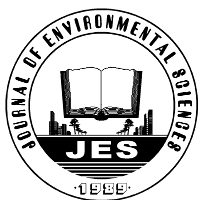

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Identification and characterization of the chromium(VI) responding protein from a newly isolated *Ochrobactrum anthropi* CTS-325

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Abstract

A Gram-negative, chromium(VI) tolerant and reductive strain CTS-325, isolated from a Chinese chromate plant, was identified as *Ochrobactrum anthropi* based on its biochemical properties and 16S rDNA sequence analysis. It was able to tolerate up to 10 mmol/L Cr(VI) and completely reduce 1 mmol/L Cr(VI) to Cr(III) within 48 h. When the strain CTS-325 was induced with Cr(VI), a protein increased significantly in the whole cell proteins. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis revealed that this protein was a superoxide dismutase (SOD) homology. The measured superoxide dismutase activity was 2694 U/mg after three steps of purification. The SOD catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide and molecular oxygen. This protein is considered to be one of the most important anti-oxidative enzymes for *O. anthropi* as it allows the bacterium to survive high oxygen stress environments, such as the environment produced during the reduction process of Cr(VI).

Key words: chromium; LC-MS/MS; superoxide dismutase; *Ochrobactrum anthropi*

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Introduction

With widespread industrial applications, chromium (Cr) has become a serious environmental pollutant. Chromium is present in the environment primarily in hexavalent form (Cr(VI)) and trivalent form (Cr(III)) (Kirpnick-Sobol *et al*., 2006). The mobile Cr(VI) is considered to be a highly toxic and carcinogenic agent compared to the relatively innocuous and less mobile Cr(III) (Aviva and Peter, 2005; Wise *et al*., 2002).

Some bacterium exposed to Cr(VI) can reduce the highly noxious and mobile Cr(VI) to the harmless and less mobile Cr(III) (Cervantes *et al*., 2001). Bacterial reduction of Cr(VI) may also result in the concomitant generation of reactive oxygen species (ROS) including hydroxyl radical (OH), singlet oxygen (O), superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Ackerley *et al*., 2004). These ROS have been demonstrated to induce a variety of DNA lesions such as single strand breaks, alkali-labile sites, and DNA-DNA or DNA-protein cross-links (Liu and Shi, 2001). In order to survive in a high oxygen stress environment, bacteria need to remove ROS (Ackerley *et al*., 2006; Brown *et al*., 2006; Fournier *et al*., 2004).

In this article we present one protein of *O. anthropi* CTS-325 that may exemplify a mechanism of chromium resistance. The expression of this protein increases significantly when the strain is induced with Cr(VI). The protein is highly homologous to superoxide dismutase (SOD) of other species, as identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) and polymerase chain reaction (PCR) related technology.

1 Materials and methods

1.1 Isolation of chromate tolerant bacterium

Sediment samples were collected from the drain of the Changsha Chromate Plant, Hunan Province, China. The samples were diluted with an appropriate amount of sterilized distilled water, placed on LB agar plates containing 10 mmol/L potassium dichromate, and incubated at 30°C for 24 h. The strains, grown on the above medium, were then selected for further evaluation of Cr reduction and toleration ability.

1.2 Identification of the strain CTS-325

Biochemical analysis and 16S rDNA sequence homology analysis were performed to identify the strain CTS-325 (He *et al*., 2007; Pattanapipitpaisal *et al*., 2001).

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1.3 SDS-PAGE analysis

LB cultures (5 mL) were inoculated with a colony and aerobically cultivated overnight at 30°C with shaking. The cultures were then inoculated (inoculation ratio 1:100, *V/V*) in 100 mL triangular flasks containing 30 mL of LB medium with 0, 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 mmol/L chromate in a horizontal shaker at 30°C for 24 h. Cells were collected, placed on ice, and lysed by sonication. Cellular debris was removed by centrifugation at 14000 ×*g* for 20 min. The denatured supernatants (10 μL each) were loaded onto a 12.5% SDS polyacrylamide linear resolving gel overlaid with a 5% stacking gel. Proteins were resolved by electrophoresis at 80 V for stacking and 140 V for resolving gel. Gels were stained with Coomassie Brilliant Blue R-250. In order to confirm whether the protein was a dimmer, some supernatants (Fig. 4a, Lanes 1 and 3) did not have dithiothreitol (DTT) added as a reducer in the loading buffer, and were not treated by heat denaturation.

1.4 Protein sequencing by LC-MS/MS

Protein bands from the 1-D SDS PAGE gels were excised for protein sequencing. In-gel trypsin digestions of the bands were performed using a procedure adapted from previous publication (Shevchenko *et al*., 1996). The digested peptides were extracted from the gel bands using 50% acetonitril and 0.1% trifluoroacetic acid in water. The extracts were subsequently dried, resuspended in 0.1% formic acid solution, and submitted for protein identification using LC-MS/MS. The peptides were analyzed on a Bruker Daltonics micrOTOF-Q (QqTOF type) mass spectrometer (Billerica, MA, USA) equipped with a nanoelectrospray interface and a Dionex ultimate 3000 nano-high performance liquid chromatography (HPLC) system (Sunnyvale, CA). A set of Dionex PepMap 100 precolumn (300 μm × 5 mm) and column (75 μm × 150 mm) packed with 5 μm C18 resins were used for peptide separation on the nano-HPLC. Peptide product ion spectra were automatically recorded during the LC-MS runs by the data-dependent analysis on the mass spectrometer. The obtained spectra were submitted for a database search using the Mascot database search engine (Matrix Science, London, UK). Since there is no sequence database available for the bacterium strain in the current study, both manual and computer-automated (RapiDeNovo®, Bruker Daltonics Inc., Billerica, USA) *de novo* sequencing of the recorded MS/MS spectra were performed (Kim *et al*., 2004). The resulting peptide sequences were submitted for a MS BLAST homology search, provided by the EMBL server (http://dove.embl-heidelberg.de/Blast2). The MS BLAST homology search identifies the known database sequences that are homologous to the sequences submitted for searching, which facilitates the identification of unknown proteins (Shevchenko *et al*., 2001; Wielsch *et al*., 2006). The assignments in both database search results were manually inspected and validated.

1.5 Obtaining full open reading frame (ORF) and flanking region sequence of the protein

Using the amino acid sequences of the peptides identified by LC-MS/MS, two degenerate primers were designed and synthesized for PCR amplification of *O. anthropi* SOD gene center sequence. The sense primer was designed based on the underlined amino acids of peptide I (MLLSHYENNYGGAVK). The anti-sense primer was designed based on the underlined amino acids peptide II (AEFLAMGK). The primer sequences are as following: the sense primer, 5'-CAG(TC)CA(TC)TA(TC)GA(AG)AA(TC)AA(TC)TA-3'; and the anti-sense primer, 5'-GCC(C)AT(TCAG)GC(TCAG)AG(AG)AA(TC)TC-3'. Amplification mixtures in a 50-μL contained 20 mmol/L Tris-HCl, 2.0 mmol/L $MgCl_2$, 200 μmol/L deoxynucleoside triphosphate (dNTP), 0.4 μmol/L of each primer, 1 μg of *O. anthropi* genomic DNA isolated using a Qiagen genome DNA kit (Qiagen, Hilden, Germany) according to the instructions, and 2.5 U of Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). A 35-cycle amplification was carried out at a melting temperature of 95°C for 30 s, an annealing temperature of 54°C for 20 s, and a polymerization temperature of 72°C for 30 s. The final elongation was 72°C for 3 min. The PCR products were electrophoresed on 1% agarose gels.

The flanking region sequence of the SOD gene was obtained using inverse PCR (IPCR) (Huang, 1994). The IPCR primer sequences were as follows: I-up: 5'-GATCAACTGTTCCCGCTTC-3'; and I-down: 5'-GATGATCCTGCATGAGGTGT-3'. The IPCR products were commercially sequenced using the amplification primers.

1.6 Purification of SOD-like protein

The strain CTS-325 was grown in the presence of 1 mmol/L Cr(VI) until the exponential anaphase. Cells were harvested by centrifugation. The pellet was resuspended in buffer A (40 mmol/L Tris/HCl, pH 7.4) and broken by sonication. Soluble extracts were recovered by centrifugation at 20000 ×*g* for 20 min. The SOD-like protein was purified in three steps. Firstly, the protein was precipitated with 65% saturated ammonium sulfate. The pellet from the ammonium sulfate precipitation was dissolved in a minimal volume of buffer A and dialyzed extensively against the same buffer. Secondly, the protein solution was loaded onto a DEAE cellulose column (Amersham, Piscataway, USA), which was equilibrated beforehand with buffer A. Fractions (3 mL each) were collected for protein concentration and SOD activity analysis. Finally, all active fractions were pooled together and loaded onto a sephadex G-75 column (Amersham, Piscataway, USA) equilibrated with buffer A and eluted at 0.6 mL/min. Active fractions were collected and concentrated by centrifugation using Ultracel PL-10 micro-concentrators (Millipore, Billerica, USA). Aliquots

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of the concentrated sample were then stored with 50% aseptic glycerol at –80°C until further use.

1.7 SOD activity measurement

The SOD activity was determined using a SOD activity assay kit (Dojindo Molecular Technologies, Gaithersburg, Japan), and was calculated from 50% inhibition of WST-1 reduction. The standard curve was generated using commercial bovine erythrocyte SOD from Sigma (St. Louis, USA). Protein concentration was determined using Bradford method (Bradford, 1976).

2 Results

2.1 Isolation and characterization of chromate-resistant microorganism

The isolated strain CTS-325 was identified as *O. anthropi* based on its biochemical properties and 16S rDNA sequence homology analysis. The sequence has been deposited in GenBank (accession No. EF493855). The strain CTS-325 was able to tolerate 10 mmol/L Cr(VI) and completely reduce 1 mmol/L Cr(VI) to Cr(III) within 48 h in liquid LB medium (Fig. 1a).

2.2 SDS-PAGE analysis

When strain CTS-325 was induced with potassium dichromate, one protein, which occurred as a dimmer of approximately identical 23 kDa subunits, increased significantly in the whole cell proteins. Within a certain concentration range (1–2 mmol/L), the higher the potassium dichromate concentration in the LB culture medium, the greater the increase of this protein in the strain CTS-325 (Fig. 1b).

2.3 Protein sequencing by LC-MS/MS

LC-MS/MS is a powerful technique for protein identification, especially for organisms without available sequence databases. Since there is no sequence database available for the bacterium strain studied, it was not expected that the Mascot database searches would return significant results. Both manual and computer-automated *de novo* sequencing of the recorded MS/MS spectra were therefore performed. The manual *de novo* sequencing results of three MS/MS spectra with doubly charged precursor ions of *m/z* 856.430(2^+) at the retention time 22.8 min is shown in Fig. 2a, 989.943(2^+) at 40.2 min is shown in Fig. 2b and 433.737 (2^+) at 24.85 min is shown in Fig. 2c. Three peptides were obtained, and showed the following peptide sequences; Peptide I: MI/L I/LSHYENNYGGAVK; Peptide II: I/LAEI/LDYENAPGFI/LI/LNGI/LK; and Peptide III: AEFI/LAMGK. Leucine and isoleucine have isobaric masses and cannot be differentiated by the low energy collision used in the current experimental setup. The corresponding mass identified in the MS/MS spectra are therefore labeled as I/L and underlined.

2.4 MS BLAST homology search and sequences alignment

Peptide sequences obtained by *de novo* were submitted for a MS BLAST homology search, provided by the EMBL server (http://dove.embl-heidelberg.de/Blast2). Among the sequences submitted for search, the three peptide sequences (listed above) matched to a set of proteins that belong to the superoxide dismutase family. The sequence alignment result is shown in Fig. 3. The algorithms of BLAST homology searching, statistical analysis, and organization of the search engine are described elsewhere (Altschul *et al*., 1997). The homology search identifies the known database sequences that are homologous to the sequences submitted for searching, which facilitates the identification of unknown proteins. The assignments in both database search results were manually inspected and validated.

2.5 Full ORF of SOD gene

Inverse polymerase chain reaction (IPCR) is an efficient approach to flank unknown DNA sequences on the basis of a small stretch of known sequence. In order to obtain the full ORF sequence of the SOD gene, PCR and IPCR technology were used. The *O. anthropi* SOD gene center part sequence was amplified at an amplified fragment size 296 bp with the degenerate primers based on peptide I and peptide II. IPCR was then performed. The IPCR product was directly sequenced with the I-up and I-down primers. The ORF sequence was deposited in GenBank (accession No. EF166091) and has 67.96% identity with the iron superoxide dismutase (Fe-SOD) of *Rasltonia metallidurans* (Juhnke *et al*., 2002; Roux and Coves, 2002).

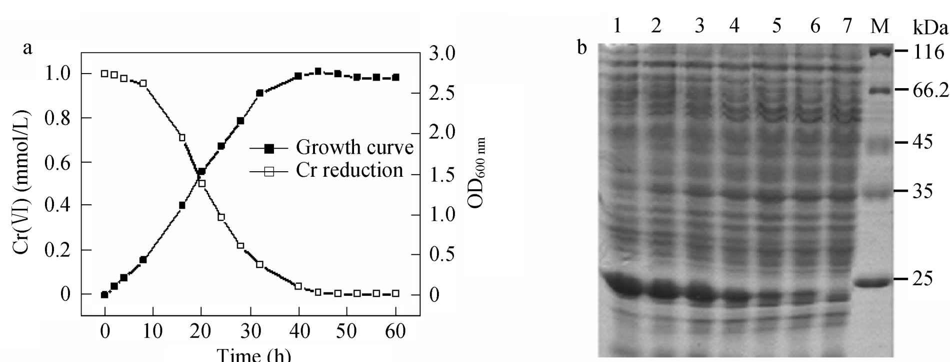

Fig. 1 Growth and Cr(VI) reduction of strain CTS-325 (a), and SDS-PAGE analysis (b). The strain CTS-325 was induced with 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0 mmol/L potassium dichromate (from lane 1 to lane 7).

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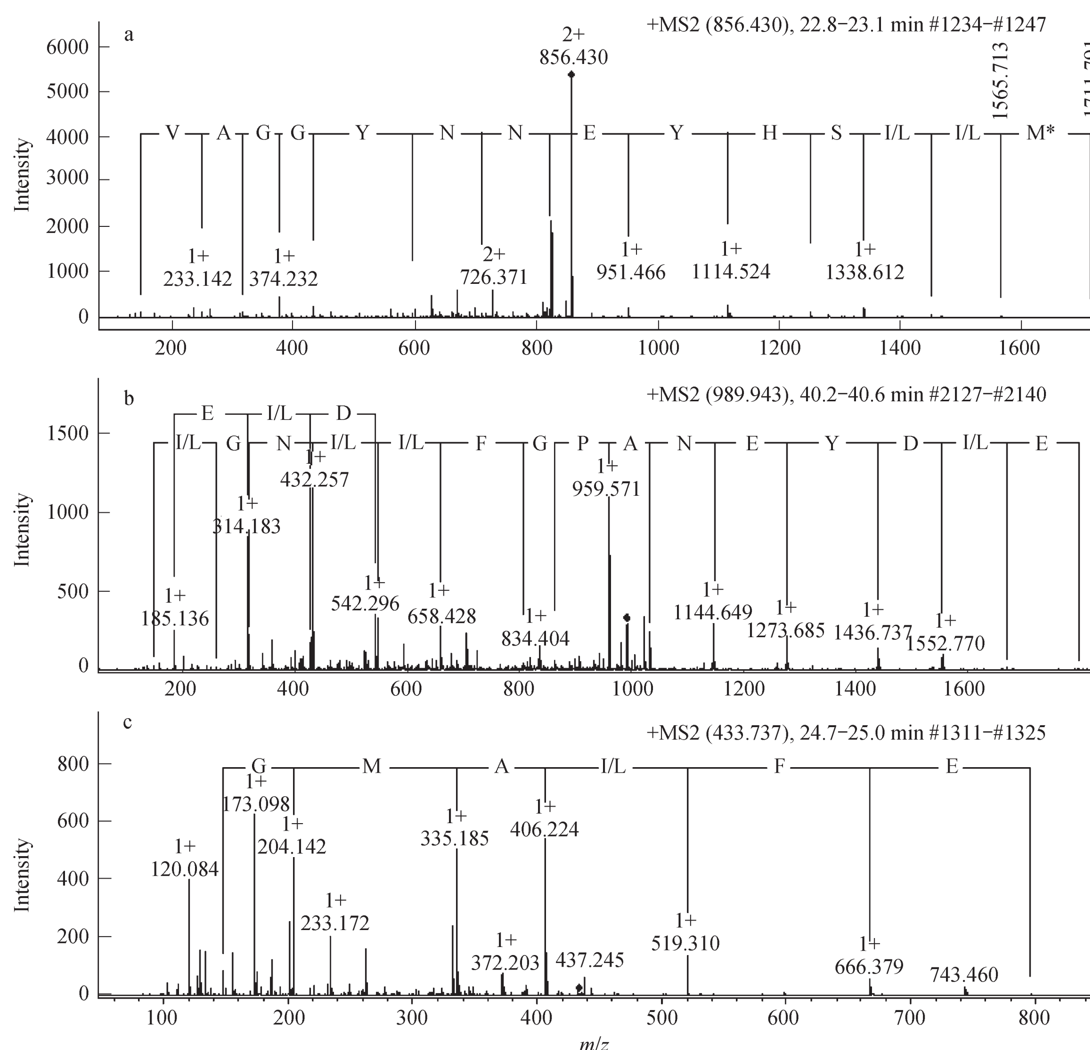

Fig. 2 MS/MS spectra of the doubly charged precursor ion with *m/z* 856.430(2^+) at the retention time 22.8 min (a), 989.943(2^+) at 40.2 min (b) and 433.737 (2^+) at 24.85 min (c).

2.6 Purification of SOD and activity measurement

The SOD (ca. 45 kDa) of *O. anthropi* CTS-325 is a homo-dimmer with two approximate 23 kDa subunits based on the molecular weight (Fig. 4a). After SOD was purified by $(NH_4)_2SO_4$, DEAE cellulose column and sephadex G-75 column (Fig. 4b), the specific activity measured during the purification procedure was 110.4, 1222.5 and 2694.6 U/mg, respectively (Table 1). The final yield was 36.6%, which corresponded to a 28.4-fold purification factor.

Table 1 Purification of SOD protein

Purification	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	132	12500	94.7	100	1
$(NH_4)_2SO_4$	53	5850	110.4	46.8	1.17
DEAE-cellulose	4.0	4890	1222.5	39.8	12.9
Sephadex G-75	1.7	4580	2694.6	36.6	28.4

3 Discussion

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is considered to be an efficient and standard method for the analysis of protein samples that have been separated by 1D or 2D gel electrophoresis with subsequent in-gel digestion (Budnik *et al*., 2004). Tandem mass spectrometry (MS/MS) produces fragment spectra that contain structural information related to the sequences of the peptides, which can then be used to identity the protein through a database search. Even if the difference is as small as one peptide, it may be sufficient for the identification of a unique protein (Ishihama *et al*., 2005). When the protein of interest is not in a database or the similarity between the unknown protein and a homologous protein of another species is low, it is essential to sequence the unknown protein by LC-MS/MS or other protein sequencing methods (Wielsch *et al*., 2006). In the present study, we endeavored to determine the sequences of three peptides of an unknown protein by LC-MS/MS and subsequently identified the protein as superoxide dismutase

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                                            Peptide I                            Peptide II
De novo  ----------------------  MIISHYENNYGGAVK  --------  LAELDYENAPGFLINGLKR  ---  31
P17550   MLYEMKPLGCEPAKLTGLSEK   LIFSHYENNYGGAVK  RLNAITAT  LAELDMATAPVFTLNGLKR  EEL  66
Q8RSK5   MPYELKPLSCDPAKLTGLSEK   LIVSHWENNYGGAVE  RLNAIEQQ  LAQLTWGAAPVFEINGLKR  EEM  66
Q89D83   MTYQSKPMPFDPKSIAGISEK   VLVSHYENNYVGAVK  RLNAIGTQ  LAELDFAKAPNFVINGLKR  EEL  66
                                 ::.**:****:***:            **:*:...**:*.:*****

                                                             Peptide III
De novo  -------------------------------------------  AEFIAMGK  ------------------  42
P17550   IATNSMILHEVYFDSLG-DGGSLDGALKTAIERDFGSVERWQ  AEFTAMGK  ALGGGSGWVLLTYSPR   131
Q8RSK5   IASGSMILHEVYFDSLGGTGGDPGGALKQAIERDFGSVDAWR  AEFTAMGK  AQGGGSGWTLLVWSPR   132
Q89D83   LASNSMILHEIYFDGLG-GAGKPSTALAEAIARDFGSMERWR  AEFSAMGK  AEGGGSGWVILSYSPR   131
                                                    ***.****

De novo  ------------------------------------------------------------------
P17550   DGRLVNQWASDHAHTLAGGTPVLALDMYEHSYHMDYGAKAAAYVDAFMQNIHWQRAATRFAAAVRD-   197
Q8RSK5   RERLVNAWAADHAHNLAGATPLVALDMYEHSYHMDFGAKAAAYVDAFMQNLSWTAAEAAFAKVEGGA   199
Q89D83   DKRLVNQWAADHTTTLAGGRPVLVLDMYEHAYHMDYGAAAAKYVDIYMEAIKWDNAATLYDQYRREA   198
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Fig. 3 Peptide sequences identified through MS BLAST homology search. "*": identical residues; ":": residues with a high level of similarity; ".": residues with a lower similarity level.

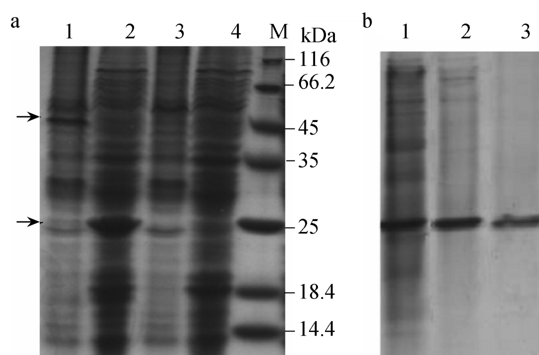

Fig. 4 SDS-PAGE analysis of superoxide dismutase like protein (a). Lanes 1 and 2 were induced with 2.0 mmol/L potassium dichromate when the strain was cultured; lanes 3 and 4 were not induced with potassium dichromate. In the SDS-PAGE analysis, in order not to reduce disulfide bond, lanes 1 and 3 were not added DTT as reducer in the loading buffer, and also not be treated by heat denaturation; lanes 2 and 4 were denatured routinely to disassociate subunits. Purification of SOD (b). Lane 1: 65% ammonium sulfate precipitation; lane 2: run through from DEAE cellulose column; lane 3: purified using Sephadex G-75 column and concentrated by centrifugation on Ultracel PL-10 micro-concentrators. M: molecular weight of DNA or protein.

homology using BLAST homology searching with the three sequences obtained. With the aid of PCR related technology, the SOD full ORF sequences can be obtained efficiently and based on this, future work related to SOD purification and functions characterization can be carried out.

According to previous research, there are three possible chromate resistance mechanisms in bacterial systems: (1) the reduction of intracellular accumulation through direct efflux chromate outside the cells as reported by Nies (2003), who demonstrated that the membrane-bound ChrA protein is essential for chromate resistance and probably responsible for chromate efflux; (2) the avoidance of chromate contact with intracellular components through the formation of a capsule on the bacterial surface (Yang *et al*., 2007; Lin *et al*., 2006); (3) the removal of ROS by anti-oxidative enzymes such as superoxide dismutase and hydrogenase (Roux and Coves, 2002; Fournier *et al*., 2004).

We did not, however, find the chromate efflux ChrA protein in the strain CTS-325 by North blotting (data not shown). Moreover, as bacterial inner and surface structures by transmission electron microscopy and atomic force microscopy were observed (Li *et al*., 2008), the results rule out the possibility of bacterial self-protection via the expression of condensed surface components. Previous research has found that the increase of SOD activity was only about 2-fold when *R. metallidurans* CH34 was stimulated by selenite (Roux and Coves, 2002). While, for *O. anthropi* CTS-325 under the stimulation of only 2 mmol/L Cr(VI), the up-regulation of the SOD was more than 10 folds. Such results indicate that a great number of ROS were produced in the chromate reduction bacteria *O. anthropi* CTS-325 under the stimulation of chromate. Scavenger ROS by SOD may be the major chromate resistance mechanism for *O. anthropi* CTS-325.

4 Conclusions

The *O. anthropi* strain CTS-325 was isolated from a Chinese chromate plant. When the strain was induced with

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Cr(VI), the superoxide dismutase increased significantly in the whole cell proteins. The superoxide dismutase catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide and molecular oxygen. It is believed that the superoxide dismutase is one of the most important anti-oxidative enzymes for *O. anthropi*.

Acknowledgments

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