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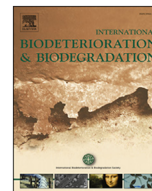

A thorough survey for Cr-resistant and/or -reducing bacteria identified comprehensive and pivotal taxa

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ABSTRACT

To better understand the bacterial potential in chromium (Cr) bioremediation, a thoroughly survey was performed to investigate the diversity and importance of Cr-resistant and/or -reducing bacterial taxa by genome mining, literature survey and laboratory isolation. Genome mining among 7887 species revealed that 1877 species distributed in 20 phyla contained Cr-resistant gene *chrA*. *Pseudomonas*, *Bacillus* and *Vibrio* were the three most abundant genera. 81 species affiliated to 4 phyla were found containing Cr(VI) reductase gene. *Bifidobacterium*, *Bordetella* and *Bacillus* were the three most abundant genera. Importantly, genome mining identified 30 genera contained both *chrA* and Cr(VI) reductase genes, while literature survey showed that 43 genera capable of Cr(VI) resistant and reducing. The two sets taxa have only four genera in overlap. We conducted an isolation work in lab in which nine Cr(VI)-resistant bacteria and one Cr(VI)-reducing *Arthrobacter* were isolated from xixi wetland sediment. Eight isolates fell in taxa identified by genome mining or literature survey, while *Lactococcus* represented a novel Cr(VI)-resistant genus, indicating that there are more Cr(VI)-resistant and/or -reducing taxa in the nature to be recognized. We present here the so-far most comprehensive Cr-resistant and/or -reducing bacterial taxa, which provide important information for Cr bioremediation.

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1. Introduction

Chromium (Cr) is one of the most widely used heavy metals in various industries such as metal smelting, electroplating, leather tanning, stainless steel manufacturing, *etc.* Cr discharge causes serious environmental pollution due to its neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity and immunotoxicity (Bagchi et al., 2002). Bioremediation of Cr pollution is considered as a green and sustainable method hence attracts lots of attention. As the most abundant microorganisms in environment, bacteria are the major resource in bioremediation. Various genera have been reported for Cr-resistant and/or -reducing including *Pseudomonas* (Song et al., 2009), *Bacillus* (Desai et al., 2008), *Shewanella* (Guha et al., 2001), *Serratia* (Zhang and Li, 2011), *Halomonas* (Focardi et al., 2012), *Enterobacter* (Rahman and Singh, 2014), *etc.* A thorough survey for bacteria capable of Cr-resistant and/or -reducing will help us better understand the bacterial potential in Cr remediation and provide technical guidelines in application.

The most common forms of Cr existing in environments are hexavalent chromium (Cr(VI)) and trivalent chromium (Cr(III)). Cr(VI) is a strong oxidizing agent and is 10–1000 times more toxic than Cr(III) which shows lower solubility and bioavailability (Katz and Salem, 1993). Bacteria have evolved various ways to survive under Cr stress such as efflux of chromate ions from cell, reduction, or SOS response, etc (Ramirez-Diaz et al., 2008; Thatoi et al., 2014). Efflux of chromate ions is the most widespread mechanism of resistance among bacteria conferred by the chromate ion transporter protein (ChrA). ChrA proteins function as chromate ions antiporters driven by proton motive force which can efflux excess chromate ions out from cell cytoplasm (Nies et al., 1998). ChrA proteins can be classified into two categories by amino acids size: short ChrA protein with a sequence length of 123–234 amino acids and long ChrA protein with a sequence length of 345–495 amino acids (Diaz-Perez et al., 2007). Long ChrA protein is predominant over short ChrA protein in number and has clear functions as chromate ion transporter (Diaz-Perez et al., 2007; Ramirez-Diaz et al., 2008), therefore only long ChrA protein was investigated in this study.

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On the other hand, chromate reduction carried out by Cr(VI) reductases from diverse bacterial species is another promising detoxification process. Cr(VI) reductases discovered so far could be classified into 6 types including nitroreductase, iron reductase, quinone reductases, hydrogenases, flavin reductases as well as NAD(P)H-dependant reductases (Thatoi et al., 2014). Among them, NAD(P)H-dependent reductase that uses NAD(P)H as cofactors, is the best studied and most widespread Cr(VI) reductase (Ramirez-Diaz et al., 2008). Enzymatic reduction of Cr(VI) has been found in diverse genera of bacteria including *Enterobacter*, *Escherichia* and *Pseudomonas*, *etc* (Thatoi et al., 2014). However an extended list of Cr-reducing bacteria is still needed.

Efflux of chromate ions through ChrA protein allows bacteria to tolerate high concentration of chromate ions and survive in harsh conditions. However, it cannot reduce the toxic effect of Cr for other organisms and clean up environments. On the other hand, Cr(VI)-reducing bacteria are able to detoxify Cr(VI) by reducing it to less toxic form but may not be able to survive under high chromate stress due to lack of resistant mechanism (Thatoi et al., 2014). For example, a *Bacillus* sp. K3 which had a high Cr(VI) reduction rate of 71 nmol mg^{-1} protein h^{-1} but showed cellular damage and sensitivity to Cr(VI) (Badar et al., 2000). Given all this, it is useful to search for strains with both resistance and reducing abilities.

The main purpose of this study is to survey and summarize the diversity of Cr(VI)-resistant and/or -reducing bacteria taxa. Nowadays, the fast growing genome database provides great information to recognize bacterial potential functions. *In silico* genome mining using long *chrA* and Cr(VI) reductase genes was carried out to identify potential Cr(VI)-resistant and/or -reducing bacteria. At the same time, a thorough literature survey and a laboratory isolation were performed to obtain actual Cr(VI)-resistant and/or -reducing bacterial taxa.

2. Materials and methods

2.1. In silico searching for Cr-resistant and/or -reducing bacteria

Date to August 2015, there were overall 28 799 genomes deposited in JGI database, affiliated to 7887 species (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). Bacteria whose genome contains long *chrA* or Cr(VI) reductase genes were regarded as potential Cr-resistant or -reducing bacteria. The keyword chromate was used in a primary screening in JGI gene searching program. The collected genes were then manually checked for authority. After confirmation, the species information and target gene/protein sequences were retrieved from JGI genome database for further analysis. The searching was done on species level, which meant, if one species contained several strains, only one positive strain was selected to represent this species.

2.2. OTU analysis of long chrA gene sequences

OTU (operational taxonomic units) clustering of long *chrA* gene sequences was done using Mothur version 1.36.1 at 60% similarity (Schloss et al., 2009). The representative sequences of each OTU exported by Mothur program were used for further phylogenetic analysis.

2.3. Phylogenetic analysis

Nucleotide or amino acid sequences were aligned by ClustalW program using MEGA version 5.1 (Hall, 2013). Phylogenetic trees of long ChrA, Cr(VI) reductase and 16S rRNA gene were constructed using the neighbor-joining method (Saitou and Nei, 1987), with bootstrap values based on 1000 replications (Felsenstein, 1985), after cutting off the redundant sequences at the end.

2.4. Literature survey for Cr(VI)-resistant & -reducing bacteria

A thorough literature survey for Cr(VI)-resistant & -reducing bacteria isolated by culture-dependent method was performed on the web of science (http://isiknowledge.com/). The keywords chromate and bacteria were used in a primary screening. From the resulted paper pool, relevant papers were read and retrieved manually one by one. The published Cr(VI)-resistant & -reducing isolates were then grouped based on taxon. The representative papers which were cited more frequently or were published in higher impact factor-journals were selected as reference of each Cr(VI)-resistant & -reducing genera.

2.5. Isolation of Cr(VI)-resistant and/or -reducing bacteria

Bacteria resistant to Cr(VI) were isolated from bank side soil of xixi wetland (30° 15′ 53.18″ N 120° 2′ 43.24″ E) in Zhejiang province, China. Soil samples were serially diluted and plated on Luria Bertani (LB) agar plates containing different concentrations (100 mg L^{-1}, 200 mg L^{-1} and 300 mg L^{-1}) of $K_2Cr_2O_7$ and incubated at 28 °C for 7 days. Individual colonies of different morphologies were picked up and purified by repeated transferring onto the same medium. Minimum inhibitory concentration (MIC) of $K_2Cr_2O_7$ for the isolates was determined on LB plates amended with different concentrations of $K_2Cr_2O_7$ (Luli et al., 1983). The concentration ranged from 100 mg L^{-1} to 1000 mg L^{-1} with intervals of 100. After incubation at 28 °C for 5 days, the highest concentration of $K_2Cr_2O_7$ which permitted growth and beyond which there was no growth was considered as MIC of $K_2Cr_2O_7$ for the isolates tested.

2.6. Bacterial identification by 16S rRNA gene

Genomic DNA of isolates was extracted using a bacterial genomic DNA extraction kit (MoBio, USA). The 16S rRNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) then sequenced by BGI Biotech Company (Shanghai, China). The 16S rRNA gene sequences were compared with sequences available in NCBI database using the BLASTN program. Reference taxa were obtained based on the highest BLASTN scores.

2.7. Measurement of Cr(VI) reduction by the isolates

To confirm reducing ability of the isolates, colony of 9 isolates were cultivated in broth cultures and on plates of LB supplemented with 100 mg L^{-1} $K_2Cr_2O_7$. The isolates which showed a color change from orange to grey green during growth were regarded as Cr(VI)-reducing bacteria. Further Cr(VI) reduction test of Cr(VI)-reducing bacteria was performed in 50 ml LB broth supplemented with 200 mg L^{-1} Cr(VI). The medium was inoculated at 28 °C and shaken at 200 rpm. During the inoculation, 1 mL culture was extracted and centrifuged. The residual Cr(VI) in the culture supernatant was measured by the 1, 5-diphenylcarbazide method (Pattanapipitpaisal et al., 2001). The uninoculated LB broth containing 200 mg L^{-1} Cr(VI) was served as negative control. All tests were performed in three replicates.

2.8. Identification of chrA or Cr(VI) reductase genes from laboratory isolates

The chrA or Cr(VI) reductase genes from the 9 isolates obtained in our laboratory were amplified by degenerate primers. Briefly, relevant gene sequences and amino acid sequences from the NCBI

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