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EPITHERMAL NEUTRON ACTIVATION ANALYSIS OF CR(VI)-REDUCER BASALT-INHABITING BACTERIA

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Цибахашвили Н. Я. и др. E14-2005-53
Эпитепловой нейтронный активационный анализ
Cr(VI)-восстанавливающих бактерий, выделенных из базальтов

Эпитепловой нейтронный активационный анализ (ЭНАА) был использован для определения элементного состава Cr(VI)-восстанавливающих бактерий, выделенных из загрязненных базальтов Грузии. Способность этих бактерий восстанавливать Cr(VI) была изучена методом электронного спинового резонанса (ЭСР), и было показано, что она различна для разных бактерий. Обсуждаются экспериментально наблюдаемые корреляции между способностью бактерий аккумулировать Cr(V) и их способностью восстанавливать Cr(V) до Cr(III). Анализ элементного состава бактерий, характерных для базальтовых пород, показывает, что они различаются по содержанию таких элементов, как K, Na, Mg, Fe, Mn, Zn, Co. Высокая степень образования Cr(III) коррелирует с концентрацией Co в бактериях. Результаты ЭНАА показывают некоторое сходство в элементном составе бактерий. Относительно высокое содержание в бактериях Fe (140–340 мкг/г сухого веса) свидетельствует об их адаптации к условиям среды, характерным для базальтов. Для каждого типа бактерий определены концентрации от 12 до 19 различных элементов в интервале 8 порядков от макро- до ультраследовых.

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Tsibakhashvili N.Ya. et al. E14-2005-53
Epithermal Neutron Activation Analysis (ENAA) of
Cr(VI)-reducer Basalt-inhabiting Bacteria

Epithermal neutron activation analysis (ENAA) has been applied to studying elemental composition of Cr(VI)-reducer bacteria isolated from polluted basalts from the Republic of Georgia. Cr(VI)-reducing ability of the bacteria was examined by electron spin resonance (ESR) demonstrating that the bacteria differ in the rates of Cr(VI) reduction. A well-pronounced correlation between the ability of the bacteria to accumulate Cr(V) and their ability to reduce Cr(V) to Cr(III) observed in our experiments is discussed. Elemental analysis of these bacteria also revealed that basalt-inhabiting bacteria are distinguished by relative contents of essential elements such as K, Na, Mg, Fe, Mn, Zn, and Co. A high rate of Cr(III) formation correlates with a high concentration of Co in the bacterium. ENAA detected some similarity in the elemental composition of the bacteria. The relatively high contents of Fe detected in the bacteria (140–340 μg/g of dry weight) indicate bacterial adaptation to the environmental conditions typical of the basalts. The concentrations of at least 12–19 different elements ranging from major- to ultratrace ones were determined in each type of bacteria simultaneously. The range of concentrations spans over 8 orders of magnitude.

The investigation has been performed at the Frank Laboratory of Neutron Physics, JINR, and Andronikashvili Institute of Physics of GAS, Tbilisi.

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INTRODUCTION

Anthropogenic activity is a source of continual influx of heavy-metal contaminants into the environment. A complex variety of abiotic and biotic processes affects their speciation and distribution [1]. Some of these processes can be applied to removing environmental pollutants. Indigenous bacteria can be successfully used to either detoxify or immobilize toxic substances [2]. Chromate-reducing bacteria are under continual investigation, and in-depth molecular understanding has been developed for some of them [3]. Bacterial strains that were tolerant to Cr(VI) showed changes in the elemental composition of cells after exposure to Cr(VI) [4, 5]. In the cells treated with other heavy metals, significant alterations in the cell composition were also observed [6]. Recently great attention has been paid to the elemental analysis of the major sites in cells, where heavy metals are accumulated [4, 7–9]. Despite the intensive studies of the problem, the dependence between the ability of bacteria to reduce or immobilize metals and their elemental compositions still is not clear.

The elemental composition of cells is studied for many bacteria which belong to various taxa [10–14]. The bacteria have been found to differ significantly in their relative contents of different elements. The elemental analysis of bacteria has been performed, in general, by inductively coupled plasma atomic emission spectrophotometry (ICP-AES), atomic absorption spectrometry (AAS), mass spectrometry, X-ray microanalysis (XRMA) with the transmission electron microscope. The epithermal neutron activation analysis (ENAA) has been found to be suitable for multielement determination in biological samples [15]. Recently, using ENAA, the multielement composition of *Arthrobacter oxydans*, which was isolated and cultivated from the Columbia (USA) basalt samples, has been characterized [16]. In the synchrotron radiation-based (SR) Fourier transform infrared (FTIR) spectromicroscopy experiments, *A. oxydans* has been demonstrated to be a Cr(VI)-tolerant bacterium that can reduce Cr(VI) to Cr(III) [17]. *A. oxydans* was used as a model Cr(VI)-tolerant and reducing bacteria in our studies [18–21]. Lately, several endolithic (rock/mineral inhabiting) bacterial strains were isolated from the most polluted regions in the Republic of Georgia [22]. Here, we focus on the establishment of elemental composition of basalt-inhabiting bacteria with Cr(VI)-reducing ability for further elucidation of Cr(VI) effect on this composition. This knowledge may turn out to be significant for understanding of mechanisms of microbial heavy metal resistance.

The aim of the present study is to determine the baseline chemical composition of different Cr(VI)-reducer bacteria isolated from the Georgian basalts using ENAA. ENAA was carried out at the IBR-2 pulsed fast reactor at FLNP, JINR,

which is characterized by a very high ratio of epithermal neutrons to thermal ones.

1. EXPERIMENTAL

Chemicals. All experimental chemicals were ACS-reagent grade and purchased from Sigma (St. Louis, MO, USA).

Sampling. The basalt samples were selected from ecologically most contaminated region of Georgia — Marneuli. In this region, the samples from Kazreti mines of copper-disulfide and copper-zinc (Cu, Fe, Cr, C, Ni, Mo, Zn, Cd, etc.), the barite-polymetallic (Pb, Zn, Cd, Ba, etc.) and gold-ore (Au, Ag, Hg) types were taken along the Mashavera River gorge [22]. All the rocks were basaltoids. From these samples, several endolithic bacteria were isolated by the method described in [22]. In the present work, the following isolates were studied: Nos. 14, 61, 151, 163. Besides, for comparison, we also tested the bacterial strain No. 224 which was isolated from the rock taken in the ecologically most pristine part of the Caucasus mountains (near the mineral water spring in the surroundings of Kazbegi). All of these bacteria were identified as Cr(VI) reducers by the ESR method. From their growth properties and morphology, they belong to the genus *Arthrobacter* (the exact identification of these bacteria by 16 s RNA is underway). *Arthrobacter* species is of interest because of its high potential for the reduction and immobilization of chromium in aerobic environments [21]. *Arthrobacter* species are the member of the high mol % G+C actinomycete-coryneform bacteria [23]. The life cycle of *Arthrobacter* is characterized by its cells change from rod to cocci (almost spherical form), i.e., in the exponential phase of growth the bacterial cells are rods that change in size and shape. In the course of exponential growth, the rods get shorter and are eventually transformed into coccoid forms characteristic of a stationary phase structure [18].

Sample preparation. The bacteria were grown in the following nutrient medium: 10 g of glucose, 10 g of peptone, 1 g of yeast extract, 2 g of caseic acid hydrolysate, 6 g of NaCl and 1 l of distilled water. Bacterial cells were grown in 250 ml Erlenmayer flasks as a suspension. The medium was inoculated with 0.1 ml of overnight broth and incubated at 21°C being shook continuously.

After being cultivated for 5 days the cells were harvested by centrifugation (10 000 rpm, 15 min, 4°C), rinsed twice in a 20-mM phosphate buffer. This wet biomass was then placed in an adsorption-condensation lyophilizer and dried following the procedure reported elsewhere [24].

The dry native biomass was finally pelletized into 5-mm pills using a special titanium press form. The elemental composition of the bacterial biomass was determined by ENAA.

To determine Cr(V) and Cr(III) content in cells by ESR, bacteria were cultivated in the same nutrient medium. At the early stationary phase of growth, 35 mg/l of Cr(VI) [as K_2CrO_4] was added to the nutrient medium. Bacterial cells were harvested in 1 and 49 h by centrifugation prior to analysis.

Analysis. *ENAA.* The ENAA measurements were carried out as described previously [16]. The bacterial samples of about 0.5 g were packed in aluminum cups for long-term irradiation and were heat-sealed in polyethylene foil bags for short-term irradiation. The neutron flux density characteristics and the temperature in the irradiation channels equipped with a pneumatic system are described in [24].

Long-lived isotopes were determined using cadmium-screened irradiation channel. The samples were irradiated for five days, repacked and then counted twice after decays of 4 and 20 days. The counting time varied from 1.5 to 10 hours.

To determine the short-lived isotopes of Mg, Al, Cl, Ca, V, Mn, and I, the conventional irradiation channel was used. The samples were irradiated for three minutes and measured twice after 3–5- and 20-min decay for 5–8 and 20 min, respectively.

Gamma-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 $3' \times 3'$ 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 a software developed at JINR FLNP [25].

Three certified reference materials (CRMs), namely, IAEA Lichen-336, IAEA Bottom Sediments SDM-2T and Nordic Moss DK-1 were used for quality assurance purposes.

ESR spectrometry. The ESR investigations were carried out on the RE 1306 radiospectrometer with 100 kHz modulation at 9.3 GHz [20]. Detection of Cr(V) was carried out at liquid nitrogen temperature (77K) to avoid a decrease in sensitivity of 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.

2. RESULTS AND DISCUSSIONS

In our earlier work, it has been shown that among 121 endolithic bacteria isolated from the most polluted regions in the Republic of Georgia, 33 bacteria are Cr(VI) reducers [22]. The reduction assay has been performed by ESR, which turned out to be a very convenient method for estimation of chromium-reducing ability of bacteria allowing fast identification of Cr(V) [22].

After mixing the bacterial cells with the chromate solution, the ESR line with a g factor of 1.980 and a width of 12 G appeared in a few minutes. This line is similar to that detected in *A. oxydans* and is characteristic of the Cr(V) complexes with diol-containing molecules [20]. Thus, Cr(VI) reduction begins at the surface of endolithic bacteria. The macromolecules at the cell wall of bacteria could act as an electron donor to Cr(VI) to form stable Cr(V) complexes.

Control experiments also revealed that some of these bacteria give a Cr(V) line more stronger than *A. oxydans*. These cultures were chosen for further investigations and tested for the time-course of both Cr(V) decomposition and Cr(III) formation in them. We observed that Cr(III) ESR line had the following parameters in all tested bacteria: $g = 2.02$ and a line width of 650 G.

The relative intensities of the ESR signals, which are proportional to the concentrations of the Cr(V) complexes accumulated during a certain period of time by isolates, are given in Fig. 1. Formation of Cr(V) and Cr(III) in different isolates are found to be time-dependent. In Fig. 1, isolates are arranged in ascending order of their Cr(V) ESR line intensities after 1 h of Cr(VI) action. As one can see, different isolates accumulated a significantly different amount of Cr(V). Though the accumulation of Cr(V) is a combined effect of the formation Cr(VI)→Cr(V) and decay Cr(V)→Cr(III) processes, one should take into account that the rate of formation of Cr(V) is much higher than that of decay. Consequently, the effect of decomposition, i.e., the decay of Cr(V) complexes and accumulation of Cr(III), manifests itself only after a long period of time (> 1 h). Since Cr(V) complexes are formed at the surface of bacteria, accumulated in large amounts Cr(V) complexes in the course of time may significantly slow down the process Cr(VI)→Cr(V), which is expected to manifest in decreasing of the amount of accumulated Cr(V) compared to the previous (1 h) level.

In fact, Fig. 1 shows that after 49 h the level of accumulated Cr(V) decreases more considerably in the isolates which accumulated more Cr(V) during 1 h than in the isolates which accumulated less Cr(V). On the other hand, according to the mechanism of Cr(V) decay into Cr(III), Cr(V) penetrates through the cell wall and is reduced to Cr(III) inside the bacteria [26]. Therefore, preventing a further penetration of Cr(V) complexes into bacteria, the accumulated Cr(III) may slow down the process Cr(V)→Cr(III) and thus may increase the level of Cr(V) accumulation as well.

Hence, the isolates characterized by a higher level of Cr(III) accumulation are expected to retain a higher level of Cr(V) accumulation as well. Indeed, the well-pronounced correlation of the amount of Cr(III) accumulated by the isolates during 49 hours with the accumulation of Cr(V) gives a sound evidence in favor of the above mechanism.

The obtained results show that at the beginning of chromate action isolate No. 61 has a higher rate of Cr(VI) reduction than other bacteria. Besides, it was observed that isolate No. 163 exhibits different dynamics of Cr(V) decomposition

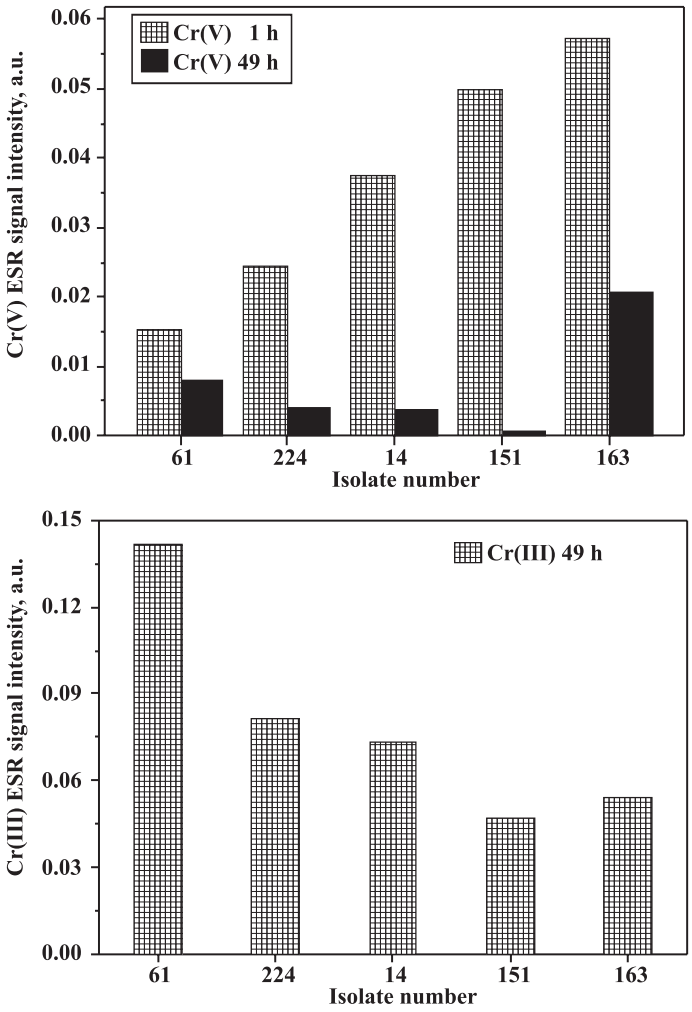

Fig. 1. Formation of Cr(V) and Cr(III) in different isolates

and Cr(III) formation. Thus we can say that the reduction of Cr(VI) proceeds differently in different bacteria. It is well-known that bacterial resistance to metals is heterogeneous in both their genetic and biochemical bases [27]. At the biochemical level, at least six different mechanisms are responsible for resistance. A detailed investigation of mechanisms of Cr(VI) reduction by bacteria from Georgian basalts is underway.

In the following set of experiments, we investigated the elemental composition of selected bacteria to gain insight into the dependence between the Cr(VI)-reducing ability of bacteria and their elemental composition. Several aspects of cellular metabolic activities of bacteria can be evaluated knowing the elemental composition of cells. For example, C/N/P ratios provide information on environmental nutrient conditions. Osmotic and energy conversion can be studied by analysis of such elements as sodium, potassium, magnesium, and calcium [7, 12]. Recently, using XRMA analysis, the haloalkaliphilic acetogenic bacteria and the alkaliphilic sulfate-reducing bacteria have been found to differ significantly in their relative contents of S, K, P, and Cl [14]. Elemental analysis of the cells also revealed that the distinctive feature of viable resting forms of bacteria was their low P/S ratio and high Ca/K ratio [7].

In the present work, we focuse on the determination of metal contents in bacteria, although the concentrations of some other elements were also established. Metals play an integral role in life processes of microorganisms. Some metals, such as Ca, Co, Cr, Cu, Fe, Na, Mg, Mn, K, Ni, and Zn, are required nutrients and are essential. Others have no definite biological function (Ag, Al, Cd, Au, Hg) and are nonessential [28].

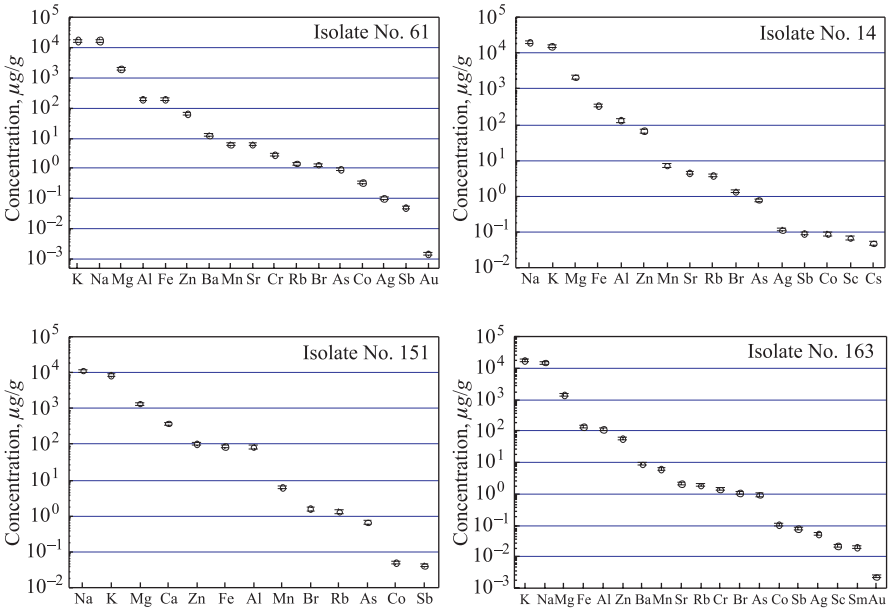

Fig. 2. Elemental distribution in lyophilized samples of different isolates

In Fig. 2, results of ENAA measurements of elemental content of bacteria isolated from the Georgian basalts are presented. The concentrations from 12

to 19 elements were determined in the bacterial cells. The maximal number of elements was detected in isolate No. 163; and the minimal one, in isolate No. 151. The concentration range was over 8 orders of magnitude, from major- to ultratrace elements.

The ENAA results revealed some similarity in the elemental composition of the isolates studied (Fig. 2). In all bacteria, potassium and sodium were the dominant elements (together more than 90% of the total content of the elements determined). The concentrations of both Na and K were in the range of 10^4 μg/g. The content of Mg was about an order of magnitude lower than those of Na and K and about an order higher than those of Fe and Al. In general, the concentration of Zn was a little less than these letters. The concentrations of other elements were much less. The trace and ultratrace elements (usually being < 1 μg/g) were distinguished in different types of bacteria.

The high content of Na and K in the bacterial cells can be explained by the fact that bacteria were freeze-dried in a Na–K phosphate buffer at pH 7, which could increase concentrations of Na and K. As known, the membranes in lyophilized bacteria lose their barrier function [29]. Therefore, during lyophilization Na and K ions must diffuse along their concentration gradients and as a result the lyophilization in media with a high concentration of any of these elements must lead to high content of this element in the cell. This result is in agreement with the data obtained recently by Mulyukin et al. [11]. It was observed that, indeed, the lyophilized *M. luteus* contained larger amounts of K than the vegetative cells and viable resting cells.

Potassium is an essential metal for living organisms and is required for regulation of intracellular osmotic pressure. K is also involved in nonspecific activation of many enzymes, in bacterial energy metabolism (as a coupling ion), and in the regulation of intracellular pH [30].

Iron is the most important metal biologically. It is a constituent of complex molecules with a wide array of functions [30]. In tested bacteria the concentration of Fe was relatively higher (in the range of 140–340 μg/g) than that in other bacteria [4, 31, 32]. For comparison, the concentration of Fe in *Pseudomonas* strain was less than 30 μg/g [4]. In autotrophic and heterotrophic bacteria, it constituted 40–50 μg/g [31]. This result suggests that the chemical composition of basalts influenced the element 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 [22]. 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).

It seems that in the tested bacteria, active transport systems exist for iron, and as a result in all the isolates high concentrations of Fe were found. As is

known, iron easily enters bacterial cells mainly by way of magnesium transport system [30]. The high content of Al (which is also present in large quantities in basalts) in isolates also confirms this consideration.

However, in basalt-inhabiting bacteria the content of magnesium, which is known to stabilize cell wall in addition to being involved in the catalysis of various reactions in the cell, was not high. This indicates that the high content of magnesium in basalts does not influence the Mg content in bacteria. In tested bacteria the concentration of magnesium was in the range detected for other bacteria (it ranged from $1.4 \cdot 10^3$ μg/g in isolate No. 151 to $2.17 \cdot 10^3$ μg/g in isolate No. 14). In most bacteria the content of magnesium ranges from 6 to 30 mM [14]. Only in halophilic microorganisms it can reach 500 mM [32]. One can presume that the obtained result indicates that the demand for magnesium is low in basalt-inhabiting bacteria. There are other types of bacteria in which demand for magnesium was insignificant [14, 33, 34]. For example, the growth of alkaliphilic bacterium *N. acetigena* did depend on the content of magnesium salts in the medium [33]. A fivefold increase of magnesium concentration in the medium stimulated *N. acetigena* growth and delayed cell lysis, although calculations showed that the amount of soluble Mg changed insignificantly when the content of magnesium salts in the medium increased five- or tenfold. Similar data were obtained for archae *Halobacterium sp.*, the growth of which was observed in the magnesium-deficient medium (less than 50 μM), although the optimal magnesium concentration ranged from 0.1 to 2 mM at pH 9.5 [34].

Thus we propose that the relatively high concentrations of Fe and Al in basalt-inhabiting bacteria may result from bacterial adaptation to environmental conditions. The similar effects are well-known in other bacteria [7, 14]. The study of cell physiology of the alkaliphilic bacteria isolated from soda lakes revealed the demands for definite elements (Mn, Co, Ni). This demand results from the bacterial adaptation to the environmental conditions typical of soda lakes [14]. The physiological adaptation of marine bacteria to high internal concentrations of chloride was also detected earlier [7]. XRMA analysis showed that growing marine bacterioplankton have an internal environment in which chloride is the dominating cation.

The concentrations of other essential metals such as Mn and Zn were also within the range detected for other bacteria [10, 14]. Zn stabilizes various enzymes and DNA through electrostatic forces. It is also a part of complex molecules with a wide array of functions. Mn is an important trace element with low toxicity [30].

The elemental analysis of basalt-inhabiting bacteria revealed a series of other metals necessary for bacterial activity. Metals displaying changes in valence, especially Ni, Cu, Cr, and Co, participate in electron transport and redox reaction in bacteria [30]. Cu was not detected in the tested bacteria. The trace amount of Ni (0.1 μg/g) was observed only in *A. oxydans* [16]. At the same time the basalt-inhabiting bacteria were found to have an obligated requirement for Co.

Co was detected in all the isolates. Intracellular Co content in the tested bacteria was significantly different. In two isolates, Nos. 61 and 163, we also detected the trace amounts of Cr (about 2 μg/g).

As, Sb, Br, Rb were determined in all bacteria. The concentrations of Br and Rb were about of 1 μg/g. The concentrations of As and Sb were much less. In some isolates, we also detected Ba and Sr and the ultratrace amounts of Au, Ag, Cs, Sm. All of these elements have no beneficial function but have to be considered by cells as toxins.

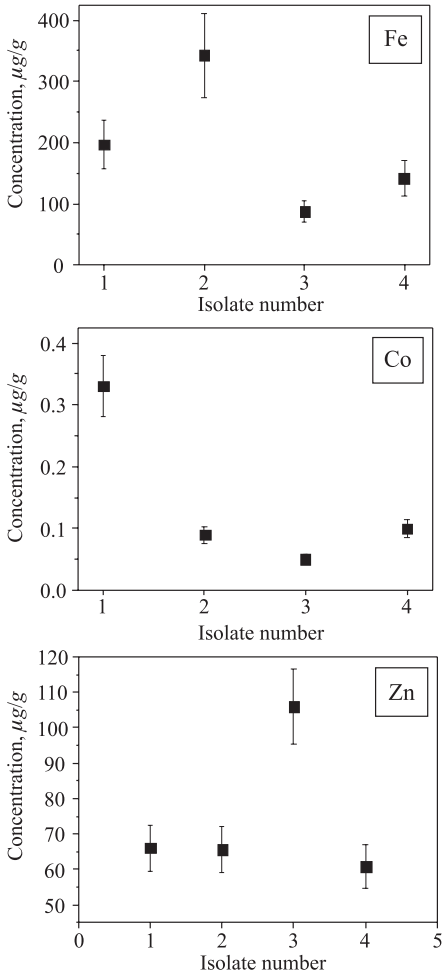

Fig. 3. Concentrations of Fe, Co and Zn in different isolates: 1 corresponds to isolate No. 61; 2, to isolate No. 14; 3, to isolate No. 15; and 4, to isolate No. 163

We did not reveal any significant difference between levels of Na, K, Mg, Fe, Zn, Mn concentrations in *A. oxydans* and in isolates from the Georgian basalts. However, in *A. oxydans* a large amount of Ca (210 μg/g) was detected [16]. The same amount of this element was observed only in isolate No. 151.

Comparison of elemental composition of different isolates revealed that, in isolate No. 61, which has the maximal rate of Cr(III) formation, the concentration of Co was much larger (0.33 μg/g) than that in other bacteria. A similar amount of Co was detected in *A. oxydans* as well [16]. It should be noted that the concentrations of Co were similar in all bacteria (Fig. 3). The concentrations of both Fe and Mn were the highest in isolate No. 14 and again in other bacteria the contents of these elements were close. In isolate No. 151, the maximal amount of Zn was observed (106 μg/g). At the same time the concentrations of Na, K, Mg, Fe, Mn, Co were the least detected. We did not detect any significant feature in the elemental composition of isolate No. 163. For more illustration, the distribution of some elements, such as Fe, Co, Zn, in different isolates is presented in Fig. 3.

The content of elements is changed in bacteria after exposure to Cr(VI). These alterations give insight into the mechanism of bacterial resistance.

CONCLUSIONS

The elemental composition of four bacterial strains isolated from polluted basalts from the Republic of Georgia has been studied by using ENAA.

The concentrations of 12–19 elements were determined in each bacterium simultaneously. The concentration range was over 8 orders of magnitude, from major- to ultratrace 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 10^4 μg/g.

The content of Mg was about an order low than those of Na and K and about an order high than those of Fe and Al. In the tested bacteria the concentrations of the other elements were much less.

The relatively high contents of Fe detected in bacteria (140–340 μg/g of dry weight) indicate bacterial adaptation to the environmental conditions typical of basalts.

Elemental analysis of these bacteria also revealed that basalt-inhabiting bacteria are distinguished by relative contents of essential metals such as Na, K, Mg, Fe, Mn, Zn, Co.

In bacterium having the maximal rate of Cr(III) formation, the concentration of Co was found to be larger. Cr(VI)-reducing ability of bacteria was tested

by ESR method, showing that different isolates have different ability to reduce Cr(VI).

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