v/v) to the domestication medium (yeast extracts 1.0 g, glucose 1.0 g, deionized water 1000 mL, natural pH, autoclaving at 121°C for 20 min) supplemented with 200 mg/L of potassium dichromate, cultured for 3 days at 28 °C by shaking at 200r/min; then the domesticated sample was transferred by 5%(v/v) to a new domestication medium supplemented with 400 mg/L of potassium dichromate, culturing for 3 days by the same condition; and the like, until the potassium dichromate concentration up to 1000 mg/L for domestication culture.

After the final domestication, the Cr(VI) tolerant bacteria were isolated by dilution plating method on four kinds of media according to the reported methods and modified by this experiment. 9K medium (Silverman et al., 1959) and 9K' medium (Wan et al., 2008) were used to isolate acidophilus bacteria from other acidic metal minerals, the modified media at pH 5.5 were used in this study for the pH of the sample is near the neutral. M5' medium (Lee et al., 2000) was reported for the isolation of actinomycete from gold-cave and used for isolation in this trail. PCA is the most commonly used medium for counting the "total" bacteria of various water medium (Greenberg et al., 1992; Marshall., 1992), in this study, we chose to use 1/5 PCA for bacteria isolation for the oligotrophy fact of the sample.

All of the media above were supplemented with 300mg/L of Cr(VI) (equals to 847.8 mg/L potassium dichromate) and 1.25g/L of sodium pyruvate for isolation. The sodium pyruvate can promote the growth of the difficult-to-culture bacteria (Lee, 1996).

The isolates were picked after culturing for 3 days at 28°C and maintained on Luria-Bertani(LB) (Bertani, 1952) agar at 4°C or in glycerol (20%, v/v) at -20°C.

1.3 Screening of chromium (VI) reducing strains

The Cr(VI) tolerant isolates were inoculated to LB (Bertani, 1952) broth containing Cr(VI) of 300, 350, 400 and 450mg/L, respectively. After shaking at 200r/min for 3days at 28°C, the growth situation were checked and the remaining concentration of Cr(VI) were measured by 1,5-diphenylcarbonhydrozide spectrophotometric method (National standard of China GB 7467-87) (Wei,2002).

1.4 Phylogenetic analysis

Phenol-Chloroform method was used to extract genomic DNA (Marmur, 1961). The 16S rRNA genes were amplified by PCR using the universal primers 27F(5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1525R(5'-AGAAAGGAGGTGWTCCARCC-3') (Lane,1991). The PCR mixture contained 1.0 µL of each primer,1.5µL of genomic DNA (about 0.1µg/µL),1µL of dNTPs (2 mmol/L each), 5µL of 10×PCR buffer, and 2U of AmpliTaq Gold DNA polymerase (Perkin Elmer), the total volume of 50µL was reached by adding pure water. Thermal cycling was carried out using a Whatman Biometra thermocycler programmed for an initial step of keeping 4 min at 97°C, followed by 30 cycles of 45 s at 95°C, 45 s at 55°C, 90 s at 72°C, and then an over extension to the last cycle for 5 min at 72°C.

The PCR products were recovered by 1.0% low-melting-point agarose gelelectrophoresis and then sequenced by Sunbiotech Corporation (Beijing).

The sequences obtained were initially estimated by the BLAST facility of NCBI (www.ncbi.nlm.nih.gov/BLAST) and then aligned with all related sequences obtained from GenBank by Cluastal W (Thompson et al., 1994). Evolutionary distance matrices were calculated by using the method of Jukes & Cantor (Jukes et al., 1969), and a phylogenetic tree was reconstructed by the neighbour-joining method (Saitou et al.,1987).

1.5 Biodiversity analysis

The alpha biodiversity of Cr(VI) tolerant bacteria of the sample was estimated basing on Shannon-wiener index and Simpson index.

2. Results and analysis

2.1 Isolation

The isolation results (Table 1) were analyzed by SPSS11.5 software. It showed that the isolated bacteria quantity of 1/5 PCA and M5' reach significant difference at 5% level comparing with modified 9K and modified 9K' media. Although the isolation effect of 1/5 PCA and M5' did not reach significant difference at 5% level, the bacteria number on 1/5 PCA is a little more than that of M5' media, and they grow faster and stronger. While the bacteria on M5' medium grow slower and relatively weaker. The results showed that Cr(VI) tolerant bacteria of the magnetite mine drainage are neutrophilic bacteria, 1/5 PCA medium is suitable for the isolation, but the modified 9K and modified 9K' media are not.

2.2 Screening result of chromium (VI) reducing strains

All the 83 Cr(VI) tolerant isolates were screened, and 10 strains with good ability to reduce Cr(VI) at the concentration of 350 mg/L were obtained. The 10 Cr(VI) reducing strains can tolerate to Cr(VI) up to a concentration of 450 mg/L and remove the Cr(VI) by efficiently after cultured for 3 days (Table 2). All the strains showed good ability to reduce Cr(VI) at the initial concentration of 350 mg/L, of which strain cp03 has the highest reduction rate of 80%. The reduction rate of the 10 strains descend at different ratios as the Cr(VI) increase.

2.3 Phylogenetic and Biodiversity analysis

All the 16S rDNA sequences of the 10 Cr(VI) reducing bacteria are submitted to GenBank with the accession numbers HQ830175 to HQ830184. Phylogenetic analysis (Figure 1) indicates that the 10 strains belong to 5 OTUs, and they all share highly homologous to α -Proteobacteria with the highest sequence similarities from 96.5 % to 99.9%. Strains cf02, cf01, cp2, cp04, cp07 and cf04 are closest to *Brevundimonas intermedia* ATCC 15262^T with the similarity of 99.9%; Strains cp03 and cp05 are closest to *Brevundimonas mediterranea* V4.BO.10^T with the sequence similarities of 99.6 % and 99.8%, respectively; Strain cp06 shares the same similarity of 97.8% with its nearest neighbors *Sphingomonas desiccabili* CP1D^T and *Sphingomonas koreensis* JSS-26; Strain cf03 shares highest homologous to *Azospirillum rugosum* IMMIB AFH-6 with the similarity of 96.5 %.

The alpha biodiversity of Cr(VI) reducing bacteria on genus level of the sample evaluated by Shannon-wiener index and Simpson index are 0.64 and 0.34, respectively, which indicate that the magnetite mine drainage sample have low richness and evenness on genus level, and the diversity is not rich.

3. Conclusion

The bacteria isolated from the magnetite mine drainage sample have strong toleration and reduction power to the heavy metal Cr(VI), and they are hopeful adopted for microbial remediation of chromium (VI) polluted environments.

Neutrophilic bacteria are the main chromium (VI) toleration groups in the analyzed magnetite mine drainage mine sample, and 1/5 PCA medium is suitable for bacteria isolation.

The alpha diversity of Cr(VI) reducing bacteria on genus level of the magnetite mine drainage sample is poor. *Brevundimonas* spp. are the dominant Cr(VI) reducing group of the sample, accompanied with little amount of *Sphingomonas* spp. and *Azospirillum* spp..

References

- Chen Su-hua, Sun Tie-hang, and Zhou Qi-xing et al. (2002). Interaction between microorganisms and heavy metals and its application. *Chinese Journal of Applied Ecology*, 13 (2): 239-242.
- Romanenko, VI, and Koren'kov, VN. (1977). Pure culture of bacteria using chromates and bichromates as hydrogen acceptors during development under anaerobic conditions. *Mikrobiologiya*, 46(3):414-417.
- Lawrence, H B, and Henry, L E. (1988). Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB300. *Archives of Microbiology*, 155: 426-431.
- Ishibashi, Y, Cervantes, C, and Silver, S. (1990). Chromium reduction in *Pseudomonas putida*. *Applied Environmental Microbiology*, 56: 2268 -2270.
- Shen, H, and Wang, Y. (1993). Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. *Applied Environmental Microbiology*, 59: 3 771-3 777.
- Long Teng-fa, Chai Li-yuan, and Zheng Su et al. (2004). Current application situation and development on detoxification of Cr (VI) by microorganisms. *Safety and Environmental Engineering*, 11(3):22-25.
- Cheng Guo-jun, Hu Guang-ji, and Li You-guo. (2008). Isolation, identification and bioreduction of a bacterium strain resistant to chromium(VI). *Journal of South-Central University for Nationalities*, 27(3):29-31.
- Silverman, M P, and Lundgren, D G. (1959). Studies on the chemoautotrophic iron bacterium *Thiobacillus ferrooxidans* (I): An improved medium and a harvesting procedure for securing high cell yields. *Journal of Bacteriology*, 77: 642-647.
- Wan Min-xi, Yang Yu, and Qiu Guan-zhou et al. (2008). Isolation and characterization of a bioleaching bacterium from cupreous complex metallic mines, *Ecology and Environment*, 17(1):122-127.
- Lee, S D, Kang, S O, and Hah, Y C. (2000). *Catellatospora koreensis* sp.nov., a novel actinomycete isolated

- from a gold-mine cave,. *International Journal of Systematic and Evolutionary Microbiology*, 50: 1103-1111.
- Greenberg, A E, Clesceri, E L S, and Eaton, A D. (Eds.) (1992). Standard methods for the examination of water and waste water(18th ed.). American Public Health Association., Washington, D.C.
- Marshall, R. (Ed.) (1992). Standard methods for the examination of dairy products(16th ed.). American Public Health Association, Washington,D.C.
- Lee, S D. (1996). Classification of novel actinomycetes from gold mine cave in Kongju, Korea. PhD thesis, Seoul National University, Seoul, Korea.
- Bertani, G. (1952). Studies on Lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology*, 62:293-300.
- Wei Fu-sheng. (2002). Monitoring analysis methods water and waste water (4th ed). Beijing: Press of Environment Science of China,p346.
- Marmur, JA. (1961). procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology*, 3: 208-218.
- Lane, D J.(1991). 16S/23S rRNA sequencing. In nucleic acid techniques in bacterial systematics (eds Stackebrandt E, Goodfellow M). Chichester: John Wiley & Sons, p115-175.
- Thompson, J D, Higgins, D G, and Gibson, T J. (1994). Clustal w: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22, 4673-4680.
- Jukes, T H, and Cantor, C R. (1969). *Evolution of protein molecules*. In *Mammalian Protein Metabolism*, Edited by H. N. Munro. New York: Academic Press, pp. 21-132.
- Saitou, N, and Nei, M. (1987). The neighbor-joining method: a new method for constructing phylogenetic trees. *Molecular Biology and Evolution*, 4: 406-425.

Table 1. Isolation result of Cr(VI) tolerant bacteria from magnitite mine drainage sample on four different media

Isolation medium	Number of bacteria colony (cfu/mL)	Difference significant
D	1.95×10^5	a
C	1.70×10^5	a
A	0	b
B	0	b

Note: A:9K;B:9K'; C:M5'; D:1/5 PCA; All dilutions were 10^{-3} , coating volume 0.2 mL

The small letters indicate the bacteria quantity of different media reach significant difference at 5% level.

Table 2. The reduction rate of chromium reducing bacterium after cultured for 3 days

Strain number	Start concentration of Cr(VI)(mg/L)	Reduction rate(%)
cf02	350	76.0
	400	65.0
	450	40.0
cf01	350	75.6
	400	65.6
	450	51.5
cp2	350	76.0
	400	64.8
	450	52.0
cp04	350	76.8
	400	65.0
	450	45.5
cp07	350	75.8
	400	64.4
	450	47.5
cf04	350	75.4
	400	64.1
	450	55.5
cp03	350	80.0
	400	63.0
	450	51.0
cp05	350	68.0
	400	59.0
	450	52.5
cp06	350	65.0
	400	55.0
	450	40.0
cf03	350	59.0
	400	45.0
	450	42.0

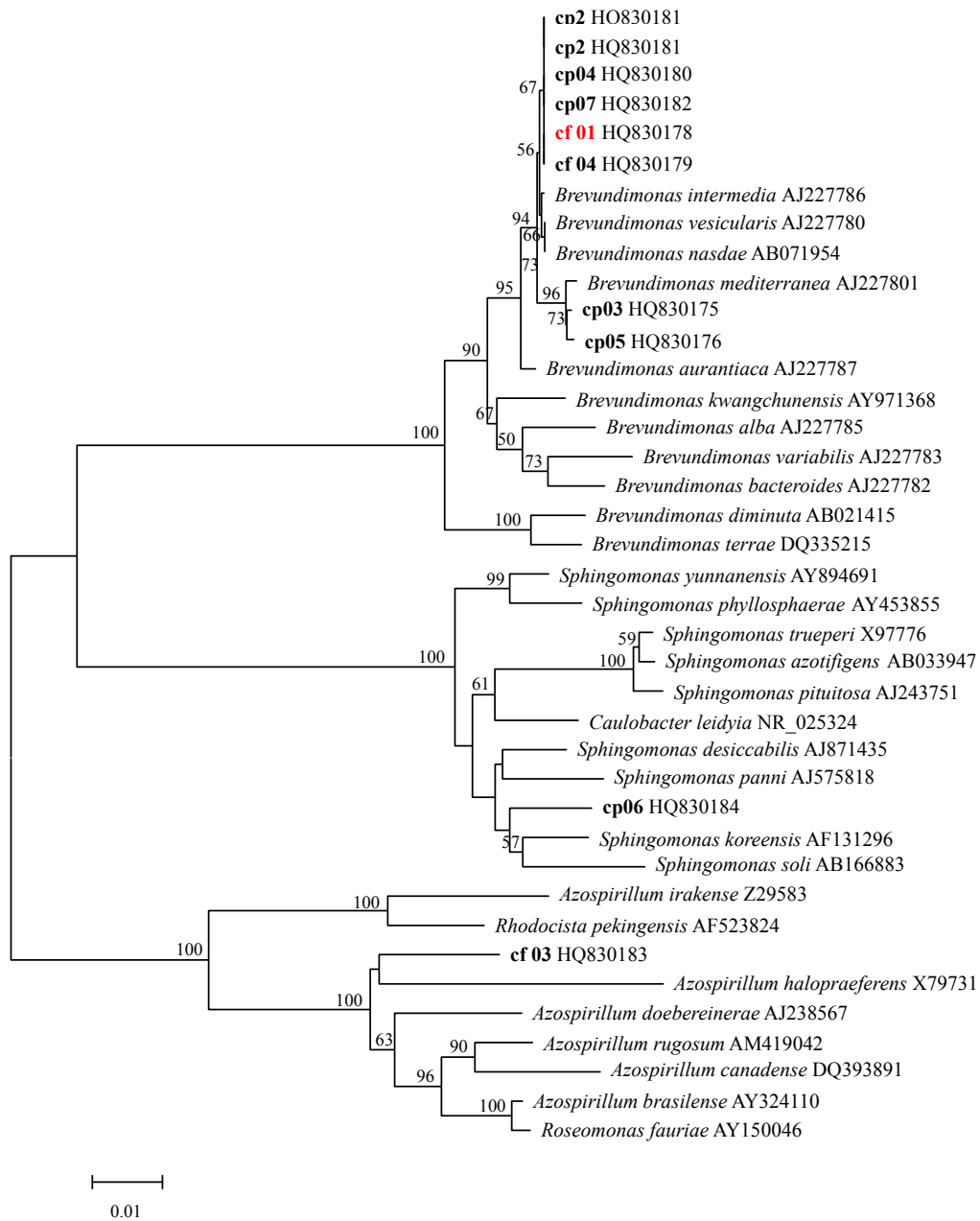


Figure 1. The phylogenetic tree of sequenced strains and their related species