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# Characterization and genomic analysis of a highly chromate resistant and reducing bacterial strain *Lysinibacillus fusiformis* ZC1

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ABSTRACT

*Lysinibacillus fusiformis* ZC1 isolated from chromium (Cr) contaminated wastewater of a metal electroplating factory displayed high chromate [Cr(VI)] resistance with a minimal inhibitory concentration (MIC) of 60 mM in R2A medium. *L. fusiformis* ZC1 showed resistances to multiple metals (Cu, Ni, Co, Hg, Cd and Ag) and a metalloid (As). This bacterium exhibited an extremely rapid Cr(VI) reduction capability. It almost completely reduced 1 mM $K_2CrO_4$ in 12 h. The Cr(VI) reduction ability of *L. fusiformis* ZC1 was enhanced by sodium acetate and NADH. By whole genome sequence analysis, strain ZC1 was found to contain large numbers of metal(loid) resistance genes. Specifically, a *chrA* gene encoding a putative chromate transporter conferring chromate resistance was identified. The chromate resistance was constitutive in both phenotypic and gene expression analyses. Furthermore, we found a *yieF* gene and several genes encoding reductases that were possibly involved in chromate reduction. Expression of adjacent putative chromate reduction related genes, *nitR* and *yieF*, was found to be constitutive. The large numbers of NADH-dependent chromate reductase genes may be responsible for the rapid chromate reduction in order to detoxify Cr(VI) and survive in the harsh wastewater environment.



## 1. Introduction

Chromium as an important industrial material is widely used in electroplating, dye and pigment manufacturing, wood preservation, leather tanning and alloy production. The uncontrolled release of industrial wastes has caused severe contamination of soil–water systems and subsequent chromium toxicosis because of its carcinogenic, mutagenic, and teratogenic potential [1]. Chromium toxicosis is associated with severe congestion and inflammation of digestive tract, kidney damage and hepatocellular deficiency [2,3]. Conventional technologies for chromium contaminated wastewater remediation including ion exchange, precipitation and adsorption on alum or kaolinite cannot be large-scale applied because of the high cost and subsequent secondary environmental pollution. Alternatively, bioremediation of toxic metal contaminated sites through bacteria is getting more and more attention because of its efficient, affordable and environmentally friendly advantages.

Bacteria have developed diverse strategies to resist chromate mainly through chromate reduction and chromate efflux. The primary role of these strategies is to depress chromate toxicity to cells. Chromate-reducing bacteria reduce bioavailable, highly soluble chromate [Cr(VI)] to thermodynamically stable and less toxic trivalent chromium [Cr(III)]. In addition, Cr(III) is easily formed as precipitate $Cr(OH)_3$ or $Cr_2O_3$ [4], thus such immobilized bacterial cells have been used to remove chromium from wastewater [5–7].

Cr(VI) reduction has been identified in various bacteria including *Leucobacter* [8], pseudomonad [9], *Streptomyces* [5], *Brucella* [10], *Bacillus* [11,12], *Intrasporangium* [6] and *Thermus* [13]. Chromate reduction takes place under both aerobic and anaerobic conditions. In the absence of oxygen, both soluble and membrane associated enzymes of the electron transfer system couple Cr(VI) reduction with the oxidation of an electron donor substrate. At the same time, Cr(VI) serves as the terminal electron acceptor of an electron transfer chain that frequently involves cytochrome b/c [14]. Under aerobic condition, chromate reduction is catalyzed by soluble enzymes encoded by genes located on chromosome [15]. These soluble enzymes include dehydrogenase in *Thermus scotoductus* SA-01 [13,16], azoreductase in *Shewanella oneidensis* [15], flavoprotein from *Pseudomonas putida* [17], NADH nitroreductase

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in *Escherichia coli* [17] and *Vibrio harveyi* KCTC 2720 [18], that were found to be associated with Cr(VI) reduction. However, none of these reductases have been found to be specifically induced by chromate so far and just a few of the enzymes responsible for chromate reduction have been purified and characterized.

Regarding chromate resistance related proteins, the chromate transporter ChrA responsible for Cr(VI) extrusion has been shown to be related to chromate resistance in *Shewanella* sp. ANA-3 [19], *Arthrobacter* sp. FB24 [20] and *Ochrobactrum tritici* 5bvl1 [21]. ChrA associated with a chromate accessory protein ChrB is essential for establishment of high chromate resistance in *O. tritici* 5bvl1 and strongly induced by both chromate and dichromate [21]. The membrane topology of ChrA located on a plasmid of *Pseudomonas aeruginosa* has been extensively studied and shown to consist of 13 transmembrane segments (TMS) with the N-terminus located in the cytoplasm and the C-terminus in the periplasmic space, which could be involved in Cr(VI) efflux [22].

In this study, we isolated a bacterial strain ZC1 from industrial wastewater with both high chromate resistance level and rapid chromate reduction ability. Thus, bacterial chromate removal efficiencies were evaluated in details including effects of cell density, initial Cr(VI) concentration and carbon sources. Bacterial identification was performed using morphological, biochemical/physiological and 16S rRNA gene analyses. Genes related to chromate and other metal(loid) resistances were identified by whole genome shotgun sequencing and reverse transcription PCR (RT-PCR) technology. The results give a first indication of the possible factors that are responsible for the high levels of chromate reduction and resistance in strain ZC1.

## 2. Materials and methods

### 2.1. Isolation and characterization of Cr(VI) resistant and reducing strain ZC1

An industrial wastewater sample obtained from an electroplating factory in Guangdong, China was used to isolate chromate resistant and reducing strains as described [6]. The total concentrations of Cr, Cu, Mn, Zn, Co, Pb, As and Cd in this sample determined by atomic absorption spectrometry were 97.12, 14.64, 4.25, 2.02, 0.34, 0.27, 0.12 and 0.014 μM, respectively. The ability of chromate-resistant bacteria to reduce $K_2CrO_4$ was determined by a spectrophotometric method using the reagent 1,5-diphenylcarbazide (DPC) [23]. Finally, several chromate-resistant bacteria were isolated and strain ZC1 was chosen for this study. The 16S rRNA gene of strain ZC1 was obtained from the whole genome sequence (see below) and analyzed by BlastN searching tools (http://www.ncbi.nlm.nih.gov/blast). Cell morphologies were examined under a scanning electron microscope (SEM; JSM-6390, JEOL, Japan) with 20,000 V accelerating voltage and 15,000 times enlargement. The elemental analysis of the cell surface of strain ZC1 after treatment with 1 mM $K_2CrO_4$ was performed using an energy dispersive X-ray spectroscope (EDS) coupled with SEM. Biochemical and physiological characteristics were analyzed using the API 20NE system (bioMérieux, Marcy l'Etoile, France).

The MIC, defined as the lowest metal(loid) concentration that completely inhibited the growth of strain ZC1, was determined in R2A medium. One liter liquid R2A medium contained: yeast extract 0.5 g, protease peptone No. 3 0.5 g, casamino acid 0.5 g, dextrose 0.5 g, soluble starch 0.5 g, sodium pyruvate 0.3 g, dipotassium phosphate 0.3 g and magnesium sulfate 0.05 g. Different concentration of $K_2CrO_4$, $CuCl_2$, $NiCl_2$, $Co(NO_3)_2$, $Na_2HAsO_4$, $NaAsO_2$, $HgCl_2$, $CdCl_2$ and $AgNO_3$ was added to the R2A medium as described by Sarangi and Krishnan [24]. After one week incubation at 37 °C on a rotary shaker at 200 rpm, ZC1 cell growth was measured at $OD_{600}$.

In a repeated-adding of Cr(VI) aliquots for ZC1, 300 ml LB medium (NaCl 3 g, tryptone 3 g and yeast extract 1.5 g) amended with 1 mM $K_2CrO_4$ was incubated as described above. After 1 mM $K_2CrO_4$ was almost completely reduced, another 1 mM $K_2CrO_4$ was added into the culture again (a total of five repeated additions of each 1 mM $K_2CrO_4$ in 72 h). All experiments were performed at least three times and the data shown are from one representative experiment performed with triplicate cultures, averaged, and standard deviation ($n = 3$) determined.

### 2.2. Effects of different cell densities and chromate concentrations on aerobic Cr(VI) reduction by Lysinibacillus fusiformis ZC1

The effects of different cell densities and chromate concentrations on the reduction of chromate by *L. fusiformis* ZC1 were investigated in triplicate samples in LB medium. The initial cell densities and Cr(VI) concentrations investigated were $4.86 \times 10^7$ to $1.26 \times 10^9$ and 2–5 mM, respectively. Cr(VI) reduction was studied under aerobic condition in 100 ml LB medium supplemented with appropriate amount of Cr(VI) (1 mM for cell density test) and inoculated desirable number of bacterial cells (1% overnight fresh inoculum for chromate concentration test), incubated at 37 °C with 200 rpm shaking. Residual Cr(VI) concentration was measured at regular intervals by a spectrophotometer using the reagent DPC as described above.

### 2.3. Cr(VI) reduction by resting cells of L. fusiformis ZC1 with different carbon sources

Bacterial cells of *L. fusiformis* ZC1 that grew about 18 h in 400 ml LB medium were harvested, washed twice with 10 mM Tris–HCl (pH 8.0), resuspended with 100 ml of the same buffer and amended with 0.1 mM $K_2CrO_4$. To each 10 ml bacterial suspension, 1% of methanol, ethanol, glucose, sucrose, lactose, sodium acetate and nicotinamide adenine dinucleotide-reduced disodium salt-trihydrate (NADH) was added, incubated at 37 °C for 12 h as described above. Heat-killed bacterial cells (100 °C, 10 min) were used as a negative control to monitor abiotic chromate reduction. At regular intervals, samples were centrifuged and measured residual Cr(VI) concentration as described above.

### 2.4. Chromate resistance and reduction tests

Exponential phase cultures induced with and without 1 mM $K_2CrO_4$ for 8 h were diluted 1:100 in tubes containing 10 ml fresh R2A medium with increasing amounts of $K_2CrO_4$, and incubated for 48 h at 37 °C with 200 rpm shaking. The $OD_{600}$ values were then determined by a spectrophotometer. For the chromate reduction assay, the uninduced and induced cultures were prepared as above and inoculated into 100 ml LB medium amended with 1 mM $K_2CrO_4$ and incubated at 37 °C with 200 rpm shaking for about 12 h. The residual Cr(VI) concentration was monitored as described above. Cell free LB medium with 1 mM $K_2CrO_4$ was incubated as a negative control to monitor abiotic chromate reduction.

### 2.5. Sequencing of L. fusiformis ZC1 genome

High-molecular-mass genomic DNA isolated from *L. fusiformis* ZC1 using Blood Cell Culture DNA Mini Kit (Qiagen, MD, USA) was used to construct 4–40 kb random genomic DNA libraries. The whole genome shotgun sequencing was performed using the next generation sequencing technologies Roche 454 GS-FLXTM at the Arizona Research Laboratory, Division of Biotechnology, University of Arizona. Genome comparison was performed through SEED server (http://rast.nmpdr.org/seedviewer.cgi) and

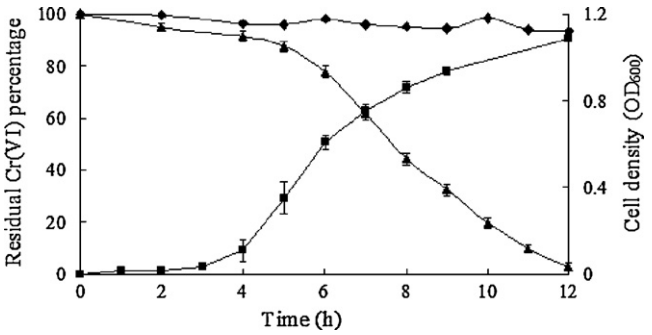


**Fig. 1.** Growth and chromate reduction curves of *L. fusiformis* ZC1: (■) growth curve of *L. fusiformis* ZC1 in LB medium without $K_2CrO_4$; (▲) Cr(VI) reduction curve of *L. fusiformis* ZC1 in LB medium (pH 8.0) with 1 mM $K_2CrO_4$; (♦) LB medium (pH 8.0) amended with 1 mM $K_2CrO_4$ without bacterial inoculation as a control to monitor abiotic chromate reduction. Error bars represent standard deviation of triplicate samples.

the subsequent results were modified manually. GC content was analyzed using CLC Main Workbench 5 program (http://www.clcbio.com). The NCBI Prokaryotic Genomes Automatic Annotation Pipeline was used for gene annotation in preparation for data submission to GenBank. (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html).

### 2.6. RT-PCR

Total RNA was obtained from mid-exponential phase strain ZC1 cells grown for 3 h in the presence or absence of 0.3 mM chromate in LB medium. Total RNA was isolated by the RNeasy Mini Kit (Qiagen) and then digested with DNase I (Fermentas, MD, USA) to remove DNA. The $OD_{260}$ values were then determined spectrophotometrically for the total RNA concentration. Equal amounts of total RNA were used to perform cDNA synthesis using iScript™ Select cDNA Synthesis Kit (Biorad, CA, USA). Standard PCR programs were used to generate amplicons from 3 μl reverse transcription reaction mixture. Primer pairs of BFC-F (5′-AAGATGGATTGGATTCATGGT-3′) and BFC-R (5′-CTAACAATCGAAAGTGCAGAT-3′), BFN-F (5′-AAAGCCCCACTGGATGTATAC-3′) and BFN-R (5′-CTACCTACGATTGCTACTACC-3′), BFY-F (5′-GGAGGAAACATCATGAAGGTA-3′) and BFY-R (5′-ATGCAGAAGTAGCATCTTTCC-3′), were designed to monitor the transcription of *chrA* (876 bp), *nitR* (752 bp) and *yieF* (626 bp), respectively. PCR amplification using RNA as template was served as a control to investigate the potential presence of DNA contamination. The positive control of RT-PCR was verified by 16S rRNA gene primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR products were analyzed by 1% agarose gel electrophoresis.

### 2.7. Deposition of nucleotide sequences

*L. fusiformis* ZC1 was deposited in The Agricultural Research Service Culture Collection, USA (NRRL http://nrrl.ncaur.usda.gov) under the accession number of NRRL B-59453. The Whole Genome Shotgun sequence of *L. fusiformis* ZC1 has been deposited at DDBJ/EMBL/GenBank under the accession ADJR00000000. The version described in this paper is the first version, ADJR01000000.

## 3. Results

### 3.1. Isolation and identification of Cr(VI) resistant and reducing bacteria

Aqueous samples collected from a site contaminated with industrial wastes of an electroplating factory, which contained as high as 97.12 μM chromium along with other metal(loid)s, were used to isolate chromate resistant and reducing bacteria. Strain ZC1 showed a MIC of 60 mM to $K_2CrO_4$ in R2A medium and rapid Cr(VI) reduction in LB medium was therefore selected for further study. This bacterium was a Gram positive, rod shaped strain. Physiological/biochemical analyses (API 20NE test) revealed that strain ZC1 was positive for catalase, oxidase, arginine dihydrolase, urease, assimilation of N-acetyl-glucosamine, gluconate and malate, but negative for β-glucosidase, protease, galactosidase, nitrate reduction, indole production, acidification of glucose, and dissimilation of glucose, maltose, arabinose, mannose, mannitol, phenyl-acetate, adipate, caprate and citrate. It was 99% identical to *L. fusiformis* isolate 24 (GenBank: EU430993) based on the 16S rRNA gene (locus_tag: BFZC1_r19938) analysis and was hereafter referred to *L. fusiformis* ZC1. *L. fusiformis* ZC1 reached log-phase after 5 to 6 h incubation in LB medium at 37 °C with shaking at 200 rpm (Fig. 1). This bacterium was also able to reduce Cr(VI) rapidly under optimum pH (8.0) and temperature (37 °C) conditions. When 1% ZC1 ($OD_{600} \approx 0.4$) was inoculated into LB broth containing 1 mM Cr(VI) and incubated at 37 °C on a rotary shaker at 200 rpm, Cr(VI) was almost completely reduced in 12 h (0.08 mM $h^{-1}$) (Fig. 1) and white precipitate was visible at the bottom of the bottle. Interestingly, after cultivation of *L. fusiformis* ZC1 for 12 h with 1 mM $K_2CrO_4$, the cell surface became very smooth (Fig. 2). No physical Cr(VI) adsorption was detected on *L. fusiformis* ZC1 cell surfaces using EDS analysis (data not shown).

The chromate reducing ability of *L. fusiformis* ZC1 was tested by five repeated additions of 1 mM $K_2CrO_4$. As shown in Fig. 3, *L. fusiformis* ZC1 reduced 94% of the first addition of 1 mM $K_2CrO_4$

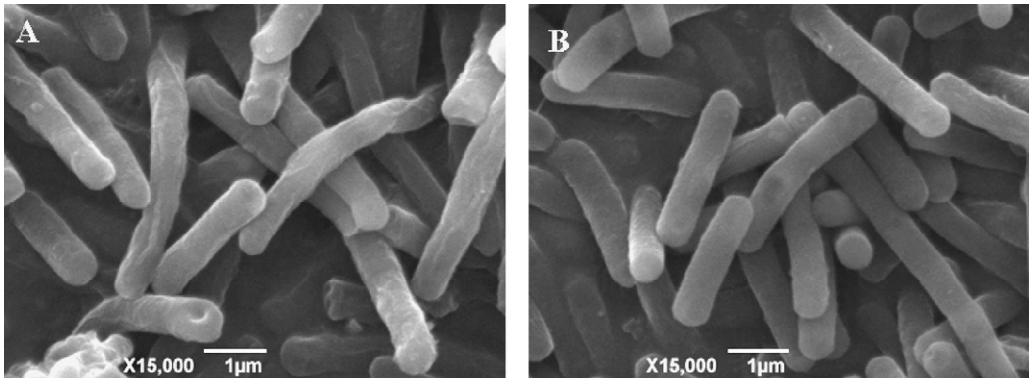


**Fig. 2.** SEM photographs of *L. fusiformis* ZC1 cells: (A) *L. fusiformis* ZC1 cells grown in LB medium for 12 h without $K_2CrO_4$; (B) *L. fusiformis* ZC1 cells grown in LB medium amended with 1 mM $K_2CrO_4$ for 12 h. Scale bars: 1 μm.

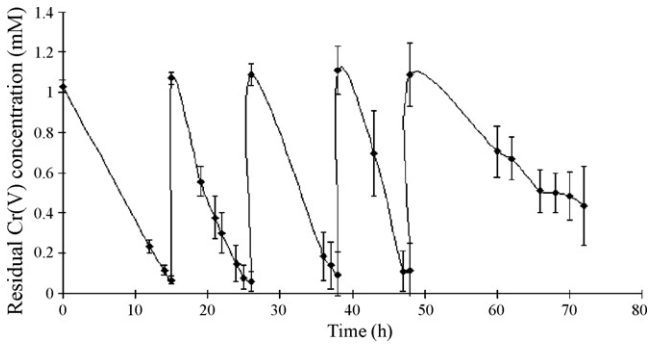


**Fig. 3.** *L. fusiformis* ZC1 chromate reduction ability tested with five times repeated spiking of 1 mM $K_2CrO_4$. The experiments were performed in LB medium (pH 8.0) and incubated at 37 °C with 200 rpm shaking for 72 h. The measured Cr(VI) concentrations of the five times $K_2CrO_4$ additions were 1.03, 1.07, 1.09, 1.11 and 1.09 mM, respectively. Error bars represent standard deviation of triplicate samples.

in 15 h, while it took another 11 h to reduce about 95% of the second aliquot of 1 mM Cr(VI). When the third aliquot of 1 mM Cr(VI) was added, Cr(VI) was reduced from 1.09 mM to 0.09 mM in 12 h. The fourth addition of 1 mM Cr(VI) was reduced from 1.11 mM to 0.11 mM in 10 h. *L. fusiformis* ZC1 could still reduce about 54% (from 1.09 mM to 0.5 mM) of the fifth addition of 1 mM Cr(VI) in 20 h (total 68 h). After 68 h, Cr(VI) reduction became less efficient.

### 3.2. Effects of initial cell densities and Cr(VI) concentrations on Cr(VI) reduction

The effect of initial cell concentration on Cr(VI) reduction was shown in Fig. 4. Cr(VI) reduction by *L. fusiformis* ZC1 increased with the increase of initial cell concentration from $4.86 \times 10^7$ to $1.26 \times 10^9$ cells $ml^{-1}$. About 0.96 mM $K_2CrO_4$ was reduced by *L. fusiformis* ZC1 in 10 h at the highest cell density ($1.26 \times 10^9$ cells $ml^{-1}$), compared to 13 h when the initial cell concentration was $4.86 \times 10^7$ cells $ml^{-1}$. The Cr(VI) reduction ability of *L. fusiformis* ZC1 was also determined by adding various concentrations of Cr(VI). As shown in Fig. 5, at an initial Cr(VI) concentration of 2, 3 or 4 mM, 91.4%, 96.5% and 96.3% of the chromate was reduced in 26, 39 and 72 h, respectively. Whereas at the initial Cr(VI) concentration of 5 mM Cr(VI), it was reduced to 2.46 mM (about 51% removed) in 84 h, with no further reduction during the following 16 h (Fig. 5).

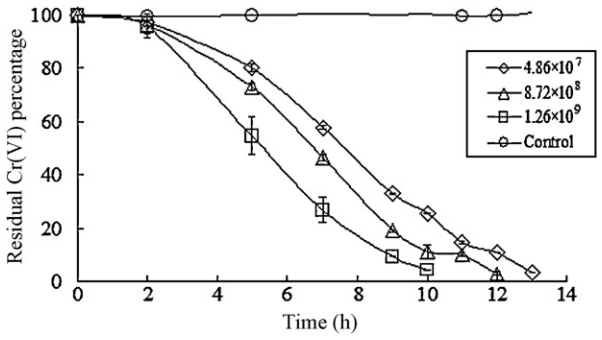


**Fig. 4.** Effects of cell densities on Cr(VI) reduction ability of *L. fusiformis* ZC1. The experiments were performed as described in Section 2. (◊), (△) and (□) represent different cell densities (cells $ml^{-1}$); (○) the abiotic control without bacterial inoculation. Error bars represent standard deviation of triplicate samples.

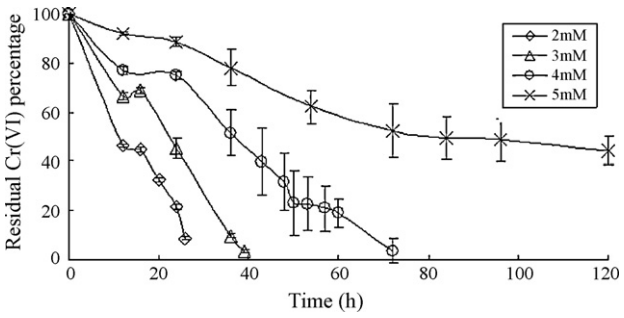


**Fig. 5.** Effects of initial chromate concentrations of 2–5 mM on Cr(VI) reduction of *L. fusiformis* ZC1. Error bars represent standard deviation of triplicate samples.

### 3.3. Cr(VI) reduction by resting cells of L. fusiformis ZC1 with different carbon sources

In Cr(VI) reduction assays using *L. fusiformis* ZC1 resting cells, sodium acetate and NADH showed effective stimulatory effects on Cr(VI) reduction (92.4% and 92.0%) compared to the control (CK+, 21%) after 12 h incubation. The presence of lactose also substantially stimulated Cr(VI) reduction by resting cells of *L. fusiformis* ZC1, while methanol, ethanol and glucose showed slight inhibitory effects on Cr(VI) reduction. No effect of sucrose on Cr(VI) reduction was observed. The heat killed bacterial resting cells (CK−) showed no obvious Cr(VI) reduction (Fig. 6).

### 3.4. General features of L. fusiformis ZC1 draft genome and genes involved in chromate metabolism

Draft genome sequence analysis of *L. fusiformis* ZC1 showed a genome size of about 4.65 Mb distributed in 126 contigs with an average GC content of 37.3%, containing 4606 putative coding sequences (CDSs). The potential origin of replication of the chromosome of *L. fusiformis* ZC1 was located on a 10.1 kb site on contig00113 including co-localization of six genes (*rpmH*, *gyrA*, *gyrB*, *recF*, *dnaN* and *dnaA*) adjacent to the origin by comparing its draft genome to the complete genomes of *Lysinibacillus sphaericus* C3-41 (GenBank: CP000817) and other *Bacillus* strains through MUMmer3.20.

A gene, *chrA*, encoding a putative chromate ion transporter (locus_tag: BFZC1_07163, 849 bp) and various genes encoding potential chromate reduction related proteins were identified from the draft genome of *L. fusiformis* ZC1 (Table S1). Two potential chromate reduction genes *nitR* (locus_tag: BFZC1_00515) and *yieF*

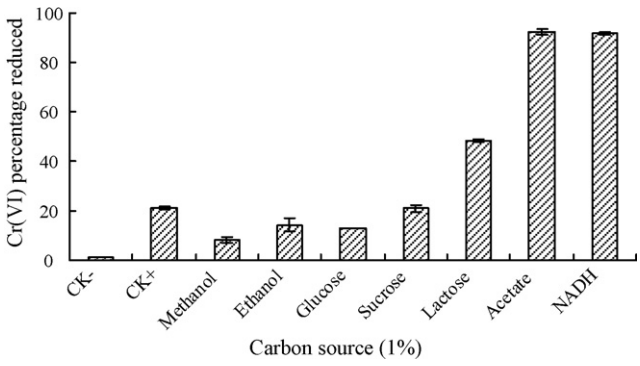


**Fig. 6.** Effects of carbon sources on Cr(VI) reduction of *L. fusiformis* ZC1 resting cells. Bacterial cells of strain ZC1 were collected and added in 0.1 mM $K_2CrO_4$ solution and further incubated at 37 °C with 200 rpm shaking for 12 h. CK−, heat killed bacterial cells (100 °C, 10 min) that used as a negative control; CK+, resting cells of strain ZC1 that previously grew in LB medium without adding a carbon source. Error bars represent standard deviation of triplicate samples.

(locus_tag: BFZC1_00520) arranged in tandem were chosen for further study because of their adjacent location and the involvement of homologs in chromate reduction.

### 3.5. Metal(loid) resistance of L. fusiformis ZC1 and identification of putative chromate resistance genes

Since *L. fusiformis* ZC1 was isolated from a site contaminated with multiple heavy metals, the MICs of strain ZC1 for these metals and metalloids were determined in R2A broth. For strain ZC1, the highest resistance was registered for As(V) (50 mM), while Ag(I) was the most toxic compared to the other metal ions. When ZC1 was incubated with increasing As(III) concentration, no viable cells were recovered at concentrations above 15 mM. The MICs of *L. fusiformis* ZC1 for Cu(II), Co(II), Ni(II), Cd(II), Hg(II) and Ag(I) were 0.70, 0.50, 0.50, 0.05, 0.04 and 0.02 mM in R2A medium, respectively. Various genes related to metal and metalloid resistances could be identified on the whole genome of *L. fusiformis* ZC1 (Table S2). The arsenic concentration of the wastewater was 0.12 μM. *L. fusiformis* ZC1 contained two arsenical resistance gene clusters, *arsRBCDA* and *arsCBR*, one gene encoding the arsenate reductase ArsC and another gene encoding the arsenic efflux pump protein ArsB to enable it to survive under such a harsh condition. Other putative heavy metals resistance genes including those for Cu, Mn, Zn, Cd, Hg, Te and Ni were also found on the draft genome of *L. fusiformis* ZC1 (Table S2).

### 3.6. Expression of chrA is constitutive

Cr(VI) resistance of *L. fusiformis* ZC1 was determined using cultures induced with and without 1 mM $K_2CrO_4$ for 8 h in R2A medium before inoculation. *L. fusiformis* ZC1 induced and not induced with chromate exhibited comparable cell densities ($OD_{600}$) at all tested Cr(VI) concentrations. The MIC of *L. fusiformis* ZC1 to chromate in both conditions was 60 mM (Fig. 7A). RT-PCR methodology was also utilized to detect *chrA* expression in the presence and absence of $K_2CrO_4$ (Fig. S1). RT-PCR products corresponding to the predicted size of 876 bp were readily amplified in chromate and chromate-free cultures, which indicate that expression of *chrA* was constitutive. A potential PCR amplification from contaminating DNA was ruled out because of the absence of products in the samples when taking RNA as a template.

### 3.7. Chromate reduction is constitutive

The difference in chromate reduction ability of *L. fusiformis* ZC1 with and without chromate induction was not significant (Fig. 7B). After 12 h incubation, both cultures emerged with approximately 93% chromate reduced. No abiotic Cr(VI) reduction was observed in LB medium. Induction of putative chromate reduction related genes, *yieF* and *nitR*, were also evaluated using RT-PCR and indicated that both genes appeared to be expressed constitutively (Fig. S1), which was in conformity with the studies on chromate reduction by *L. fusiformis* ZC1 with and without chromate induction.

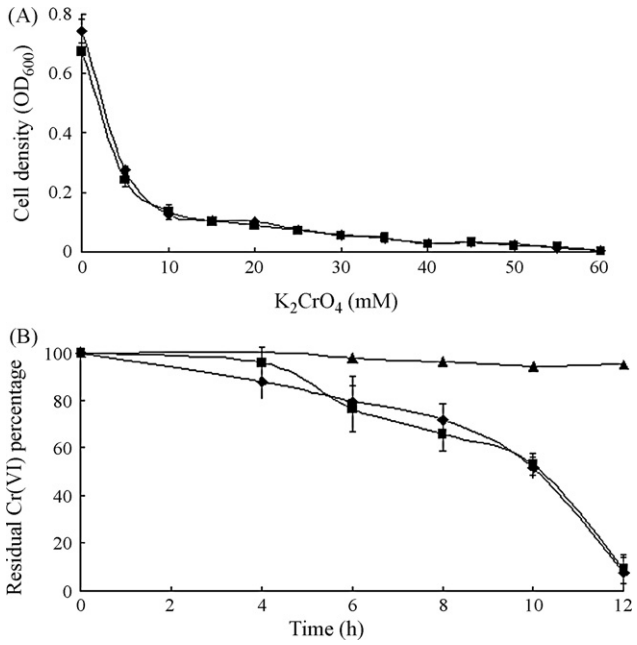


**Fig. 7.** Chromate resistance (A) and reduction (B) analysis of *L. fusiformis* ZC1 induced with (■) and without (♦) 1 mM $K_2CrO_4$ for 8 h before bacterial inoculation. For chromate resistance determination (A), strain ZC1 was incubated for one week at 37 °C with 200 rpm shaking, and cell growth was measured at $OD_{600}$. The chromate reduction (B) was performed as described in Fig. 1. (▲) LB medium (pH 8.0) amended with 1 mM $K_2CrO_4$ without bacterial inoculation as a control. Error bars represent standard deviation of triplicate samples.

## 4. Discussion

Chromium as a trace element is required for life in all organisms but is toxic in excess [10]. Biotransformation of chromium by chromate reducing bacteria offers an economical as well as eco-friendly option for detoxification and bioremediation [25]. In this work, *L. fusiformis* ZC1 with high chromate resistance and extremely rapid chromate reducing ability was isolated from industrial wastewater. *L. fusiformis* ZC1 showed a MIC of 60 mM to $K_2CrO_4$ in R2A medium. The resistance of *L. fusiformis* ZC1 to $K_2CrO_4$ was on a very high level, perhaps the highest recorded so far in R2A medium, compared to other microorganisms. More importantly, *L. fusiformis* ZC1 showed the highest Cr(VI) reduction rate (0.08 mM $h^{-1}$) compared to other microorganisms that has been reported so far and thus makes it a suitable candidate for bioremediation (Table 1). In addition, *L. fusiformis* ZC1 showed an efficient and persistent Cr(VI) reducing ability (4.5 mM $K_2CrO_4$ was reduced in 68 h) in a five times repeated-adding of Cr(VI) aliquot experiment. It is much more efficient than the reduction of 3.29 mM $K_2CrO_4$ in 136 h by *Intrasporangium* sp. Q5-1 [6], which makes it a more economical candidate for future Cr(VI) detoxification application.

**Table 1**
Chromate resistance and reduction rates in different organisms.

| Organism | MIC of chromate (mM) | Chromate reduction rate (μM $h^{-1}$) | Reference |
|---|---|---|---|
| *Lysinibacillus fusiformis* ZC1 | 60 | 80 | This study |
| *Intrasporangium* sp. Q5-1 | 17 | 38 | [6] |
| *Brucella* sp. DM1 | 19.2 | 18 | [10] |
| *Ochrobactrum intermedium* SDCr-5 | 288 | 53 | [27] |
| *Burkholderia cepacia* MCMB-821 | 19.2 | 39 | [31] |
| *Providencia* sp. UTDM314 | 19.2 | 64 | [32] |
| *Bacillus sphaericus* AND303 | 15.4 | 5 | [33] |
| *Pseudomonad* CRB5 | 10 | 37 | [9] |

The chromate reduction ability of bacteria is affected by a number of factors. In this study, the Cr(VI) reduction ability of *L. fusiformis* ZC1 increased with the increase in initial cell concentrations. A similar trend was also observed in *Pseudomonas* CRB5 [26] and *Ochrobactrum intermedium* SDCr-5 [27]. The chromate reduction rates of *L. fusiformis* ZC1 were 0.07, 0.07 and 0.05 mM $h^{-1}$ when the initial Cr(VI) concentration was 2, 3 and 4 mM, respectively. In contrast, they were 0.05, 0.06 and 0.04 mM $h^{-1}$ in *Intrasporangium* sp. Q5-1 [6]. The decrease in chromate reduction rate with the increase of initial Cr(VI) concentration might have been due to chromate toxicity.

In the Cr(VI) reduction assays using resting cells of *L. fusiformis* ZC1, sodium acetate was the most effective carbon source which is in agreement with data reported for *Intrasporangium* sp. Q5-1 [6]. The relatively affordable price makes sodium acetate a useful stimulator in large scale Cr(VI) bioremediation for *L. fusiformis* ZC1. Furthermore, NADH showed almost the same stimulatory effect on Cr(VI) reduction as sodium acetate for *L. fusiformis* ZC1. Our work supports other studies that have reported NADH showing a high stimulatory effect on Cr(VI) reduction in *Bacillus* sp. G1DM20, *Bacillus* sp. G1DM22, *Bacillus* sp. G1DM64 and *Bacillus* sp. ES 29 [28,29]. The high stimulatory effect of NADH on Cr(VI) reduction was most likely due to the fact that the "Cr(VI) reductase" of *L. fusiformis* ZC1 is an NADH-dependent enzyme. This hypothesis was consistent with the result that the putative reductases detected in the ZC1 genome were also NADH-related (gene locus_tags: BFZC1_03873 BFZC1_10272, BFZC1_03508, BFZC1_17639 and BFZC1_15935).

Due to the high chromate resistance of *L. fusiformis* ZC1 even in low nutrient medium (R2A, 60 mM), we reasoned that this strain must contain genes that contributed to the high tolerance of Cr(VI). This anticipation was confirmed by the analysis of the whole genome of *L. fusiformis* ZC1, in which a *chrA* gene, encoding a putative chromate transporter that conferred chromate resistance was identified. Furthermore, we also identified a *yieF* gene and several reductases that could possibly be involved in chromate reduction. Nitroreductase, flavin oxidoreductase, FMN reductase and quinone oxidoreductase have been reported in *V. harveyi* [18], *Thermus scotoductus* SA-01 [16], *P. putida* [30] as potential chromate reductases. In *L. fusiformis* ZC1, all of these enzymes were found in the draft genome. Even though these proteins may not be specific for chromate reduction, the large numbers of potential chromate reductases may be responsible for the observed rapid chromate reduction. Furthermore, the reduction of Cr(VI) to less toxic Cr(III) may make a significant contribution to the high chromate resistance of *L. fusiformis* ZC1. Given the extraordinary properties of *L. fusiformis* ZC1 to be both highly chromate resistant and able to reduce very high concentrations of toxic Cr(VI), as well as the presence of multiple metal(loid) resistances, this bacterium provides a great potential for Cr(VI) removal from wastewater systems.

## 5. Conclusions

*L. fusiformis* ZC1 isolated in the present study was found to be highly chromate resistant and efficient in reduction of Cr(VI). Higher initial cell concentration, lower initial Cr(VI) concentration and addition of some carbon sources, such as sodium acetate and NADH increased the chromate reduction ability of *L. fusiformis* ZC1. The presence of the chromate transporter gene *chrA*, potential chromate reduction genes coupled with a great number of other metal(loid) resistance related genes in the whole genome sequence makes *L. fusiformis* ZC1 a suitable candidate for bioremediation in multi-metal(loid) contaminated environments. Furthermore, our research provides valuable information to better understand the mechanism of chromate resistance which may be helpful for construction of bio-engineered bacteria in the future.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2010.09.072.

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