Journal of Hazardous Materials 185 (2011) 682–688

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

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

Minyan He[a,b], Xiangyang Li[a], Hongliang Liu[a], Susan J. Miller[c], Gejiao Wang[a,*], Christopher Rensing[b,**]

[a] State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China
[b] Department of Soil, Water and Environmental Science, The University of Arizona, Tucson, AZ 85721, USA
[c] Biotechnology Computing Facility, Arizona Research Laboratories, The University of Arizona, Tucson, AZ 85721, USA

ARTICLE INFO

Article history:
Received 14 June 2010
Received in revised form
17 September 2010
Accepted 18 September 2010
Available online 29 September 2010

Keywords:
Lysinibacillus fusiformis
Chromate reduction
Chromate resistance
Chromate reductase
Whole genome sequence

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

* Corresponding author. Tel.: +86 27 87281261; fax: +86 27 87280670.
** Corresponding author. Tel.: +1 520 6268482; fax: +1 520 6211647.
E-mail addresses: au4818285@yahoo.cn (M. He), lixiangyang1984@yahoo.com.cn (X. Li), kydarling@126.com (H. Liu), sjmiller@email.arizona.edu (S.J. Miller), gejiaow@yahoo.com.cn (G. Wang), rensingc@ag.arizona.edu (C. Rensing).

0304-3894/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2010.09.072

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 4.86×10^7 to 1.26×10^9 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