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Membrane-Associated Hexavalent Chromium Reductase of *Bacillus megaterium* TKW3 with Induced Expression

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Abstract Hexavalent chromium (Cr^{6+}) is a highly harmful pollutant, which can be detoxified and precipitated through reduction to Cr^{3+}. *Bacillus megaterium* TKW3 previously isolated from chromium-contaminated marine sediments was capable of reducing Cr^{6+} in concomitance with metalloids (Se^{4+}, Se^{6+}, and As^{5+}). Notwithstanding approximately 50% inhibition, it was the first report of simultaneous bacterial reduction of Cr^{6+} and Se^{4+} (to elemental Se). No significant difference was observed among electron donors (glucose, maltose, and mannitol) on Cr^{6+} reduction by *B. megaterium* TKW3. The reduction was constitutive and determined to be non-plasmid mediated. Peptide mass fingerprints (PMF) revealed a novel aerobic membrane-associated reductase with Cr^{6+}-induced expression and specific reductive activity (in nmol Cr^{6+}/mg protein/min) of 0.220 as compared with 0.087 of the soluble protein fraction. Respiratory inhibitor NaN_3 did not interfere with the reductase activity. Transmission electron microscopy with energy dispersive X-ray (TEM-EDX) analysis confirmed the aggregation of reduced chromium along the intracellular membrane region. Future identification of the *N*-terminal amino acid sequence of this reductase will facilitate purification and understanding of its enzymatic action.

Key words: *Bacillus megaterium*, bioremediation, chromate reductase, metalloid, proteomics, selenium.

Hexavalent chromium (Cr^{6+}) draws serious public health and legislative concerns because of its extremely high toxicity, mutagenicity, and carcinogenicity. The structural similarity of chromate (CrO_4^{2-}), a predominant Cr^{6+} oxyanion, to sulfate (SO_4^{2-}) allows it, as an analogue substrate, to penetrate cells through the sulfate-transport system in the membrane. Its strong oxidizing power damages cellular materials and generates reactive radicals attacking nucleic acids [10, 25]. Because of its impressive corrosion resistance, Cr^{6+} is widely applied in electroplating, wood preservation, leather-tanning, and alloy production [6, 18]. Furthermore, the high solubility of Cr^{6+} enhances mobility and bioavailability, posing extensive hazards to humans and ecosystems. Conventional physicochemical treatments are prohibitively expensive for large-scale *in situ* cleaning up. Alternatively, bioremediation through sorption, accumulation, and transformation are of increasing interest. Selective microorganisms have been found to be capable of reducing Cr^{6+} to nontoxic insoluble Cr^{3+} [commonly as $Cr(OH)_3$]. Bioreduction can be achieved indirectly with metabolites, such as ascorbic acid and H_2S, or through direct enzymatic reactions [4, 9, 28].

The majority of aerobic Cr^{6+} reductases reported hitherto, mainly from *Pseudomonas* spp., *Escherichia coli*, and *Bacillus* sp. ES29, are soluble in the cytoplasm [4, 12, 20]. Two of these soluble reductases, namely ChrR and YieF isolated from *P. putida* and *E. coli*, respectively, have recently been purified to homogeneity [1, 17]. *P. maltophilia* O-2 was the only reported obligatory aerobic Cr^{6+} reducer involving membrane-associated reductase [3]. In contrast, Cr^{6+} under anaerobic condition frequently serves as a terminal electron acceptor in the respiratory chain with membrane-associated reductase, similar to sulfate-reducing bacteria (SRB) and *Enterobacter cloacae* [15, 27]. In our previous study, *Bacillus megaterium* TKW3 was isolated and characterized from Cr^{6+}-contaminated marine sediments [7]. Intact cells were found to reduce Cr^{6+} in the presence of oxygen. Optimal growth conditions and the effects of various physical parameters, including temperature, salinity, initial Cr^{6+} concentration, and cell density, were investigated [7]. The objectives of the present research were to enhance the capacity of *B. megaterium* TKW3 to reduce Cr^{6+} with

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different electron donors and concomitant toxic metalloids, and to isolate its functional aerobic Cr^{6+} reductase.

MATERIALS AND METHODS

Culture and Cell Fractions

B. megaterium TKW3 was enriched and isolated from marine sediments inundated with Cr^{6+} and other metals, as previously reported [7]. Cells were grown overnight in 400 ml of nutrient broth (NB) (Difco Lab., Detroit, MI, U.S.A.), containing (in g/l): 1.0 beef extract, 2.0 yeast extract, 5.0 peptone, and 5.0 NaCl, at 30°C with orbital shaking of 150 rpm. Cell pellet was harvested at the mid-exponential phase by centrifugation at 8,000 ×*g* and 4°C for 15 min, and then washed twice with phosphate buffer (5.3 g/l K_2HPO_4 and 5.2 g/l KH_2PO_4, pH 7.2). The pellet was resuspended in 10 ml of prechilled phosphate buffer and lyzed in an ice-bath with a Microtip-probe sonifier (Model 250, Branson Ultrasonic, Danbury, CT, U.S.A.). Power was supplied 5 times in 1-min pulse at 50 W. The sonicate was centrifuged at 27,000 ×*g* and 4°C for 40 min to yield the soluble extract (S_{27k}) in the supernatant and the membrane-associated fraction (*M*) in the pellet, and the latter was washed and resuspended in 10 ml of phosphate buffer. Both fractions were immediately analyzed for protein concentration with a modified Bradford assay [8] by adding 200 µl into 2.5 ml of Coomassie Brilliant Blue reagent (100 mg Coomassie Brilliant Blue G250, 50 ml 95% ethanol, 100 ml 85% H_3PO_4, 1 l distilled water), and then absorbance at 595 nm (A_{595}) was measured by a spectrophotometer (Model UV-1201V, Shimadzu, Kyoto, Japan) after 5 min. The standard bovine albumin protein (Sigma-Aldrich, St. Louis, MO, U.S.A.) showed a linear relationship between 2.5 and 30 µg/ml.

Effect of Electron Donors and Concomitant Metalloids

The effect of various electron donors on Cr^{6+} reduction by *B. megaterium* TKW3 was also studied. One ml of active cell culture of *B. megaterium* TKW3 was inoculated into 30 ml of minimal salt medium (MSM) containing 0.8 mM K_2CrO_4 (Fisher Scientific, Pittsburgh, PA, U.S.A.), and 20 mM each of glucose, maltose, or mannitol supplemented as the sole carbon source. The constituents of MSM included (in g/l): 0.8 K_2HPO_4, 0.2 KH_2PO_4, 0.05 $CaSO_4 \cdot 2H_2O$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, and 1.0 $(NH_4)_2SO_4$ [11]. The inoculated culture was incubated at 30°C and 150 rpm with orbital shaking. One ml of sample was taken from each culture at regular time intervals for the assessment of bacterial growth with a modified Bradford assay and Cr^{6+} concentration with the colorimetric diphenylcarbazide (DPC) method [24]: Three-hundred µl of subsample was transferred into 9.7 ml of 0.4 M H_2SO_4 buffered with 25 µl H_3PO_4, then 500 µl of DPC reagent (0.025 g 1,5-diphenylcarbazide in 10 ml of acetone) was added for a 5-min reaction before measuring A_{540} on a spectrophotometer (Shimadzu UV-1201V). To study the effect of concomitant metalloid oxyanions on Cr^{6+} reduction by *B. megaterium* TKW3, 1.0 ml of active culture of *B. megaterium* TKW3 was inoculated into 20 ml of NB (Difco Lab., Detroit, MI, U.S.A.) amended with sodium salt of 0.32 mM arsenate (AsO_4^{3-}), 0.58 mM selenite (SeO_3^{2-}), or 0.53 mM selenate (SeO_4^{2-}). The inoculated cultures were incubated as described above, and 1.0 ml of sample was taken at regular time intervals for quantification of bacterial biomass and Cr^{6+} concentration. All procedures were conducted aseptically in duplicate.

Reduction by Cell Fractions and Localization

Cr^{6+} reductase activity was assayed in 20 ml phosphate buffer containing 3.6 mM NADH, 1.2 mM Cr^{6+}, and 1.0 ml cell fractions (intact cells, S_{27k}, and *M*) at 30°C with 150 rpm orbital shaking. One-ml sample at regular time intervals was withdrawn and quantified for the remaining Cr^{6+}. The effect of respiratory inhibitor NaN_3 (1 mM) on Cr^{6+} reduction by cell fractions was investigated to elucidate their functional role in the process.

Transmission electron microscope equipped with energy dispersive X-ray (TEM-EDX) analyzer was used to determine the localization of reduced chromium deposits. Cell pellet pregrown on Cr^{6+} was first fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) for 1 h at 4°C. It was resuspended in cacodylate buffer with 0.1 M sucrose for 30 min at 25°C and postfixed with 1% OsO_4 in cacodylate buffer for another hour, followed by centrifugation with an equal volume of 2% agar at 2,500 rpm for 10 min to generate the gel block. A 1 mm cube was then prepared for dehydration in an ethanol series of 50%, 70%, and 90% each for 5 min and 100% thrice for 10 min, followed by propylene oxide twice for 5 min. Thereafter, it was infiltrated with a mixture of 1:1 (v/v) epoxy resin and propylene oxide mixture, and epoxy resin at 37°C for 1.5 h and 1 h, respectively, and then embedded in epoxy resin and polymerized at 90°C for 2 h. Semithin section with 0.4-µm thickness was cut with a Reichert ultramicrotome and stained with 0.5% toluidine blue in 1% sodium borax for 30 s on a hot-plate to facilitate drying. Ultrathin section with 100-nm thickness was finally prepared, with the target portion confirmed under a light microscope, and stained with 2% (w/v) uranyl acetate for 20 min and lead citrate for 15 min. It was observed under TEM (Model H-600, Hitachi) and analyzed for the atomic percentage of chromium at selected spots with EDX (Link eXL) and its prescribed software.

Proteome Profile upon Cr^{6+} Exposure

Two-dimensional electrophoresis (2-DE) was conducted to investigate the proteome profile of *B. megaterium* TKW3

upon exposure to Cr^{6+}, and to identify the induced protein(s) with possibility of being the Cr^{6+}-reductase. Cells pregrown in control and 1.2 mM Cr^{6+} added NB (Difco Lab, Detroit, MI, U.S.A.) were lyzed with a sonifier (Branson 250), and then centrifuged at 8,000 $\times g$ at 4°C for 15 min to remove unbroken cells and debris. Supernatants were concentrated by ultrafiltration through an Amicon YM-3 membrane (Amicon, Bedford, MA, U.S.A.).

Two kinds of extraction reagent were used: (i) Tris buffer (40 mM Tris base), which extracts the soluble protein fraction, and (ii) Urea-thiourea cocktail buffer {5 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 2% (w/v) *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (SB 3-10), 40 mM Tris, 0.2% (w/v) Bio-lyte 3/10 ampholyte, and 1% (v/v) tributyl phosphine (TBP)}, which extracts membrane-associated proteins. An immobilized pH gradient (IPG) strip of linear pH 3–10 (Amersham Pharmacia Biotech Asia Pacific Ltd, Hong Kong) was used in the initial one-dimensional electrophoresis (1-DE). Eighty μg of sample was rehydrated before loading onto the IPG strip for isoelectric focusing with Protean-2D-cell (Bio-Rad, Hercules, CA, U.S.A.) in the following manner: 13 h at 50 V, 2 h at 100 V, 2 h at 200 V, 1 h at 500 V, 1 h at 1,000 V, 2 h at 4,000 V, and 6 h at 8,000 V. The IPG strip was then placed in a screw-cap tube added with 4 mM TCEP in 10 ml of equilibration solution [50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), and traces of bromophenol blue] for 15 min with gentle rotary shaking. The first equilibration buffer was then replaced with 10 ml of 260 mM iodoacetamide in a similar manner.

After equilibration, the IPG strip was rinsed with electrophoretic buffer and placed on the top edge of the resolving polyacrylamide gel overlaid with molten 0.5% agarose solution in Tris/Glycine/SDS electrophoresis buffer for two-dimensional electrophoresis (2-DE). Electrophoresis was performed at 12.5 mA for 30 min and 25 mA for 3.5 h, followed by silver staining for visualization. The gel was fixed for 2 h in 40% ethanol and 10% acetic acid, sensitized with 30% ethanol, 0.2% (w/v) sodium thiosulfate, 6.8% (w/v) sodium acetate, and 0.125% glutaraldehyde, followed by washing thrice with distilled water for 5 min each time. For image development, staining was performed for 20 min in 0.25% (w/v) silver nitrate with 0.015% formaldehyde before washing twice with distilled water for 1 min each time. It was finally developed in 2.5% (w/v) sodium carbonate containing 0.0074% formaldehyde, and then terminated with 1.5% (w/v) EDTA. Duplicates of 2-DE gel analysis for each extraction set were highly reproducible.

Protein Identification

Protein spots with differential expression between the cultures supplemented with Cr^{6+} and those without were selected for the determination of peptide mass fingerprint (PMF) with matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS). Following the separation of proteins with 2-DE, 0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid and 40% ethanol (v/v) was used for staining overnight (instead of silver staining) and destaining with the identical solvent. Selected protein spots were excised and preserved in acetonitrile for tryptic digestion and MALDI-TOF-MS, which were conducted by the Genome Research Centre, The University of Hong Kong. Mass spectra were analyzed with the computer software Data Explorer for PMF, and the database was then searched against MASCOT (Matrix Science) at http://www.matrix-science.com.

Extraction of Plasmid

Small-scale extraction of plasmid DNA from culture pregrown in the presence of Cr^{6+} was performed with alkaline lysis, as described by Sambrook *et al.* [19], and confirmed with the QIAprep spin miniprep kit (Qiagen Inc., California). Plasmid DNA was loaded onto a 0.7% horizontal agarose gel, resolved at 5 V per cm, and then stained with ethidium bromide before being photographed on an ultraviolet transilluminator. Detailed procedures are available elsewhere, with fluorescent band of extracted plasmid DNA visible in the profile [26].

RESULTS

B. megaterium TKW3 was resistant to metalloids Se^{4+}, Se^{6+}, and As^{5+}, and also of utilizing a broad range of electron donors and reducing Cr^{6+} [7]. The effect of electron donors on Cr^{6+} reduction was further investigated with

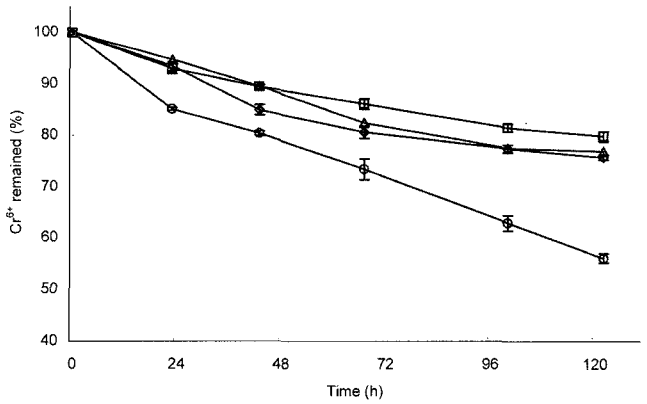

Fig. 1. Effect of electron donors on Cr^{6+} reduction by *B. megaterium* TKW3.
The concentration of Cr^{6+} was quantified with a colorimetric diphenylcarbazide method. One ml of live culture was inoculated into 30 ml of minimal salt medium amended with 0.8 mM Cr^{6+} and supplemented with 20 mM glucose (□), maltose (△), mannitol (◇), or nutrient broth (○). Error bars indicate standard deviation from duplicate.

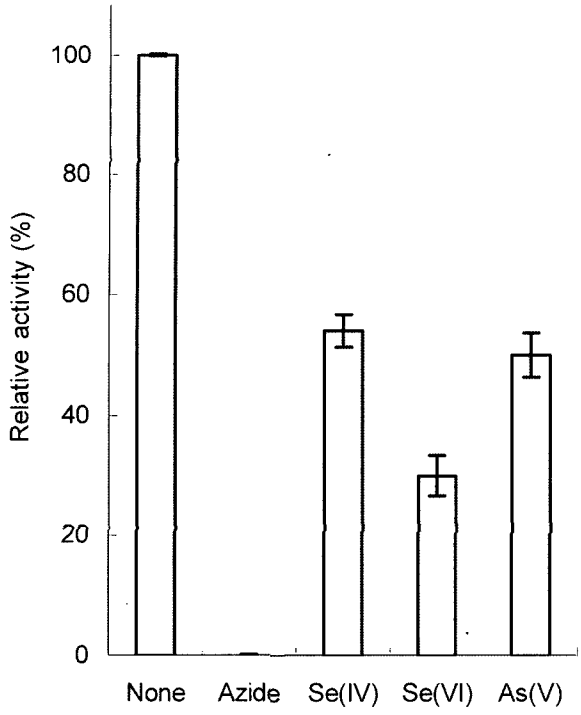

Fig. 2. Effect of metalloids (Se^{4+}, Se^{6+}, and As^{5+}) and respiratory inhibitor (NaN_3) on the relative activity of Cr^{6+} reduction by *B. megaterium* TKW3.
Error bars indicate standard deviation from duplicate.

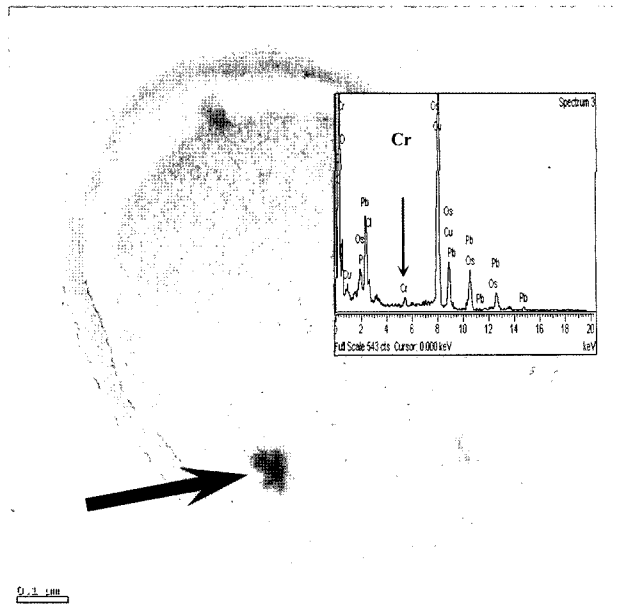

Fig. 3. Transmission electron micrograph of cross-section of *B. megaterium* TKW3 grown in NB amended with 1.2 mM Cr^{6+}, with inset illustrating the energy dispersive X-ray spectrum of the electron-dense deposit (arrow) along the intracellular membrane region.
Peaks of Os and Pb originated from reagents in the TEM preparation, whereas the Cr peak represents the elemental composition of the deposit (scale bar= 0.1 μm).

different carbon sources to support bacterial growth, including glucose, maltose, and mannitol. At an initial concentration of 0.8 mM Cr^{6+}, the percentage of Cr^{6+} reduced was similar: mannitol (25%), maltose (23%), and glucose (21%). In comparison, the percentage reduction facilitated by NB was 1-fold higher than the single carbon source (Fig. 1). Although the metalloids tested did not inhibit the growth of *B. megaterium* TKW3, Se^{4+} and As^{5+} decreased Cr^{6+} reduction activity by half, whereas Se^{6+} retarded the activity by nearly 70% (Fig. 2). Red precipitates were observed in culture supplemented with Se^{4+}, indicating its reduction to amorphous elemental Se^{o}. Respiratory inhibitor NaN_3 completely ceased metabolism of *B. megaterium* TKW3 (Fig. 2). However, no inhibitory effect of NaN_3 on Cr^{6+} reduction was found by cell fractions S_{27k} or *M* (data not shown). Specific reductive activity (in nmol Cr^{6+}/mg protein/min) of the membrane-associated fraction *M* (0.220) was significantly higher than the soluble fraction S_{27k} (0.087); the activity of intact cell was 0.297 (Table 1). TEM-EDX analysis revealed the aggregation of chromium deposits along the intracellular membrane region (Fig. 3). Similar to the intact cells, the cell fraction *M* was capable of reducing Se^{4+} to elemental Se^{o} as red precipitates.

The exposure to Cr^{6+} was found to alter the proteome profiles extracted with both Tris buffer and Urea-thiourea cocktail buffer. In the soluble fraction, Cr^{6+}-induced expression of at least 7 protein spots were identified in clusters A, B, and C of Fig. 4A, whereas suppression by Cr^{6+} was seen in clusters D and E of Fig. 4B. The membrane-associated fraction displayed 2 prominent spots with Cr^{6+}-induced expression in cluster A of Fig. 5A, whereas spots in clusters B and C were present exclusively without Cr^{6+} stress (Fig. 5B). Bioinformatic PMF search indicated

Table 1. Hexavalent chromium reductase activity in cell extracts of *B. megaterium* TKW3, initially with 1.2 mM Cr^{6+} and 3.6 mM NADH as electron donor in 20 ml of phosphate buffer.

Cell extracts	Protein conc. (mg/ml)	Cr^{6+} reduced (%)	Specific reductive activity (nmol Cr^{6+}/mg protein/min)
Intact cells	0.386	53.4	0.297
Soluble fraction (S_{27k})	0.312	16.8	0.087
Membrane-bound fraction (*M*)	0.532	69.5	0.220

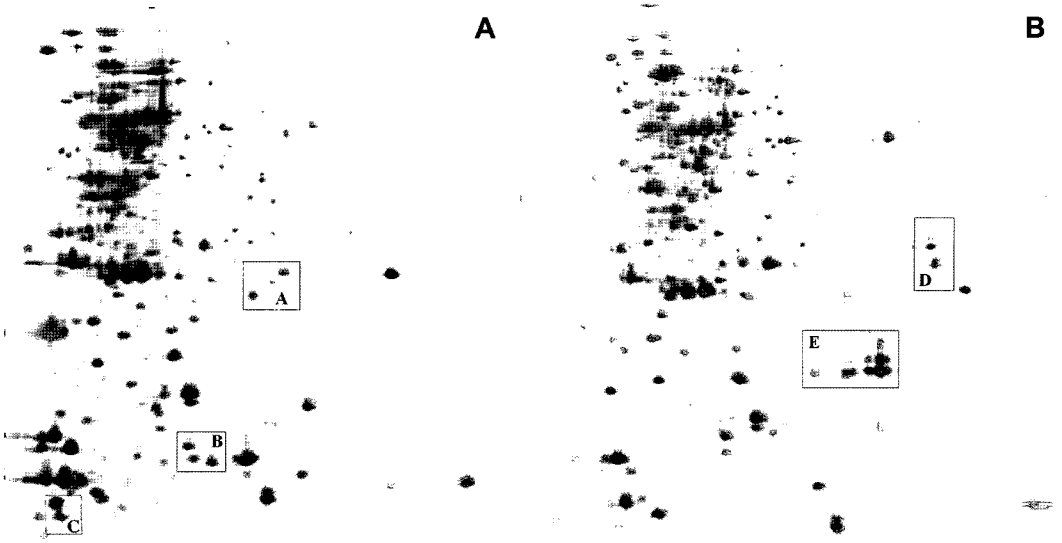

Fig. 4. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of soluble proteome profile of *B. megaterium* TKW3 extracted by Tris buffer

A. Culture exposed to 1.2 mM Cr^{6+}, with clusters A to C indicating the induced protein spots; **B**. culture without Cr^{6+} stress, with clusters D to E indicating the suppressed proteins. Conditions of 2-D PAGE were initial first horizontal-dimension with immobilized pH 3–10 gradient, and then second vertical-dimension with 12.5% PAGE.

that the multiple protein spots in cluster B of Fig. 5B suppressed by Cr^{6+} were isoforms of 3-ketoacyl-CoA reductase PhaB (Table 2), specific for *B. megaterium* fatty acid metabolism, with high MASCOT score of 82, indicating that the identification was significant (p= 0.0062). On the other hand, the 2 spots in cluster A of Fig. 5A might be isoforms, but with no significant matching of reported proteins, indicating a new enzyme

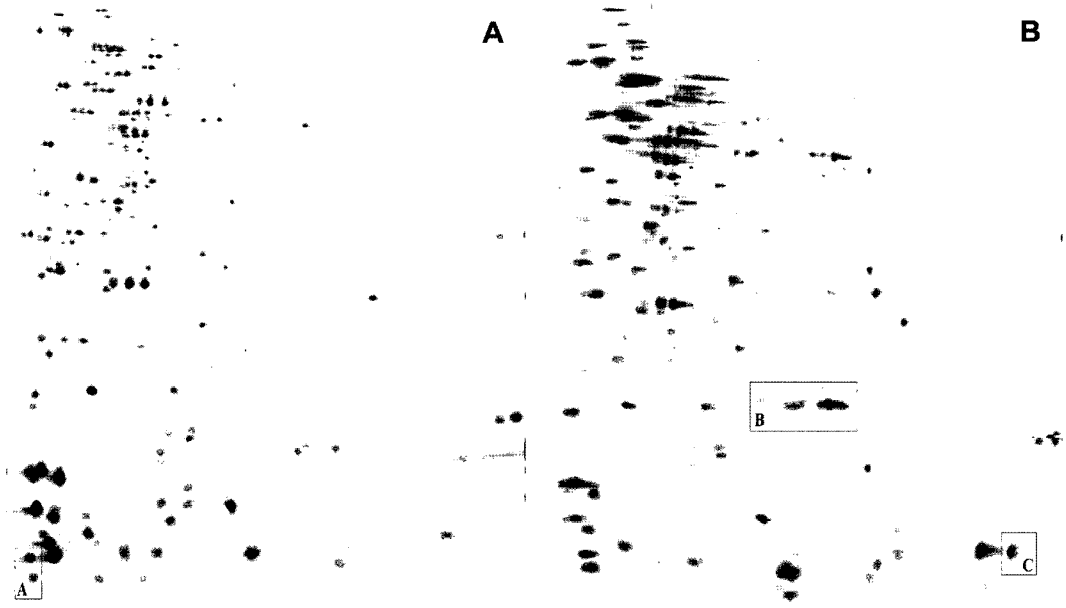

Fig. 5. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of membrane-associated proteome profile of *B. megaterium* TKW3 extracted by Urea-thiourea cocktail buffer.

A. Culture exposed to 1.2 mM Cr^{6+}, with cluster A indicating the induced protein spots; **B**. culture without Cr^{6+} stress, with clusters B to C indicating the suppressed proteins. Conditions of 2-D PAGE were initial first horizontal-dimension with immobilized pH 3–10 gradient, and then second vertical-dimension with 12.5% PAGE.

Table 2. A sequence of peptide mass fingerprints was obtained by MALTI-TOF-MS from the multiple protein spots in cluster B of Fig. 5B, observed peptide masses (±0.2 Da) were compared with those theoretically calculated, and were identified as isoforms of 3-ketoacyl-CoA reductase PhaB. Matched peptides covered 32% of the total length. A MASCOT score of 82 (greater than 72) indicates that the protein identification is significant (p=0.0062, <0.05).

Observed mass (Da)	Calculated mass (Da)	Position	Peptide sequence
2,420.05	2,419.04	50–72	EIKDNGGEAIAVQADVSYVDQAK
1,772.91	1,771.96	80–96	AAFGQLDILVNNAGITR
1,004.49	1,003.50	103–110	LGEEDWKK
2,296.06	2,295.05	138–160	VINISSIIGQAGGFGQTNYSAAK
1,151.59	1,150.62	213–222	RLGHAEEIAR

potentially conferring Cr^{6+} reductive activity of the membrane-associated fraction *M* (Table 1). Its closest match was an integrase, commonly known to mediate gene recombination in *Pseudomonas* spp. [5, 21], of *P. syringae* with a low MASCOT score of 66 (p=0.26). Furthermore, no plasmid was detected in the electrophoresis gel of *B. megaterium* TKW3 culture grown with Cr^{6+} using standard procedures with both the plasmid positive and negative control strains of bacteria (data not shown). These results confirmed that plasmid was not involved in Cr^{6+} reduction by *B. megaterium* TKW3 for the reductive activity, and that the reductive activity was a constitutive property of the bacterium.

DISCUSSION

Cr^{6+}-reducing bacteria are ubiquitous in soil and sediment, and biostimulation of indigenous bacteria shows potential for *in situ* remediation. However, concomitant pollutants might restrict the efficacy [23]. *B. megaterium* TKW3 has been shown to be capable of reducing Cr^{6+} with common substrates (e.g., glucose, maltose, and mannitol) in the concurrent presence of toxic metalloids (Se^{4+}, Se^{6+}, and As^{5+}). Simultaneous reduction of Cr^{6+} and Se^{4+} further demonstrated its versatility for *in situ* cleanup of multiple contaminations. Unlike metals that inhibit Cr^{6+} reduction with increased susceptibility of *E. cloacae* HO1, such as Hg^{2+}, Ag^{+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} [13], no direct relationship between Cr^{6+} reduction and viability of *B. megaterium* TKW3 has been established, indicating an alternative inhibitory mechanism of these metalloids other than toxicity. In the present study, the structural similarity of metalloid oxyanions, SeO_4^{2-} in particular, to CrO_4^{2-} might have led to competitive inhibition, as observed with SO_4^{2-} [29]. Similar to *Bacillus* sp. ES29, NaN_3 showed no inhibitory effect on Cr^{6+} reduction by cell extracts of *B. megaterium* TKW3, illustrating the independence of Cr^{6+} reduction from metabolism [4].

In contrast to the soluble Cr^{6+} reductase of *Bacillus* sp. ES29 with activity stimulated by Cu^{2+} while noncompetitively inhibited by Hg^{2+} [4], a membrane-associated reductase was isolated from *B. megaterium* TKW3. Hence, multiple Cr^{6+} reduction mechanisms appear to be present among Bacillaceae species. Membrane-associated Cr^{6+} reductase was first discovered by Wang *et al.* [27] in anaerobe *E. cloacae*, which utilizes Cr^{6+} as a terminal electron acceptor in the respiratory chain. In an oxygenated environment, with the exception of *P. maltophilia* O-2 with membrane-associated reductase that has not further been investigated [3], all reported obligatory aerobic Cr^{6+} reductases are soluble in nature. Two aerobic Cr^{6+} reduction mechanisms with the coupling of reductase and electron donor such as NADH, NADPH, and endogenous reserve have been identified: (i) direct reduction of Cr^{6+} to Cr^{3+} with three-electron transfers catalyzed by reductase YieF (from *E. coli*), and (ii) transient two-step reduction of Cr^{6+} to intermediate Cr^{5+}/Cr^{4+} followed by instant transformation to Cr^{3+}. Some nitroreductases and Cr^{6+} reductase ChrR (from *P. putida*) were found to mediate the second pathway [1, 2, 14].

Ishibashi *et al.* [12] partially purified a Cr^{6+} reductase from *P. putida* PRS2000, and another Cr^{6+} reductase was purified 38-fold from *P. ambigua* G-1 [22], which was later found to be homologous to a nitroreductase in *Vibrio harveyi* KCTC 2720 [14]. Since the reductase from *P. ambigua* G-1 was not deposited in GenBank at first, Park *et al.* [17] employed strain MK1 of *P. putida*. Following sequential extraction of functional fractions, complemented with 1-DE for protein isolation, they purified the Cr^{6+} reductase ChrR to homogeneity (600-fold). The ChrR-coding gene, known as *chrR*, was cloned [16], and ChrR was identified as a flavoprotein of about 50,000 Da [1]. Furthermore, a previously uncharacterized open reading frame (ORF) *yieF* from *E. coli* was found to code for Cr^{6+} reductase YieF [16]. YieF is an obligatory 4-electron reducer that mediates direct transformation of Cr^{6+} to Cr^{3+}, with an additional electron transferred to molecular oxygen, thus preventing generation of harmful intermediate Cr^{5+}/Cr^{4+} and free radicals [2]. Both ChrR and YieF are soluble Cr^{6+} reductases. The isolation of a novel membrane-associated Cr^{6+} reductase from *B. megaterium* TKW3 may provide an opportunity to investigate an alternative mechanism of aerobic bacterial Cr^{6+} reduction.

In summary, aerobic Cr^{6+} reduction is an intrinsic property of *B. megaterium* TKW3, which does not involve plasmid, and is mediated by a novel membrane-associated Cr^{6+} reductase whose expression is induced upon Cr^{6+} exposure. Application of reductase in bioremediation has the advantage to protect the culture sensitive to ambient toxicants. Purification and characterization of reductase are expected to allow genetic and protein engineering that potentially enhances operation efficiency. The biomass of reductase involved is minimal [16]. Future studies to identify the *N*-terminal amino acid sequence of this reductase would facilitate the purification and understanding of its enzymatic action on Cr^{6+} reduction, thus bioremediation.

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