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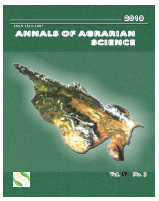

Proteomic responses of oxidative stress-resistant *Arthrobacter globiformis* 151B to Cr and Mg and the influence of Mg on the uptake process of Cr(III) and Cr(VI)

O. Rcheulishvili[a,b*], N. Metreveli[a], L. Tsverava[a] , R. Solomonia[a], A. Rcheulishvili[b], L. Tugushi[b], M. Gurielidze[c]

[a]Ilia State University; 3/5, Kakutsa Cholokashvili Ave., Tbilisi, 0162, Georgia

[b]Ivane Javakhishvili Tbilisi State University; 6, Tamarashvili Str., Tbilisi, 0177, Georgia

[C]Agricultural University of Georgia; 240, David Agmashenebeli Alley, Tbilisi 0159,Georgia

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ABSTRACT

In nature, there are some heavy-metal and oxidative stress resistant bacterial species, which can be used effectively for the environmental biotechnological purposes. They can participate in bioremediation processes: degrade aromatic compounds, uptake radioactive or heavy metals and reduce their carcinogenic activity. Different metals reveal synergist or antagonist effects on each other during their bioaccumulation processes. Knowing of the effects of a metal on another metal uptake processes by bacteria, is one of the actual issues for environmental protection biotechnologies. *Arthrobacter globiformis* 151B is an aerobic, basalt dwelling bacteria, isolated from heavy metal polluted Kazreti region (Georgia). Here we report the influence of magnesium (Mg) ions on the uptake capability of Cr(III) and Cr(VI) by *A. globiformis* 151B. The Influence of Mg(II) and Cr(VI) ions on the proteome of *A. globiformis* 151B have been studied. For identification the significantly differentially expressed proteins, two-dimensional (2-D) gel electrophoresis and Liquid chromatography mass spectrometry (LC-MS/MS) methods were used. We demonstrated that Mg(II) increased the accumulation of Cr(III) by *A. globiformis* 151B and decreased the uptake process of Cr(VI). Significant changes in protein expression involve different groups of proteins. Most remarkable changes were associated with proteins participating in oxidation-reduction processes. According to our earlier and present data, *A. globiformis* 151B is reactive oxygen species (ROS) resistant bacteria with great potential for bioremediation purposes.

Keywords: *Arthrobacter globiformis* 151B, Cr(VI), Mg(II), Bioremediation, Heavy metals, Metal-resistance.

*Corresponding author: Olia Rcheulishvili; E-Mail address: olia.rcheulishvili.1@iliauni.edu.ge

Introduction

Heavy metals, most important industrial contaminants and systemic toxic agents, exert harmful effects on living organisms. Cr(VI) is a highly water soluble, toxic and carcinogenic metal, which is released into the environment due to different industrial and antrophogenic factors [1- 3]. Cr(VI) occupies the prior place between the most acute environmental pollutants [4, 5]. It can easily penetrate the biological membranes of any living cell. Different cellular components are capable to reduce Cr(VI) using the specific or nonspecific reductants such as glutathione, cysteine, NADH and others [6, 7]. In the cell, Cr(VI) can be reduced to its subsequent Cr(V)→Cr(IV) →Cr(III) forms [7]. During the reduction process, the reduction by products: free radicals, reactive oxygen species (ROS), H_2O_2, reactive nitrogen species (RNS) are formed, which attack the DNA, RNA, protein or lipid molecules, damage them and cause mutations [7]. Reduced Cr(III) form is less toxic and less soluble and it is even essential for different living systems [8, 9]. Different types of living cells are experiencing difficulties to counteract oxidative stress processes caused by heavy metals such as Cr(VI). By this point of view,

there is a high interest in the Cr(VI) resistant endogenous bacterial species of *Arthrobacter* genera. *Arthrobacter globiformis* 151B is an aerobic, basalt-dwelling bacteria, isolated from the metal contaminated (Cr, Pb, Zn, V, Ni, Mo) Kazreti region (Georgia) [10], which reduces toxic Cr(VI) into Cr(III) form even of its high concentrations (Cr(VI) >1000 mg/l) and survives [11]. Since the soil microorganisms are influenced by many different metals concomitant action, it is important to study the simultaneous action of diverse metals on resistant bacterial species, during metal accumulation processes. Some metals cause synergic or negative effects upon the accumulation/uptake capabilities by different bacteria. It is shown, that zinc can increase Cr(VI) reduction and accumulation intensity by *A. globiformis* 151B [10]. Magnesium (Mg) is among the most abundant element in the earth's crust and plays an important role for all living systems. Natural habitats are often characterized by the coexistence of Cr and Mg. As a cofactor, Mg participates in different energy-producing enzymatic reactions, in oxidative phosphorylation or glycolysis [12, 13]. Does the presence of Mg(II) ions influence capacity of *A. globiformis* 151B to accumulate and reduce Cr(VI)? This issue is very important for polluted environments. The purpose of the present study was to determine the effect of Mg(II) ions on *A. globiformis* 151B Cr(VI) and Cr(III) accumulation properties and to study the proteomic responses of bacteria under the influence of Mg(II) and Cr(VI) joint action.

Objects and Methods

Reagents and Chemicals

K_2CrO_4, $MgCl_2$, $CrCl_3$, TSB (Tryptic Soy Broth), TSA (Tryptic Soy Agar), reagents for 2-D gel-electrophoresis and mass spectrometry analyses were purchased from Sigma and Thermo Fisher (all ACS grade).

Sample Preparation for Cr(VI), Cr(III) and Mg(II) Uptake Processes, for Proteomic and Spectral Analyses

Bacterial cells of *A. globiformis* 151B were grown aerobically in 250-ml Erlenmeyer flasks as a 100-ml suspension in TS broth at 26˚C. The cells were grown with constant shaking conditions during 40 hours (at a speed of 100 rpm).

To examine the uptake capability of research elements by *A. globiformis* 151B, we conducted two sets of experiments:

First type of experiments were carried out on the following groups of bacterial cells of *A. globiformis* 151B which were grown during 40 hours in the culture medium containing: (**A)** 7.1 μg/ml Cr(III) and 4.1 μg/ml Mg (controls); (**B)** 40 μg/ml Cr(VI) and 4.1 μg/ml of Mg; (**C)** 40 μg/ml Cr(VI) and 50 μg/ml of Mg; (**D**) 7.1 μg/ml Cr(III) and 50 μg/ml of Mg. After 40 hours of metals exposure, bacterial cells were harvested from the nutrient medium by the centrifugation (3,000g, 15 min, 4˚C), rinsed three times in bi-distilled water for atomic absorption spectroscopy (AAS), 2D-gel electrophoresis and Mass spectrometry (MS) analysis. Bacterial culture growth proceeded without medium renewal. Culture growth was monitored by measuring optical density at 490 and 590nm and by weighing of dried bacterial biomass after their centrifugation and lyophilization.

For the second type of experiments we decided to study the effect of high concentration of Mg(II) on Cr(III) accumulation process on different time points by AAS method. The concentration of Mg(II) was 1600 μg/ml and the concentration of Cr(III) was 7.1 μg/ml in the TS broth for *A. globiformis* 151B. Bacterial cells were incubated at 26°C, during the different time points: 16 hours, 24 hours, 48 hours, 96 hours, and 144 hours. After, they were harvested and prepared for spectral analysis. K_2CrO_4, $CrCl_3$, $MgCl_2$ salts were used respectively for both types of experiment.

AAS measurements

For the determination of the concentrations of Cr and Mg(II) by bacteria itself, wet biomass of bacterial pellet (after centrifugation and washing procedures) was placed in an adsorption-condensation lyophilizer and dried following the procedure reported in [14, 15]. Dried cells were ashed in nitric acid, diluted with bi-distilled water and analyzed by AAS method in an acetylene air flame. Analyst 800 (Perkin Elmer) was used. The detection was carried out at 357.9 nm for Cr and at 285.2 nm for Mg(II).

Statistical analysis

The changes in the concentration of Cr and Mg was analyzed separately with one-way analysis of variances (ANOVA) with factor- cultivation media. Two-way ANOVA was applied used for the analysis of data of Cr(III) uptake in time. The factors were: Time and cultivation media. Planned comparisons

were carried out with student t-test. All comparisons were two-tailed. For the statistical analysis GraphPad Prism 5 software was used.

Bacterial cell wall lysis and protein extraction for 2-D Gel Electrophoresis and MS Analyses

The sample preparations for 2-D electrophoresis and MS analysis were carried out essentially as described in [15, 16]. Bacterial pellets were resuspended in buffer (20 mM Tris-acetate, pH 7.8, 20 mM NaCl, 2 mM EDTA, 100μg/mL lysozyme). Samples were incubated for 30 min at 37°C with intermittent vortexing. 9M Urea, 4% Tween 40, 2% Pharmalyte, 2% Mercaptoethanol, 2% protease inhibitor (bacterial) were added and lysates were centrifuged at 15,000 × g for 30 min at 4°C. Protein concentration in supernatants was quantified by a micro-BCA kit (Pierce, Thermo Scientific) in quadruplicate. Appropriate buffer controls were used.

Isoelectric focusing, Equilibration, SDS Electrophoresis, Staining, Scanning, In-gel Digestion and MS analysis were carried out according the [15, 16]. MS/MS spectra data were analyzed using SEQUEST (Proteome Discoverer 1.4), searching against UniProt UniRef 100 *Arthrobacter* species protein databases.

Results and Discussion

It is known, that Mg(II) ion is the important co-factor-component of different macromolecules such as glutathione, enzymes, polycarbohydrates, ATP, RNA, etc. These molecules actively participate in almost all redox reactions occuring in the cell. We examined the changes in bacterial proteome under the influence of Mg(II) and Cr(VI)+Mg(II) as compared to control cells, using 2-D gel electrophoresis and MS analyses. MS analysis were used to determine the identity of the excised proteins. The 2-D gel electrophoresis of *A.globiformis* 151B protein extracts were carried out initially with two pH gradients: 3.0–11.0 and 4.0–7.0. The majority of the proteins on the 3.0 – 11.0 pH gradient gels were concentrated between pH 4.0 and 7.0. Thus, for the better resolution and identification of differentially expressed bands 2-D gel electrophoresis were continued using strips with a pH gradient from 4.0 to 7.0 (Fig. 1 A, B, C, D). During comparative studies of the influence of Cr(VI) and Mg(II) ions action using 2D electrophoresis on the bacterial proteome, reveal that Mg(II) and Cr(VI) joint action can increase protein content on the 2D electrophoregrams, when 40 μg/ml Cr(VI) and 50 μg/ml Mg(II) were added in a growth medium.

Differently expressed bands were identified by MS. The list of differentially expressed proteins are given in the Table (1). Different microorganisms have their diverse protection capabilities from heavy-metal toxicity by various mechanisms such as uptake, methylation, oxidation or reduction [17]. The disturbed cellular metal ion homeostasis, caused by the increased Cr(VI) and Mg(II) level in the growth medium and further reduction process of Cr(VI) inside the cell, produced the formation of ROS and RNS in high quantity, which may cause oxidative damage to biological macromolecules. The 40 hours exposure of *A. globiformis* 151B cells with the concomitant action of Cr(VI) and Mg(II) or single Mg(II) action, resulted in statistically significant differential expression of 30 proteins out of 556 (Table 1). Functions of statistically significantly differentially expressed proteins between Mg(II) and Cr(VI) treated bacteria (**C**) versus control groups (**A**), or between Mg(II) treated bacteria (**D**) versus control groups (**A**) are associated with different activities. The function of proteins, which appeared or increased in Mg(II) or Mg+Cr(VI) treated cells characterized by oxido-reduction activity, metal ion binding, metal ions transport activities, cell SOS-response, nucleotide -excision repair activities, electron-transport activity, riboflavin synthase complex synthesis activity (which is one of the important reductant in the cell) and the other cellular activities related to the oxidative stress response (Table). During Cr(VI) reduction to Cr(III) form a wide spectra of ROS (superoxide anions, hydrogen peroxide, hydroxyl radicals) are generated and as a result the activities of the enzymes involved in the detoxification of reactive oxygen species are changed [18].

The biomass of *A. globiformis* 151B cells were significantly decreased after the treatment of single Cr(VI) or joint Mg(II)+Cr(VI) ions action due to the elevated oxidative stress (Fig. 2). Between the four combinations of metal mixtures (A, B, C or D), 40 μg/ml Cr(VI) in growth medium (**B**) revealed detrimental effect upon the bacterial biomass and decreased it by 1.8 fold. Mg(II) and Cr(VI) joint (**C**) combination decreased bacterial growth by 1.5 fold. The combination (**D**) decreased bacterial biomass only by 1.3 fold.

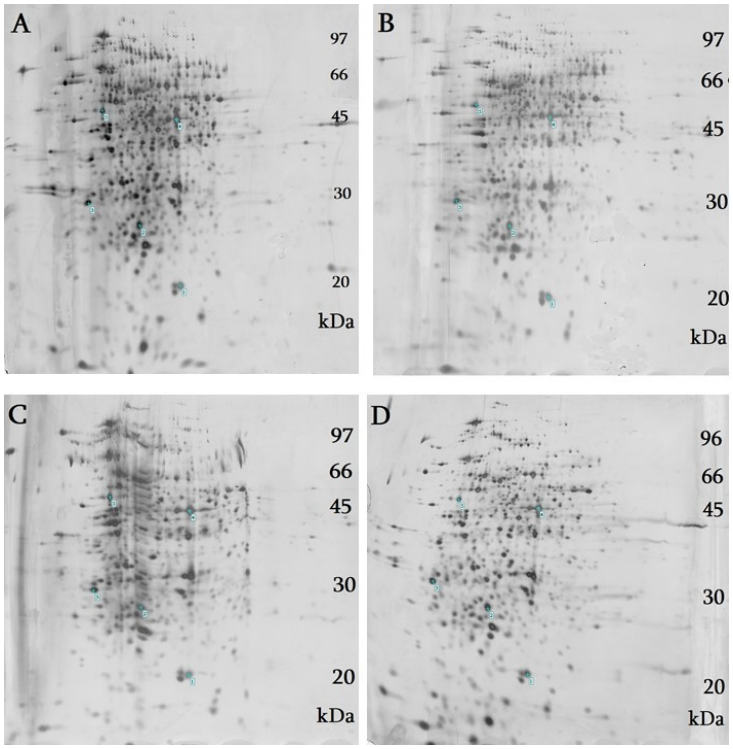

Fig. 1. *Representative images of silver-stained 2-D gel electrophoresis gels on strips with pH linear gradient 4.0 - 7.0 of A. globiformis 151B protein extracts of 40 hours of growth. (A) protein extract from low concentrations of Cr(III) –(7.1 µg/ml) and Mg – (4.1 µg/ml) treated cells (controls); (B) protein extract from 40 µg/ml Cr(VI) and 4.1 µg/ml of Mg treated cells; (C) protein extract from 40 µg/ml Cr(VI) and 50 µg/ml of Mg treated cells; (D) protein extract from 7.1 µg/ml Cr(III) and 50 µg/ml of Mg treated cells. light green color points represent the programme marks done by ImageMaster 2-D platinum 7.0 software. They indicate the matched starting identical points (ID similarities) for matching the rest of the protein ID-s, to find the significantly diferentially appeared protein bands (dots) between A, B, C or D gel-figures. Different ones are cut and analysed by MS for the identification.*

Table. *The list of statistically significantly differentially expressed proteins extracted from Mg(II)+Cr(VI) treated cells versus control cells and Mg(II) treated cells versus control cells of A. globiformis 151B.*

Proteins appearing and their expression increasing in Mg(II)+Cr(VI) treated cells ↑	Molecular/biological functions	**Proteins appearing in Mg(II) treated cells ↑ their expression increases in Mg treated cells**	Molecular/biological functions
Carboxymuconolactone decarboxylase	carboxymuconolactone decarboxylase activity; peroxiredoxin activity, thiol-containing-reductant: hydroperoxide oxidoreductase activity	30S ribosomal protein S1	Nucleic acid binding
DNA-directed RNA polymerase subunit beta	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substratesMolecular functions: DNA binding; DNA-directed 5'-3' RNA polymerase activity ribonucleoside binding	Dimethyl sulfone monooxygenase	Monooxygenase activity, oxidorductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
Capsular glucan synthase	Transferase activity, glucan biosynthetic process	SAM-dependent methyltransferase	Metal ion binding, methyltransferase activity

Aldehyde dehydrogenase	aldehyde dehydrogenase (NAD) activity; aldehyde: NADP+ oxidoreductase, glyceraldehyde-3-phosphate dehydrogenase (NAD+) (non-phosphorylating) activity; NAD binding; NADP binding; Ligand for NAD and NADP, Oxidoreductase activity	NUDIX hydrolase	Hydrolase activity
HNH endonuclease	endonuclease activity; nucleic acid binding; is an integral component of membrane	Two-component sensor histidine kinase	ATP binding, phosphorelay sensor kinase activity
Phenylacetate-CoA oxygenase	2 iron, 2 sulfur cluster binding; electron transfer activity; metal ion binding	UDP-glucose-6-dehydrogenase	NAD binding, oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor polysaccharide biosynthetic process.
ABC transporter	ATPase activity; ATP binding; DNA binding; exonuclease ABC activity; zinc ion binding; nucleotide-excision repair; SOS response	PHB domain-containing protein	integral component of membrane
Glycosyltransferase	guanosine phosphorylase activity; purine-nucleoside phosphorylase activity; pyrimidine-nucleoside phosphorylase activity; thymidine phosphorylase activity; uridine phosphorylase activity	Thiamine-monophosphate kinase	Magnesium ion binding, ATP binding, thiamine-biosynthetic process
ABC transporter substrate-binding protein	metal ion transport	DNA modification methylase	DNA binding, N-methyltransferase activiy, DNA-mediated transposition activity
FAD-linked oxidoreductase	FAD binding; oxidoreductase activity	Transposase	Transposase activity, DNA binding
Undecaprenyl-diphosphate phosphatase	diacylglycerol diphosphate phosphatase activity; phosphatidate phosphatase activity; phosphatidyl glycerophosphatase activity; undecaprenyl-diphosphatase activity	Glutamate synthase	Glutamate synthase activity
1-acyl-glycerol-3-phosphate acyltransferase	1-acylglycerol-3-phosphate O-acyltransferase activity; phospholipid biosynthetic process; an integral component of membrane;	4-aminobutyrate--2-oxoglutarate transaminase	4-aminobutyrate transaminase activity, pyridoxal phosphate binding
6,7-dimethyl-8-ribityllumazine synthase	riboflavin biosynthetic process; 6,7-dimethyl-8-ribityllumazine synthase activity; riboflavin synthase complex	Putative glutamate--cysteine ligase	Glutamate cysteine ligase activity, ATP binding
		FAD-binding protein	D-arabino-1,4-lactone oxidase activity, FAD binding
		L-asparaginase	Asparaginase activity
		Putative oxygenase	Oxidoreductase activity, iron ion binding, 2 iron, 2 sulfur cluster binding
		DNA helicase	ATP binding, DNA binding, DNA helicase activity

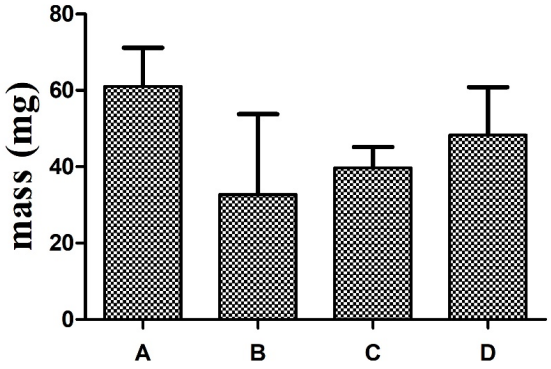

Fig. 2. *The effects of metals on the biomass of A. globiformis 151B. Cultivation time 40 hours. Biomass of the cells which were grown in the culture medium: (A) 7.1 µg/ml - Cr(III) and 4.1 µg/ml Mg (controls); (B) 40 µg/ml Cr(VI) and 4.1 µg/ml of Mg; (C) 40 µg/ml Cr(VI) and 50 µg/ml of Mg; (D) 7.1 µg/ml Cr(III) and 50 µg/ml of Mg .*

By our observations with AAS method, the effect of cultivation medium was significant on the Cr accumulation [$F_{3,11}$=124.09, P=0.0001], whereas Mg(II) accumulation was not changed significantly [$F_{3,11}$=1.87, P=0.214]. *A. globiformis* 151B effectively increased accumulation of Cr(VI) from the growth medium, where the Cr(VI) concentration was 40 µg/ml and Mg(II) concentration was low (Fig. 3, condition **B**) [T = 24.98 P= 0.0001 DF = 4 as compared to condition (**A**)]. Increase in the concentration of Mg(II) ions up to 50 µg /ml (see Fig.3, condition **C**) significantly decreased the uptake of Cr(VI): [condition (**C**) vs condition (**B**) T= 3.93 P = 0.017 DF = 4], whereas the uptake of Cr(III) was strongly enhanced by the same Mg(II) concentration: condition (**D**) vs condition (**A**) [T = 25.18 P= 0.0001 DF = 4]. It seems, that at this time point divalent Mg decreases Cr(VI) assimilation capability by bacteria, but by the other hand Mg(II) ions increased Cr(III) uptake significantly.

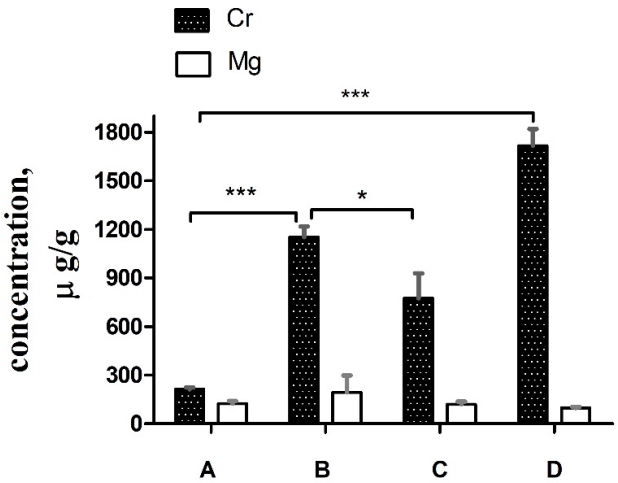

Fig. 3. *Concentration of Cr (dark bars) and Mg(II) (white bars) in A. globiformis 151B from different metal containing medium.*

*The mean values ± sem of Cr and Mg(II) concentrations are indicated on y axis. Culture mediums are denoted on x axis. Culture medium contains: (A) 7.1 µg/ml - Cr(III) and 4.1 µg/ml Mg (controls); (B) 40 µg/ml Cr(VI) and 4.1 µg/ml of Mg; (C) 40 µg/ml Cr(VI) and 50 µg/ml of Mg; (D) 7.1 µg/ml Cr(III) and 50 µg/ml of Mg. For statistical analysis one-way ANOVA with factor- culture medium were made. Planned comparisons were carried out with student t-test. All comparisons were two-tailed. For Cr accumulation, factor- culture medium was significant $F_{3,11}$ = 124.09, P=0.0001, For Mg(II) accumulation, factor- culture medium was not significant: $F_{3,11}$ = 1.87, P=0.214. All the significant differences were marked as follows: *p = 0.017 and ***p = 0.0001. Cultivation time 40 hours.*

The effect of time as well as cultivation medium on Cr(III) uptake was significant [$F_{4,29}$ = 71,73, P=0.0001 and $F_{1,29}$ = 85.92, P=0.0001 correspondingly (Fig.4)]. The interaction of factors was also significant [$F_{4,29}$=45.98, P=0.0001]. The uptake of Cr(III) without Mg(II) displayed significant decrease (e.g. 48h). The addition of Mg(II) shifts uptake uniformly to strong increase with the highest point at 144h (Fig.4). We suggested that, Cr(III) concentration increased in cells, when 1600 μg/ml Mg(II) was added in the growth medium (Fig. 4). Cr concentration significantly (p = 0.0005) increased at the cultivation time of 48 hours (increased by 2.3 fold). Significant changes were observed at the 96 hours of cultivation, when Cr concentration increased by 1.3 fold (p = 0.0099).

Based on our present data, we can suggest, that Mg(II) increases Cr(III) assimilation capability for *A. globiformis* 151B and decreases Cr(VI) accumulation. During Cr(VI) and Mg(II) concomitant action expression of certain group of proteins are increased. Differentially expressed protein bands functions are mainly associated with oxido-reductase and metal ions or different functional groups transport activities. According to the data, *A. globiformis* 151B belongs ROS resistant bacterial species and is promising candidate for bioremediation purposes.

Conclusion

We conclude, that Mg(II) and Cr(VI) joint action caused oxidative stress in *A. globiformis* 151B. Bacteria effectively scavenged ROS and other free radicals produced during Cr(VI) reduction process. Mg(II) ions increased the bioaccumulation of Cr(III) ions in time but at the same time they decreased the uptake process of Cr(VI) by bacteria.

Acknowledgement

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Conflict of Interest

The authors declare no conflict of interest.

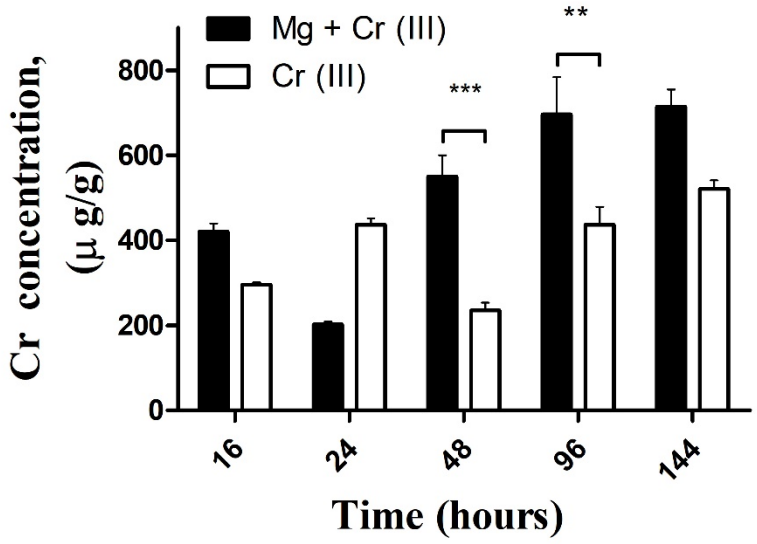

Fig. 4. *The Effect of Mg (1600 μg/ml) on Cr(III) uptake capability by A. globiformis 151B in time. The mean values ± sem of Cr(III) concentrations are indicated on y axis. The time of cultivation is denoted on x axis. Black bars indicate Cr concentration in cells, which were triated by 1600 μg/ml of Mg(II) and 7.1 μg/ml Cr(III); White bars indicate Cr concentration in cells, which were triated by 7.1 μg/ml Cr(III); Two-way ANOVA was applied for the analysis of data of Cr(III) uptake. The factors were: Time and cultivation medium and their effects on Cr(III) uptake were significant. Planned comparisons were carried out with student t-test. All comparisons were two-tailed. The effect of factor- time: $F_{4,29}$ = 71,73, P=0.0001; The effect of factor-cultivation medium: $F_{1,29}$ = 85.92, P=0.0001. The interaction of factors was also significant $F_{4,29}$=45.98, P=0.0001. All the significant differences were marked as follows: **p = 0.0099, ***p = 0.0005.*

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