EPITHERMAL NEUTRON ACTIVATION ANALYSIS FOR BACTERIAL TRANSFORMATIONS OF CHROMIUM

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Abstract: Most powerful primary analytical technique, neutron activation analysis, was applied to study indigenous bacteria, namely, *Arthrobacter* genera which can be successfully used in detoxification and immobilization of toxic substances. In the present study the effect of Cr(VI) on the elemental content of these bacteria has been examined. The concentrations from 12 to 19 elements such as Na, Al, Cl, K, Fe, Co, Zn, As, Br, Rb, Sr, Sb, Ba, Tb, Th, U were determined in the bacterial cells. The high rate of Cr accumulation in the tested bacterial cells was shown. In bacteria treated with chromate some similarity in the behaviour of the following essential elements − potassium, sodium, chlorine − was observed. Such non-essential elements as Ag, As, Br and U were determined in all bacteria and have to be considered by cells as toxins.

Keywords: neutron activation analysis, electron spin resonance spectrometry, *Arthrobacter*

Introduction

Environmental pollution by chromium is widespread in soils, sediments and groundwater [1]. Being a transition element, it occurs in a number of oxidation states from Cr(II) to Cr(VI). However, only two stable, trivalent and hexavalent, species are significant in environmental systems. Hexavalent chromium compounds are highly soluble and toxic [2]. Epidemiological**,** animal and cellular studies have also established that Cr(VI) compounds are carcinogenic [3]. In contrast to Cr(VI), most Cr(III) compounds are less soluble and less toxic. Moreover, trace amounts of Cr(III) appear to be essential for animal and human glucose and lipid metabolism. The biological effect of Cr(VI) is generally attributed to cellular uptake, because Cr(VI), unlike Cr(III), is easily uptaken by cells via SO_4^{2-} and HPO_4^{2-} channels [4]. The toxicity of Cr(VI) compounds is ascribed to reactive intermediates (such as Cr(V), Cr(IV), radicals) generated during their reduction by living cells. Relatively, long-lived Cr(V) intermediates have been detected in the reduction of Cr(VI) both in vitro and in vivo [4,5]. However, to the best of our knowledge, there have been only a few studies that examined the in vivo formation of Cr(V) (in algae and fangs) [5,6] and none on the dynamic detection of Cr(V) and its effect in bacteria. Toxic chromium compounds can be removed by chemical reduction, which is both difficult and expensive.

Indigenous bacteria can be successfully used to either detoxify or immobilize toxic heavy metals. These bacteria are under continuous investigation, and in-depth molecular understanding has been developed for some of them. However, up to date the dependence between the ability of bacteria to reduce or immobilize metals and their elemental compositions is not clear yet [7, 8].

In the present study the effect of Cr(VI) on the elemental content of these bacteria has been examined. Specifically, we tested three Gram-positive bacterial strains of *Arthrobacter* genera – *A. oxydans* (isolated in the USA from polluted Columbia basalt rocks), *Arthrobacter* sp. (61 B), and *A. globiformis* (151 B) (isolated from the most polluted regions in the Republic of Georgia).

Materials and Methods

Sample cultivation and preparation for analysis

The bacteria were grown aerobically in the following nutrient medium: 10 g of glucose, 10 g of peptone, 1 g of yeast extract, 2 g of caseic acid hydrolysate, 5 g of NaCl, and 1 liter of distilled water. To provide the chromium concentration of 35 and 200 mg/L, Cr(VI) [as K_2CrO_4] was added to the nutrient medium at an early stationary phase of growth.

After being cultivated for 4 days the cells were harvested by centrifugation (10,000 rpm, 15 min, 4 °C), rinsed twice in a 20 mM phosphate buffer and analyzed by NAA method.

To prepare bacterial samples for NAA, wet biomass was placed in an adsorption-condensation lyophilizer, dried, and pelletized to 5 mm pieces (~ 0.5 g) by means of titanium press form.

Instrumental Neutron Activation Analysis (INAA) at the reactor IBR-2

ENAA was conducted at the IBR-2 pulsed fast reactor in FLNP, JINR, Dubna, which is characterized by a very high ratio (~100) of epithermal neutrons to thermal ones. The IBR-2 reactor provides activation with the whole fission spectrum: thermal, epithermal, and fast neutrons. Thermal NAA takes advantage of the high intensity of neutrons available from the thermalization of fission neutrons and the large thermal neutron cross sections for most isotopes.

Epithermal is taken to be neutrons with energies from the Cd "cutoff" of 0.55 eV up to ~1 MeV. ENAA is a useful extension of thermal (conventional) NAA in that it enhances the activation of a number of trace elements relative to the major matrix elements. ENAA is particularly advantageous for radionuclides produced from a stable isotope with a high resonance activation integral relative to its thermal neutron activation cross section. In general, the activation cross sections of the matrix elements of environmental samples are inversely proportional to the neutron energy (1/*v* law). The trace elements also follow this general trend, but many of them have large activation cross sections at specific energies in the epithermal energy region. In our case, the following advantages are evident: (i) improved detection limits for As, Br, Rb, Sr, and Sb; (ii) reduction of high matrix activity, e.g., from ^{28}Al, ^{56}Mn, ^{24}Na, ^{46}Sc, ^{60}Co.

Bacterial samples of about 0.5g were heat–sealed in polyethylene foil bags for short–term irradiation and for long-term irradiation were packed in aluminum cups. To determine the short–lived isotopes Cu, I, Br, Mn, Mg, Na, V, K, Cl and Ca, channel 2(Ch2) was used. Samples were irradiated for 3 minutes and measured twice after 3−5 and 20 minutes, respectively for 5 and 15 minutes. In case of long–lived isotopes of Na, K, Sc, Cr, Fe, Co, Ni, Zn, As, Se, Br, Rb, Sr, Ag, Cd, Sb, Cs, La, Ce, Sm, Tb, Yb, Hf, Au, Th and U, channel 1 (Ch1) was used. Samples were irradiated for five days, re-packed and then measured twice after 4−6 and 20 days, respectively. Measure time varied from 1−3 hours. γ-Ray spectra were measured using a large-volume Ge(Li) detector with a resolution of 1.96 keV at the 1332.4-keV line of ^{60}Co with an efficiency of 30% relative to a 3 in.× 3 in. NaI detector for the same line. The data processing and element concentration determination were performed on the basis of certified reference materials and comparators using software developed in FLNP JINR [9].

Three certified reference materials (CRMs), namely, IAEA Lichen-336, Donnye SL-1and Coal, fly ash SRM-1633b were used for quality assurance purpose.

Electron Spin Resonance Spectrometry

The ESR investigations were carried out on a RE 1306 radiospectrometer with 100 kHz modulation at 9.3 GHz [10]. Detection of Cr(V) was carried out at liquid nitrogen temperature (77 K) to avoid a decrease in sensitivity of the ESR spectrometer caused by the water content in bacterial samples. The detection of the broad line for Cr(III) was complicated at low temperatures due to the presence of oxygen impurity in liquid nitrogen, which shifts the zero line. To avoid this problem, we measured Cr(III) at room temperature after drying the samples at 100 °C. The typical settings for the registration of Cr(V) and Cr(III) are described in detail in [10].

Results and discussions

Metals play an integral role in life processes of microorganisms. In the present work, we focused on the determination of metal contents in bacteria cells. Some metals, such as Co, Cr, Fe, Na, K, and Zn, are required nutrients and are essential. Others have no definite biological function (Ag, Al, Cd) and are non-essential [11].

The concentrations from 12 to 19 elements such as Na, Al, Cl, K, Fe, Co, Zn, As, Br, Rb, Sr, Sb, Ba, Tb, Th, and U were determined in the bacterial cells of 3 strains of *Arthrobacter*.

Data on chromium shows the high rate of Cr accumulation in tested bacterial cells (**Fig.1**). The chromium content in the control cells was less than 10 *μg/g*, while the same values in the treated cells were much higher. For example, in *Arthrobacter* sp. it reached to 3 10^3 *μg/g* after exposure to 35 *mg/L* of Cr(VI) for 4 days. Reduction of Cr(VI) to Cr(III) begins at the surface of bacteria with the formation of Cr(V) complexes **(Fig.2)** and the main part of reduced chromium (Cr(III hydroxide) is tightly bound to bacterial cells. Our current ENAA data provide evidence that one part of chromium penetrates inside cells as well.

Bacteria can use chromium (VI) as terminal electron acceptor during oxidation of organic compounds. The capability of Cr (VI) reduction is not uncommon among Cr(VI)-resistant microorganisms. Investigation of oxidation-reduction potential (ORP) of nutrient medium during microbial chromate reduction has shown that Cr(VI) reduction takes place effectively at ORP level +400 – +200 mV. This is a potential range of aerobic processes. Standard

electrode potential (E_0) of reaction: $Cr_2O_4^{2-} + 8H^+ + 3e = Cr^{3+} + 4H_2O$ is 1477 mV. The potential is higher then potential of oxygen reduction process: $O_2 + 4H^+ + 4e = 2H_2O$ (E_0=1228 mV). The reactions take place in bacterial cells during respiration. Therefore, the presents of oxygen is necessary to supply high OPR level in cultural medium. Thus, the reduction of Cr(VI) to Cr (III) has to take place in aerobic condition [12].

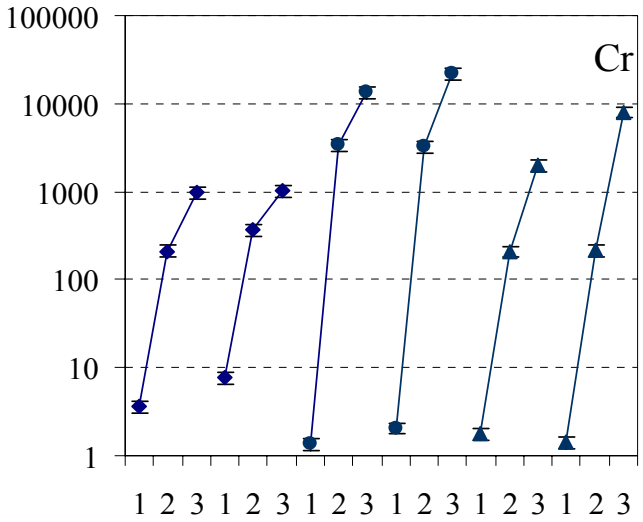

Fig. 1. Concentration of chromium (*μg/g*) in different species of *Arthrobacter*: 1 – control; 2 – 35 *mg/L* Cr(VI); 3 – 200 *mg/L* Cr(VI)

◆ *Arthrobacter oxydans*; ■ *Artrobacter* sp. (61B); ▲ *Arthrobacter globiformis* (151B)

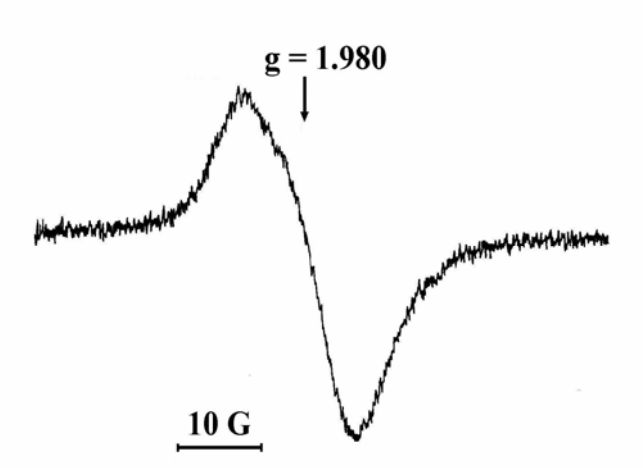

Fig. 2. An ESR spectrum of Cr(V) from tested bacteria after growing in the nutrient medium containing Cr(VI), line width = 12 gauss and g-factor = 1.980

In bacteria treated with chromate some similarity in the behaviour of the following essential elements – potassium, sodium, chlorine – was observed (**Fig. 3)**.

Potassium is known to play an important role in maintaining cellular osmotic pressure; it is involved in non-specific activation of many enzymes, in bacterial energy metabolism (as the coupling ion), and in the regulation of intracellular pH. Potassium is the principal positively charged ion (cation) in the fluid inside of cells, while sodium is the principal cation in the fluid outside of cells. Potassium concentrations are about 30 times higher inside than outside cells, while sodium concentrations are more than ten times lower inside than outside cells.

The concentration differences between potassium and sodium across cell membranes create an electro-chemical gradient known as the membrane potential. A cells membrane potential is maintained by ion pumps in the cell membrane, especially the sodium, potassium−ATPase pumps. These pumps use ATP (energy) to pump sodium out of the cell in exchange for potassium [11].

First, exposure to Cr(VI) caused a lower potassium concentrations in cells and the decrease of K content was almost equal at both low (35 mg/L) and high (200 mg/L) concentrations of Cr(VI). Second, concentrations of sodium and chlorine changed in a parallel way to each other, but in an opposite way to that of potassium. Decrease of K content, in other words extrusion of K from cells to maintain the acidity of their cytosol, concomitant with increase of Na (and correspondingly Cl) content, suggests that, one part of Cr(V)-diols (as well as Cr(VI) ions (via HPO_4^{2-} channels)) were able to penetrate inside bacterial cells.

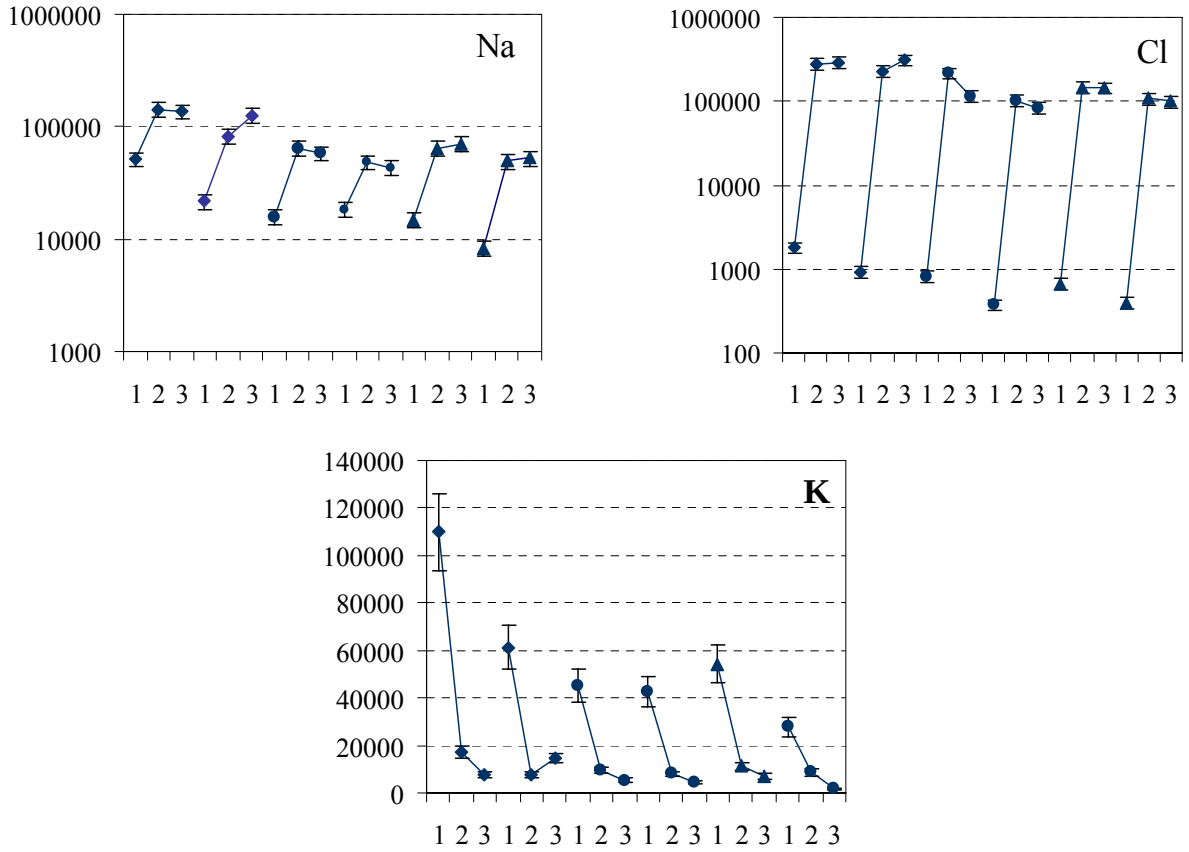

Fig. 3. Concentration of Na, Cl and K (*μg/g*) in different species of *Arthrobacter* under different Cr(VI) loadings

NAA measurement of iron content in bacteria supports this conclusion **(Fig. 4.)**. As is known, iron is the most important metal biologically. It is a many-functional constituent ofcomplex molecules. **Fig. 4** demonstrates that in the tested bacteria the Fe content significantly increased in response to Cr(VI) loading, indicating that the bacterial protective system was activated significantly against chromium toxic impact.

In *A. globiformis*, contrary to *A. oxydans* and *Arthrobacter* sp, the content of iron increased almost linearly with increase of Cr(VI) dose. It seems that Cr(VI) transformation mechanism is rather different in *A. globiformis* than in *Arthrobacter oxydans* and *Arthrobacter* sp.

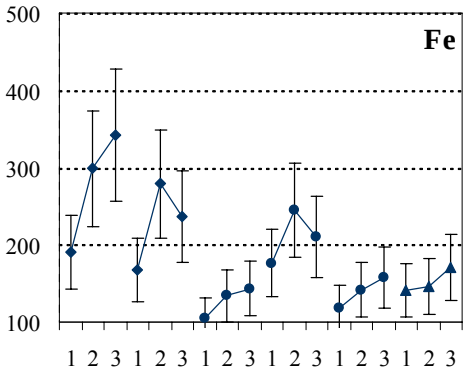

Fig. 4. Concentration of Fe (*μg/g*) in different species of *Arthrobacter* under different Cr(VI) loadings

This result suggests what the chemical composition of basalts influenced the elemental composition of bacteria. Really the Georgian basalt samples from the studied sites are rocks with high content of total iron (Fe_2O_3+FeO), which is due to the abundance of ferromagnesial minerals-pyroxenes [(Ca, Na, Mg, Fe)(Al, Si)O_3], olivine ($Mg_{1.8}Fe_{0.2}SiO_4$), magnetite($Fe^{2+}Fe_2^{3+}O_4$). Ferrous monoxide form is predominant in all samples (FeO = 4.2-8.4%), while ferrous oxide is also present in quantity (Fe_2O_3= 3.0-5.9% of iron) [13].

As, Br, Nd, Rb, Sb, U were also determined in all bacteria (**Fig. 5**). These non-essential elements have no beneficial function and have to be considered by cells as toxins, however their behavior illustrates that the permeability of bacterial cell wall changed after treatment with chromium.

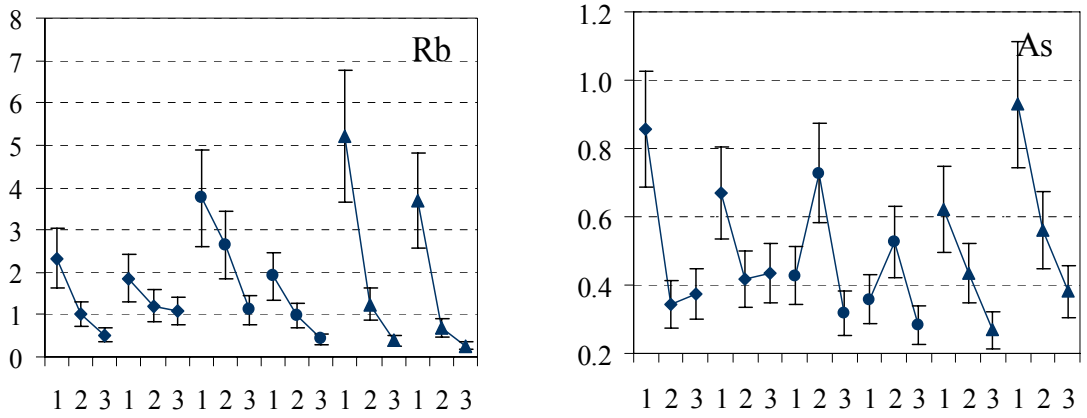

Fig.5. Concentration of As and Rb (*μg/g*) in different species of *Arthrobacter* under different Cr(VI) loadings

Conclusions

- By application of INAA and ESR spectrometry the behavior of chromium in basalt-inhabiting bacteria of *Arthrobacter* genera exposed to high concentrations of Cr(VI) was studied. It was shown that the tested bacteria of *Arthrobacter* genera can efficiently detoxify high concentrations of Cr(VI).
- The concentrations of 12−19 elements were determined in each bacterium simultaneously. The concentration range was over 8 orders of magnitude, from major- to ultra trace elements. Some similarity in the elemental composition of bacteria was observed.
- In all bacteria, potassium and sodium were the dominant elements. The concentrations of both Na and K were in the range of 10^5*μ*g/g. In the tested bacteria the concentrations of the other elements were much less.
- The relatively high contents of Fe detected in bacteria indicate bacterial adaptation to the environmental conditions typical for basalts.
- Elemental analysis of these bacteria also revealed that basalt-inhabiting bacteria are distinguished by relative contents of essential metals such as Na, K, Fe, Zn, Co.

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