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Chromate Interaction with the Chromate Reducing Actinobacterium *Intrasporangium chromatireducens* Q5-1

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This study was conducted to determine the microbe-chromate [Cr(VI)] interaction and the effect of quinoid analogue anthraquinone-2-sulfonate (AQS) on aerobic Cr(VI) reduction by *Intrasporangium chromatireducens* Q5-1. The addition of redox mediator AQS, which might expedite the electron transfer, promoted Cr(VI) bioreduction. Addition of carbon sources, such as maltose, acetate, sucrose and lactose stimulated the AQS-promoted Cr(VI) reduction of strain Q5-1. Induction experiment clarified that the enzyme involves in the Cr(VI) reduction is constitutive. Energy-dispersive spectroscopy (EDS) spectra showed the existence of trace Cr distributed on the cell surface. X-ray photoelectron spectroscopy (XPS) analysis revealed that the Cr(III) complex was bound to the cell surface (0.87%, atomic percent). The spectra shifts detected by Fourier transform infrared (FTIR) spectroscopy indicated that Cr(III) was bound to the carbonyl and amide groups. In addition, Cr(VI) reduction by different cell fractions showed that Cr(VI) reduction was occurred extracellularly rather than intracellularly. The results disclosed that Cr(VI) detoxification of strain Q5-1 was mainly associated with extracellular Cr(VI) reduction process in combination with trace Cr(III) adsorption on the cell surface. A schematic figure depicting the interactions between strain Q5-1 and Cr(VI) was presented. This study enhanced the understanding of the microbe-Cr(VI) interaction mechanism and revealed the AQS-promoted aerobic Cr(VI) reduction of strain Q5-1. Such strain and quinoid analogue-mediated bacterial Cr(VI) reduction may facilitate the bioremediation for Cr(VI)-polluted environment.

Keywords: biosorption, Cr(VI) reducing actinobacterium, *Intrasporangium chromatireducens* Q5-1, microbe-Cr(VI) interaction, redox mediator, spectroscopy

Introduction

The extensive use of chromium (Cr) and its compounds in various industrial processes generates a large amount of toxic Cr-containing effluents, which causes increasingly serious environment pollution (He et al. 2010; He et al. 2011; Liu et al. 2012a). Hexavalent Cr [Cr(VI)] and trivalent Cr [Cr(III)] are the most common valence states in natural system (Megharaj et al. 2003). These two states differ in both physicochemical and toxic property. Cr(VI) is classified as a priority pollutant of soil and groundwater due to its mobility, together with its carcinogenic and mutagenic effects (Costa and Klein 2003). In contrast, Cr(III) has low toxicity and low mobility. Moreover, Cr(III) is an essential element in human metabolism and performs an important role in sugar and lipid metabolism (Anderson 1997). Microbes capable of reducing and adsorbing Cr(VI) can potentially be used as feasible tools to remedy Cr(VI) contamination (Kamaludeen et al. 2003; Ramírez-Díaz et al. 2008).

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Microorganisms have developed diverse resistance strategies to counter the Cr(VI) toxicity including enzymatic reduction (chromosome encoded), Cr(VI) efflux system (plasmid/chromosome encoded), biosorption (cellular functional group mediated), bioacculmulation (precipitation related), ROS scavenging, SOS response, enzymatic DNA repair system, sulphur metabolism, sulphate transport and regulation of iron uptake etc. (Ahemad 2014; Ramirez-Diaz et al., 2008; Viti et al 2014).

Reduction of Cr(VI) have been reported in a wide variety of microbial species under aerobic or anaerobic conditions (Christl et al. 2012; He et al. 2010; He et al. 2011; Liu et al. 2012a; Yang et al. 2009). Two direct enzyme-mediated Cr(VI) reduction processes have been described: (i) In the presence of oxygen, microbial Cr(VI) reduction is commonly associated with NAD(P)H, soluble and extracellular Cr(VI) reductases such as ChrR, NfsA/NfsB and NemA (Ackerley et al. 2004; Park et al. 2000; Robins et al. 2013) except in *Pseudomonas maltophilia* O-2, *Bacillus megaterium* TKW3 and *Amphibacillus* sp. KSUCr3, which utilize membrane-associated reductases (Blake et al. 1993; Cheung et al. 2006; Ibrahim et al. 2012), and ii) under anaerobic conditions, Cr(VI) was reduced both by soluble and membrane-associated enzymes, which was frequently shown to be associated with cytochrome families (Cheung and Gu 2007). In

anaerobic reduction, Cr(VI) can function as a terminal electron acceptor in the electron transport chain for numerous electron donors, including carbohydrates, amino acids, proteins, fats, hydrogen, NAD(P)H, vitamins and organic acids (Kanmani et al. 2012; Zhu et al. 2008). In addition, Cr(VI) can be also reduced indirectly by nonspecific chemical reactions associated with redox intermediate compounds such as glutathione, amino acids, sugars, vitamins or organic acids (Ramírez-Díaz et al. 2008; Robins et al. 2013).

Microbial systems like bacteria, fungi and algae have been successfully used as efficient biosorbents for removal of Cr (Kang et al. 2007; Khambhaty et al. 2009; Murphy et al. 2009). Diverse chemical groups including carboxyl, sulfhydryl and hydroxyl have been shown to be responsible for heavy metal biosorption (Congeevaram et al. 2007; Vegilo and Beolchini 1997). These functional groups serve as binding sites for metals relying on factors such as the quantity of sites, their accessibility and the affinity between sites and metals (Murphy et al. 2009).

The only known Cr(VI) transporter named ChrA, specifically responsible for Cr(VI) extrusion, has been shown to confer Cr(VI) resistance in numerous bacteria including *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*, Nies et al. 1990), *Pseudomonas aeruginosa* (Cervantes et al. 1990), *Shewanella* sp. (Aguilar-Barajas et al. 2008), *Ochrobactrum tritici* (Branco et al. 2008), *Arthrobacter* sp. (Henne et al. 2009), *Bacillus cereus* (He et al. 2010) and *Lysinibacillus fusiformis* (He et al. 2011). The *chrA* gene can be located on plasmid or chromosomal DNA or both, and they are generally clustered in operons with other *chr* genes including *chr BCF* (Viti et al. 2014). ChrA associated with a putative regulatory protein ChrB is essential for Cr(VI) resistance in *O. tritici* 5bvl1 and strongly induced by Cr(VI) (Branco et al. 2008; Branco and Morais 2013).

Recent studies indicate that Cr(VI) reduction by bacterial strains leads to the production of soluble Cr(III) end products but not generally recognized $Cr(OH)_3$ precipitate (Dogan et al. 2011). Humic substances and quinone analogues can shuttle electrons from bacterial cells to electrophilic pollutants and facilitate their biotransformation (Van der Zee and Cervantes 2009). The addition of redox mediator anthraquinone-2-sulfonate (AQS) has been shown to enhance Cr(VI) reduction by resting cells under anaerobic culture (Guo et al. 2012; Liu et al. 2010). However, the study on AQS-promoted aerobic Cr(VI) reduction is limited.

Currently, there is increasing concern in the microbe-Cr(VI) interactions and studies on the interactions mainly focused on the following aspects: (i) the reduction mechanism; (ii) the related transformation process of Cr(VI) and the valence states of the reduzate; (iii) the sequestration situation of Cr(III) and (iv) the biosorption mechanism (Chen et al. 2012). Nevertheless, the knowledge is mostly theoretical and a comprehensive understanding of microbe-Cr(VI) interactions is still lacking. In a previous study, strain Q5-1 was shown high Cr(VI) resistance with a minimum inhibitory concentration (MIC) of 883.9 mg L^{-1} (17 mM). Free and immobilized Q5-1 cells could both reduce Cr(VI) under aerobic condition (Yang et al. 2009). In 2012, we identified this strain as *Intrasporangium chromatireducens* Q5-1 which represented a new actinobacterium species in genus *Intrasporangium* (Liu et al. 2012b). In this study, *I. chromatireducens* Q5-1 was chosen as a model to further study the microbe-Cr(VI) interaction process. This study represents a global characterization of the cellular response to acute Cr(VI) exposure and effects of AQS and different carbon sources on Cr(VI) reduction.

Materials and Methods

Reagents, Bacterial Strain and Growth Conditions

All the chemicals and reagents used in this study were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd and Aladdin Chemical Co. Ltd. (Shanghai, China). Potassium chromate (K_2CrO_4) was used as a source of Cr(VI). A filter-sterilized Cr(VI) stock solution was added to obtain the desired concentration of Cr(VI) before inoculation. Strain Q5-1 was selected in this study for 3 reasons: (i), strain Q5-1 can endure high concentration of toxic Cr(VI); (ii) strain Q5-1 could reduce Cr(VI) efficiently, which facilitates its Cr(VI) resistance; (iii) among the type strains in *Intrasporangium* species, only strain Q5-1 could reduce Cr(VI) (Liu et al. 2012b). Seed liquid of strain Q5-1 was prepared in Luria-Bertani (LB) broth. Cr(VI) reduction test was performed under the optimum condition of 37°C and pH 8 (Yang et al. 2009) in this study. In microscopic and spectroscopic analyses, 125 mg L^{-1} (~2.4 mM) Cr(VI) was added in the medium and incubated for 48 h. Cr(VI) reduction by strain Q5-1 is largely more efficient in rich medium than in nutrient-deficient minimal medium, so we lowered an order of magnitude of Cr(VI) concentration (12.5 mg L^{-1}, ~0.24 mM) in assays of Cr(VI) reduction in minimal medium (see below).

AQS-Mediated Aerobic Cr(VI) Reduction and Cr(VI)-Preculture Induction

The 250-mL shake flask containing 100 mL LB medium was supplemented with 125 mg L^{-1} Cr(VI) and 155.1 mg L^{-1} (~0.5 mM) AQS. Q5-1 cells was inoculated and rotated at 180 rpm for 60 h. Negative controls without bacterial inoculation were simultaneously tested. The samples were aseptically taken at about every 6 h, centrifuged at 8,000 rpm for 10 min. Cr(VI) concentration in the supernatant was measured using 1,5-diphenylcarbazide (DPC) reagent at OD_{540} by a UV spectrophotometer (DU800, Beckman, USA; APHA 1999). The growth of the bacterium was determined synchronously at OD_{600}. Induction experiment was performed as described below: (i) strain Q5-1 was pre-cultured in LB medium supplemented with 26 mg L^{-1} Cr(VI) [as Cr(VI) induced] or without Cr(VI) [as non-Cr(VI) induced] for 24 h and collected by centrifugation; (ii) the cell pellets were washed twice with 0.1 M potassium phosphate buffer (PPB, pH = 8.0) and resuspended in the same buffer to yield seed liquids of strain Q5-1; (iii) the induced and noninduced seed liquid were inoculated separately in the LB medium supplemented with 104 mg L^{-1} Cr(VI) and incubated for 60 h;

(iv) the remaining Cr(VI) concentration in the supernatant was determined at timed intervals.

Effects of Cr(VI) Concentration and Carbon Sources on AQS-Promoted Cr(VI) Reduction

K_2CrO_4 solution of 260 mg L^{-1} (~5 mM) was added in LB medium supplemented with 155.1 mg L^{-1} AQS. Q5-1 cells was inoculated and rotated at 180 rpm for 120 h. Negative controls without bacterial inoculation were simultaneously tested. The samples were aseptically taken at timed intervals, centrifuged at 8,000 rpm for 10 min and measured for the Cr(VI) concentration. The effects of seven carbon sources, including maltose, sucrose, citrate, glucose, lactate, lactose and acetate on AQS-promoted Cr(VI) reduction were also examined. Strain Q5-1 was inoculated in a chemical defined medium (CDM; Weeger et al. 1999) supplemented with 10.4 mg L^{-1} Cr(VI) and 5 g L^{-1} of each carbon source. The inoculant was incubated as described above for 96 h, when Cr(VI) concentration in the supernatant was determined. A negative control without adding carbon source was simultaneously tested.

Assays of Cr(VI) Reduction by Different Cell Fractions

To determine the major Cr(VI) reduction site of strain Q5-1, we performed Cr(VI) reduction tests using different cell fractions as follows: (i) Resting cells of strain Q5-1 were prepared following the method of Yang et al. (2009) with slight modifications. Overnight grown bacterial cultures without the addition of Cr(VI) were harvested by centrifugation. Cell pellets were washed twice with 10 mL of 0.1 M PPB (pH = 8.0) and resuspended in the same buffer to yield resting cells. The remaining culture supernatant was filtered through 0.22-μm filters to yield supernatant filtration (Millipore, USA); (ii) Permeabilized cells and cell-free extracts of strain Q5-1 were prepared by following reported protocols with minor modifications (Soni et al. 2012). Two equal parts of overnight grown cells were harvested and resuspended.

Permeabilized cells were attained by treating one of the cell suspensions with 0.2% (v/v) Triton X-100. Another cell suspension was placed in an ice bath and disrupted with an ultrasonic probe (Sonic VC-750, USA) at 120 W with 5-s pulse and 5 s off mode for 30 min. Sonicated extracts was centrifuged at 10,000 rpm and 4°C for 30 min to precipitate insoluble cell pellets and membrane fractions. The supernatant containing soluble fractions of cytoplasm and membrane was sterilized by filtration to yield cell-free extract. Each cell fraction was supplemented with 12.5 mg L^{-1} Cr(VI). Aliquots (1 mL) were withdrawn at regular time interval (6h) and analyzed for Cr(VI) reduction. Abiotic Cr(VI) reduction in PPB was served as a negative control.

SEM-EDS and TEM Analyses

Raw cells and Cr-exposed cells were separately fixed in 2.5% glutaraldehyde and then dehydrated in a graded ethanol series (30–100%). Lyophilized raw control and Cr-laden samples were mounted on an aluminum stub using carbon tape and coated with gold under vacuum in an argon atmosphere. The surface microstructure of samples was visualized by a SEM (JSM-6390, JEOL, Japan) at a voltage of 20 kV. Analyses of desired elements distributed at the cell surface were performed by an EDS analyzer (JSM-6390/LV, NTC, Japan) simultaneously (Liao et al. 2013). As for TEM analysis, samples were pre-fixed in 2.5% glutaraldehyde for 12–18 h and post-fixed for 2 h in 1% osmium tetraoxide at 4°C. The specimens were washed in fresh buffer, followed by dehydration in graded acetone solutions (30–100%). The ultrathin sections were prepared by an ultramicrotome and the sections were separately stained in lead citrate and uranyl acetate before examining by a TEM (H-7650, HITACHI, Japan).

XPS Analyses

XPS was used to investigate the oxidation state of the Cr bound to the biomass during Cr(VI) reduction. Lyophilized samples were separately mounted on insulating double-sided tape attached to a stainless steel holder. The holder was then transferred to the analysis chamber of a VG Multilab 2000 spectrometer (Thermo Electron Corporation, USA) with a monochromatic AlKα source (1486.6 eV). The system was operated at a base pressure of 10^{-7} mbar. The X-ray source was operated at 300 W, and spectra were recorded at 12.5 kV. Calibration of the binding energy of the spectra was referred to the C 1s peak at 284.6 eV of the surface aliphatic carbon.

FTIR Analyses

Infrared spectra of the raw and Cr-laden biomass were performed with a FTIR spectrometer (Thermo Nicolet, Avatar, USA). Lyophilized samples were separately mixed with KBr and then ground in an agate mortar at an appropriate ratio of 1:100. The translucent disks were prepared by pressing the abrasive material at 10 tons. The flake was immediately analyzed by FTIR spectrometer in the range of 4000–400 cm^{-1}. The background obtained from the scan of pure KBr was automatically subtracted from the sample spectra.

Results

AQS Promoted Aerobic Cr(IV) Reduction

The aerobic Cr(VI) reduction by strain Q5-1 cultured in the absence and presence of redox mediator AQS are shown in Figure 1. In the presence of AQS, Cr(VI) reduction by strain Q5-1 was enhanced greatly (Figure 1a). Within 30 h cultivation, strain Q5-1 incubated with AQS reduced more than 95% of total Cr(VI), whereas strain Q5-1 in AQS-free incubation reduced less than 45% of total Cr(VI). Growth status was not influenced by the addition of AQS (Figure 1a). High concentration of Cr(VI) ($\geq$ 260 mg L^{-1}) did not show obvious AQS mediated stimulation (Figure 1b). The abiotic control showed no obvious Cr(VI) reduction (Fig. 1). After Cr(VI) reduction, we collected the supernatant by centrifugation, and recovered

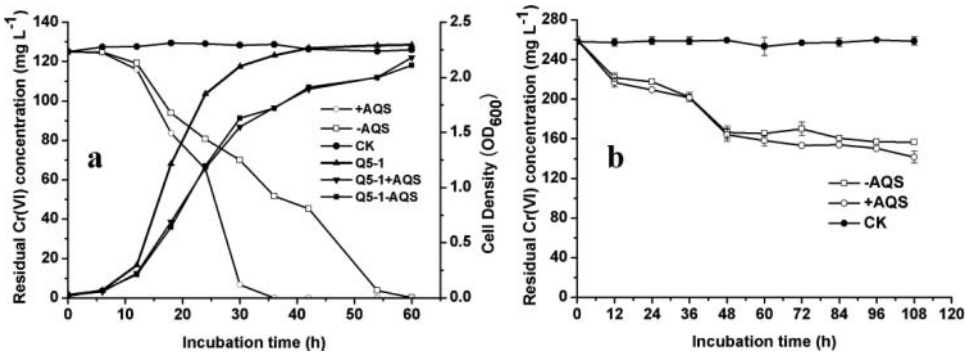

Fig. 1. Cell growth curve and AQS-mediated Cr(VI) reduction of strain Q5-1: (a) Cell growth curves of strain Q5-1 in LB medium amended with neither Cr(VI) nor AQS (▲), with 125 mg L^{-1} Cr(VI) and 155.1 mg L^{-1} AQS (▼), and with 125 mg L^{-1} Cr(VI) alone (■); Cr(VI) reduction by strain Q5-1 in LB medium amended with (○) and without (□) AQS, and abiotic Cr(VI) reduction in LB medium amended with AQS as a negative control (•). (b) Strain Q5-1 reduced 260 mg L^{-1} Cr(VI) in LB medium amended with (○) 155.1 mg L^{-1} AQS or (□) without AQS. (•) represents abiotic Cr(VI) reduction in LB medium with 155.1 mg L^{-1} AQS. Error bars represent standard deviation of triplicate tests.

the reduced Cr(VI) in the supernatant by potassium permanganate oxidation (Saltzman 1952). More than 96% of initially added Cr(VI) was recovered (data not shown). Thus, we found that the concomitant formation of Cr(III) in LB medium was almost soluble Cr(III) complexes, which was in agreement with the reported Cr(VI) reduction with *Pseudomonas* strains (Dogan et al. 2010).

Appropriate Carbon Sources Stimulate AQS-Mediated Cr(VI) Reduction

The reduction of Cr(VI) by strain Q5-1 in CDM was tested with and without additional carbon sources (Figure 2). Maltose and acetate appears to be the most effective carbon sources for stimulating AQS-mediated Cr(VI) reduction. Sucrose, lactose, glucose and lactate moderately stimulated the Cr(VI) reduction, while citrate showed no enhancement of Cr(VI) reduction. Strain Q5-1 showed minor Cr(VI) reduction in AQS-supplemented CDM when no carbon source was added (Figure 2; CK). In addtion, Cr(VI) preculture of strain Q5-1 exhibited no induction effect on Cr(VI) reduction (Supplementary material Figure S1).

Cr(VI) Reduction by Strain Q5-1 Mainly Occurred Extracellularly

Resting and permeabilized cells, culture supernatant and cell-free extracts were used to reduce Cr(VI) and the results were presented in Figure S2. All of the resting cells, permeabilized cells and culture supernatant showed obvious Cr(VI)-reducing activities but the cell-free extracts showed no Cr(VI) reduction. The PPB solution alone exhibited no Cr(VI) reduction. Comparing to the resting cells, permeabilization of cells further increased the Cr(VI) reduction rate. The Cr(VI) reduction

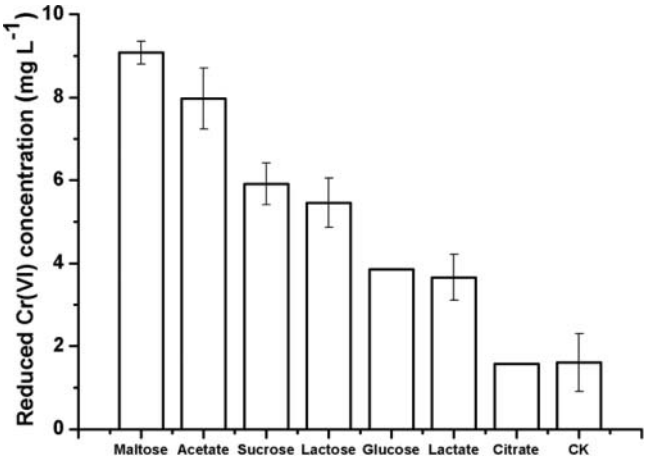

Fig. 2. Effects of carbons sources on the AQS-promoted Cr(VI) reduction. Strain Q5-1 reduced 10.4 mg L^{-1} Cr(VI) in CDM medium with the addition of 155.1 mg L^{-1} AQS and different carbon sources (maltose–citrate). CK represents Cr(VI) reduction in CDM medium amended with AQS. Error bars represent standard deviation of triplicate tests.

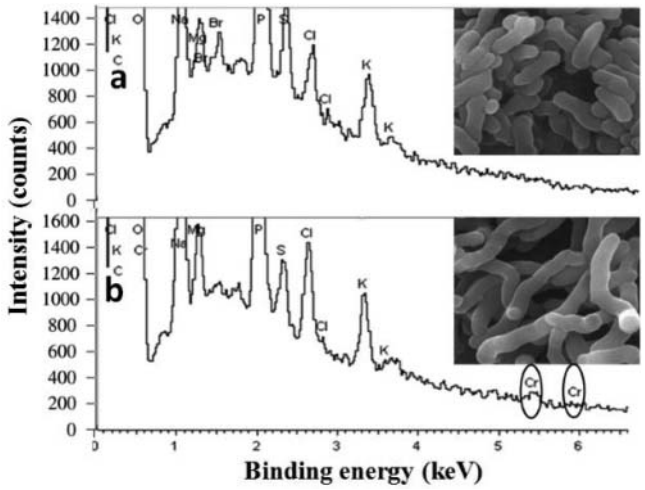

Fig. 3. EDS analyses for strain Q5-1 interacted with Cr(VI). EDS spectra of strain Q5-1 incubated for 48 h (a) without Cr(VI) and (b) with the addition of 125 mg L^{-1} Cr(VI). Oval circles indicated the presence of Cr. Insets of (a) and (b): SEM images of strain Q5-1 used for EDS analysis.

capacity of strain Q5-1 incubated in nutrient-absent condition [~12.5 mg L^{-1} Cr(VI) was completely reduced within 66 h, Figure S2] was far below that (~125 mg L^{-1}, 60h, Fig. 1a) in rich medium. In addition, TEM micrographs did not show the appearance of Cr(III) precipitate microstructure changes in the cytoplasm (Supplementary Figure S3).

SEM-EDS Analyses Confirmed Cr Biosorption and XPS Spectra Revealed the Cellular-Bound Cr was Mainly Cr(III)

After cultivation of strain Q5-1 in LB medium for 48 h with and without addition of 125 mg L^{-1} Cr(VI), the SEM micrographs (inserted pictures on the upper right area in Figures 3a and 3b) revealed considerable differences in morphological characters of Cr(VI) exposed cells, which is in agreement with a previous observation (Yang et al. 2009, 26 mg L^{-1}, 20h). The SEM-EDS equipment was used to examine the cell surface of strain Q5-1 and to analyze the elements of the cell surface. The marked EDS spectrum showed the existence of trace Cr distributed on the cell surface (Figure 3b), which was accounting for about 0.15% (At.%, atomic percent) of the detected elements (Supplementary Table S1). The XPS spectrum presented in Fig. 4b displayed one Cr 2p3/2 peak. The peak in the spectrum of the Cr-laden biomass is located at 575.2 eV, which is close to the binding energy of the references in Cr(III) complexes (Li et al. 2010). The Cr(III) complexes bound to the cell surface was accounted for 0.87% of the detected elements (At.%, Table S2 and Figure 4).

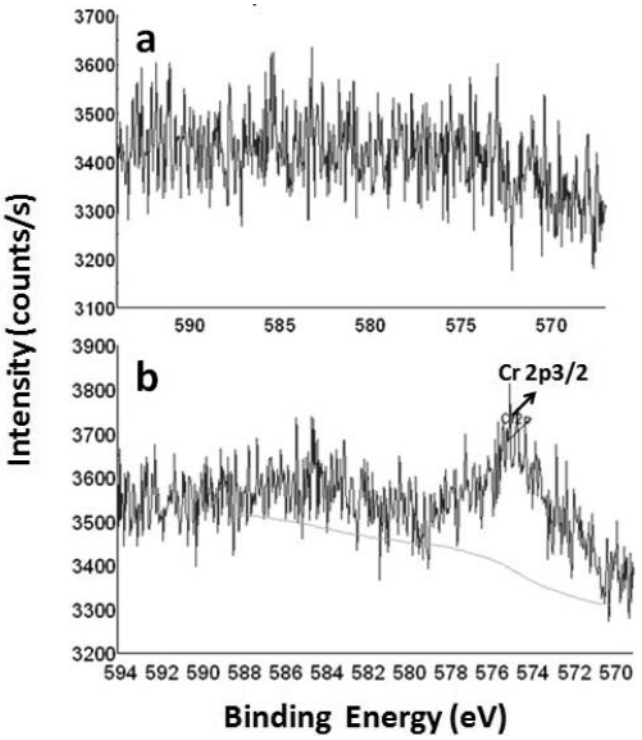

Fig. 4. XPS analyses for strain Q5-1 interacted with Cr(VI). XPS spectra of strain Q5-1 incubated for 48 h (a) without Cr(VI) and (b) with the addition of 125 mg L^{-1} Cr(VI). Oval circles indicated the presence of Cr. The arrow in (c) shows the characteristic peak of Cr(III) species.

FTIR Analysis Indicated That Cr(III) was Bound to the Cellular Carbonyl and Amide Groups

To ascertain which functional groups might act on the Cr(III) adsorption during Cr(VI) reduction, the FTIR spectra analysis of raw and Cr-laden biomass was performed. As shown in Figure 5a, the spectra of raw sample exhibited a number of absorption peaks indicating the complex nature of the examined biomass. The spectrum of raw biomass exhibited in the range 3,430–3,280 cm^{-1} represented the —OH and —NH stretching groups, most probably from glucose and proteins; the peak at ~2,927 cm^{-1} represented C—H stretching of $—CH_2$ groups; the absorption band between 1,655 and 1,540 cm^{-1} might be corresponded to —NH bending of primary and secondary amide from the peptide bond coupled with —COO anion, respectively (Dhal et al. 2010); the region between 1,540 and 1,000 cm^{-1} is the fingerprint region, which contains the C-O bending vibration (~1,380 cm^{-1}) and C—OH stretching vibration (~1,080 cm^{-1}) (Li et al. 2010). By contrast, the spectra of Cr-laden biomass displayed a number of peak shifts (lowered by 3–15 cm^{-1}) as well as the appearance of new peaks indicating of Cr binding to the functional groups (Figure 5b). The shifts of the peaks at ~1,655 cm^{-1}, ~1,540 cm^{-1} and especially ~1,080 cm^{-1} to a lower frequency (Figure 5b) indicated carbonyl and amide groups playing a leading role in Cr biosorption. The appearance of low intensity peaks at ~800–700 cm^{-1} (Fig. 5b) potentially represented Cr-O vibration (Dhal et al. 2010).

Discussion

Various studies have shown the effectiveness of the microbes in reducing Cr(VI) to less toxic Cr(III), but the mechanisms of microbe-Cr interaction are far from being elucidated (Chen et al. 2012). A previously identified type strain *I. chromatireducens* Q5-1, with capabilities of Cr(VI) reduction/resistance, was chosen as a model for this study of microbe-Cr(VI) interactions. Our results demonstrated that the redox mediator AQS could notably enhance the aerobic reduction of Cr(VI) with the concentration less than 208 mg L^{-1} by

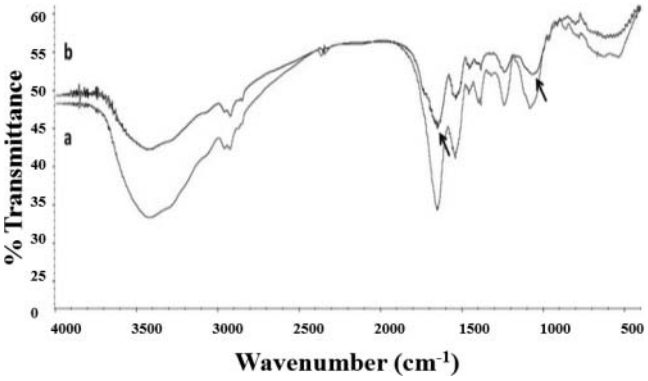

Fig. 5. FT-IR spectra of lyophilized biomass of strain Q5-1. Strain Q5-1 was incubated for 48 h (a) without Cr(VI) and (b) with the addition of 125mg L^{-1} Cr(VI). The arrows show shifted peak.

strain Q5-1 (Figure 1). Enhanced bioremediation of various electrophilic pollutants in the presence of redox mediators has been reported (Liu et al. 2010), and it was proved that soluble quinone-like redox mediators could also serve as electron shuttles between bacteria and Cr(VI) under anaerobic condition (Fredrickson et al. 2000; Guo et al. 2012). Our results showed that under aerobic condition, the transformation of Cr(VI) was also promoted by the redox mediator. A possible explanation for AQS-promoted Cr(VI) reduction by strain Q5-1 was that AQS-accelerated extracellular electron shuttle might further facilitate the electron transfer to Cr(VI). This finding suggested the potentiality of applying quinone analog as an electron mediator in the microbial incubation for the fast elimination of Cr(VI) contaminations.

Microbial Cr(VI) reduction is an electron-requiring process and effects of carbon sources such as maltose, acetate, glucose, sucrose, lactose, lactate and citrate on AQS-promoted Cr(VI) reduction was studied. Maltose and acetate were the most effective carbon sources for stimulating Cr(VI) reduction of strain Q5-1 in minimal medium (Figure 2). Without the addition of carbon sources, the Cr(VI) reduction rate declined greatly even in the presence of AQS (Figure 2). This finding suggested that AQS-mediated Cr(VI) reduction might require electron donors such as different carbon sources. Electron donors are abundant in nutrient medium, thus AQS-promoted Cr(VI) reduction in LB medium is explicable and reasonable. In two *Bacillus* species G1DM20 and G1DM64, adding acetate increased the reduction of Cr(VI) (Desai et al. 2008a). In other report, fructose and lactose were very effective for stimulating Cr(VI) reduction (Zhang and Li 2011). Cr(VI) was transformed to Cr(III) via a reduction reaction, during which electrons transmitted from electron donor to Cr(VI). The additional carbon source can be acted as electron donor in the nutrient-absent minimal medium for Cr(VI) reduction. Therefore, it may be possible to stimulate Cr(VI)-reducing microbes in situ remediation using relatively cost-effective carbon source or its by-product.

Cr(VI) reduction performed with the resting cells, permeabilized cells, culture supernatant and cell-free extracts of strain Q5-1 (Figure S2) indicated that the Cr(VI) reduction was primarily occurred extracellularly. Indirect reduction of Cr(VI) to Cr(III) by bacterial surfaces under nonnutrient conditions was probably attributed to the oxidation of organic molecules within the cell wall that serve as electron donors (Fein et al. 2002). A recent study on Cr(VI) reduction by aerobically grown granular bacterial biofilm revealed that there was no Cr(VI) reduction under non-nutrient conditions, whereas they could efficiently reduce Cr(VI) from minimal media in the presence of acetate (Nancharaiah et al. 2010).

The Cr(VI) reduction efficiency was much higher in rich medium (Figure 1) than that in nutrient-absent condition (Figure S2), which implied that Cr(VI) toxicity and reduction level were depending on the growth medium. The energy and reducing power originated from normal growth and metabolism of bacteria in enriched medium were essential to counteract the toxic effects of Cr(VI) exposure (Thompson et al. 2010; Viti et al. 2014). After Cr(VI) reduction, the Q5-1 cells became elongating and irregular with appearance of protrusions on the surface (Figure 3), which might be attributed to the toxicity of Cr(VI), Cr(III) or both. Changes in morphology of microbe might indicate adaptive features to the hostile environment.

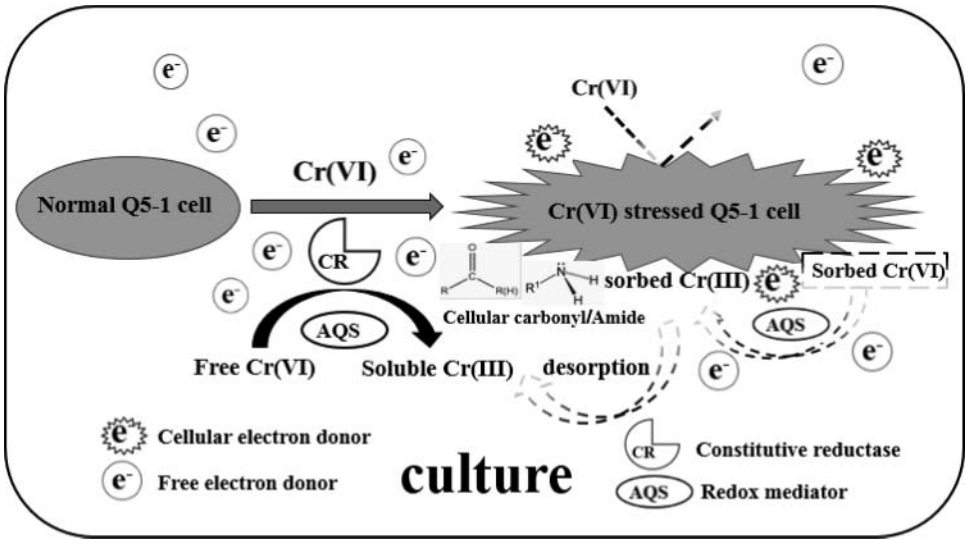

Fig. 6. Schematic depiction of interactions between strain Q5-1 and Cr(VI). Cr(VI) detoxification of strain Q5-1 seemed to be largely an extracellular reduction-based process, and biosorption plays a minor role. In an enrichment culture, Free Cr(VI) was almost reduced aerobically to a soluble Cr(III) species by constitutive reductases. Cellular-sorbed Cr(VI) reduction was hypothesized to be a combined process of sorption-reduction-desorption. The binding characteristics of the Cr mainly involved the carbonyl and amide groups on the biomass. Cr(VI) could induce marked changes in the cellular microstructure and component, but no Cr(III) precipitates were sequestrated intracellularly. Moreover, AQS promoted Cr(VI) reduction of Q5-1 might be attributed to the electron transfer acceleration between electron donor (carbon sources or other organic substances) and Cr(VI).

The EDS spectrum analysis confirmed Cr-sorption by strain Q5-1 during Cr(VI) reduction (Figure 3 and Table S1). These results indicated that Cr(VI) reduction process by strain Q5-1 was more complex than the reported direct production of insoluble $Cr(OH)_3$ (Desai et al. 2008b). The formation of soluble organo-Cr(III) complexes is possibly an integral part of the biogeochemical cycle of Cr (Dogan et al. 2011; Puzon et al. 2005). The valence state of Cr bound to the biomass was characterized using XPS. In a previous study, Cr $2p_{3/2}$ orbitals were assigned at ~577.2 eV ($CrCl_3$) and ~576.2–576.5 eV (Cr_2O_3) for Cr(III) compounds, while Cr(VI) forms are characterized by higher binding energies such as ~578.1 eV (CrO_3) or ~579.2 eV ($K_2Cr_2O_7$) (Dambies et al. 2001). The XPS spectrum of Cr(VI)-laden samples in this study showed only a Cr 2p3/2 peak (Figure 4). The peak value in the spectrum of the Cr-loaded biomass is supposed as the binding energy of the Cr(III) references. The bound Cr(III) was accounted for 0.87% of atomic percent (Table S2), which is not directly proportional to the total percentage of added Cr sorbed to the cells.

FTIR revealed substantially unchanged peak shape of Q5-1 biomass after Cr(VI) reduction, but some of the peaks drift moderately. Peaks drift in the region of ~1,655–1,080 cm^{-1} suggested amide and carbonyl groups of lipids, proteins and polysaccharides might contribute to Cr biosorption by strain Q5-1 (Figure 5). Amide was very susceptible to oxidation and might play a part role in Cr(VI) reduction during Cr sorption. Carbonyl was a mainly integral part of functional groups in aldehyde, ketone, carboxylic acid, and carboxylic acid derivatives which might function as electron donors within the cell wall. It was reported that under aerobic conditions, NAD(P)H-dependent soluble reductases are produced extracellularly by the bacterial cells to reduce Cr(VI) to Cr(III) that is removed by reacting with functional groups locating on cell surface (Ahemad 2014). Induction experiment indicated that these extracellular soluble reductases are produced constitutively by strain Q5-1 (Fig. S1). Cr(VI) reduction mediated by such reductases was supposed to be an energy-requiring and a highly regulated process (Ahemad 2014).

Based on the above analyses, we speculated that Cr(VI) detoxification of strain Q5-1 seemed to be largely a reduction-based process, in which biosorption played a minor role. A summary figure demonstrating what the overall conclusions of the interactions between strain Q5-1 and Cr(VI) was presented in Figure 6. The extracellular Cr(VI) reduction is advantageous for bacterial cell as it neither needs to uptake Cr(VI) into the cell nor to export the Cr(III) into the medium, and it may protect the cell from Cr(VI)/Cr(III)-induced intracellular oxidative stress and DNA damage. Moreover, using cells that reduce Cr(VI) extracellularly is particularly favorable for cellular separation from an exhausted medium and reutilization in the bioreactor system.

Abbreviations

Cr, chromium; Cr(VI), chromate or hexavalent Cr; Cr(III), trivalent Cr; SEM, scanning electron microscopy; EDS, energy-dispersive spectroscopy; XPS, X-ray photoelectron spectroscopy; FTIR, Fourier transform infrared spectroscopy; AQS, anthraquinone-2-sulfonate; PPB, potassium phosphate buffer

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Supplemental Material

Supplemental materials for this article can be found on the publisher's website.

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