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HEAVY METAL ACCUMULATION AND THE EXPRESSION OF THE *copA* AND *nikA* GENES IN *Bacillus subtilis* AG4 ISOLATED FROM THE SOTK GOLD MINE IN ARMENIA

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The growth response, heavy metal bioaccumulation ability and the expression of the *copA*, *nikA* and *czcD* genes in the presence of different concentrations of a combination of Cu(II), Ni(II), Zn(II) and Cd(II) metals of *Bacillus subtilis* AG4 isolated from the Sotk Gold Mine (Armenia) have been studied. Minimal inhibitory concentrations of Cu(II), Ni(II), Zn(II) and Cd(II) metals were 3.5 mM, 4.5 mM, 1 mM and 0.5 mM respectively. The concentrations of metal ions in the medium up to 16 μM Cu(II), 17 μM Ni(II), 10 μM Cd(II) and 15 μM Zn(II) were found not to affect bacterial growth. It was shown that *B. subtilis* AG4 accumulated Cu(II) and Zn(II) to 6.8 and 3.0 mg/g wet weight of biomass, respectively. The *nikA* and *copA* genes (but not *czcD*) were identified by PCR amplification using specific primer sets. The highest expression of the *nikA* and *copA* genes was observed at 16 μM Cu(II), 17 μM Ni(II), 10 μM Cd(II) and 15 μM Zn(II) by using RT-qPCR. The expression of the *nikA* and *copA* was inhibited at higher metal ion concentrations.

Heavy metal resistance – metal accumulation – RT-qPCR – nikA – copA – czcD

Ուսումնասիրվել է Սոթքի ոսկու հանքից (Հայաստան) մեկուսացված *Bacillus subtilis* AG4 շտամի աճը, մետաղները կենսակուտակելու ունակությունը, ինչպես նաև *copA, nikA* և *czcD* գեների էքսպրեսիան միջավայրում Cu(II), Ni(II), Zn(II) և Cd(II) տարբեր կոնցենտրացիաների համակցությունների պայմաններում: Cu(II), Ni(II), Zn(II) և Cd(II) մետաղների աճն արգելակող նվազագույն կոնցենտրացիաները կազմել են համապատասխանաբար 3,5 մՄ, 4,5 մՄ, 1,0 մՄ և 0,5 մՄ: Սննդամիջավայրում 16 մկՄ Cu(II), 17 մկՄ Ni(II), 10 մկՄ Cd(II) և 15 մկՄ Zn(II) կոնցենտրացիաների համակցությունը բակտերիայի աճի վրա էական ազդեցություն չի թողնում: Cu(II) և Zn(II) կենսակուտակումը կազմել է *B. subtilis* AG4-ի բջիջների խոնավ քաշում 6.8 մգ/գ և 3.0 մգ/գ համապատասխանաբար: Հատուկ փրայմերներով ՊՇՌ ամպլիֆիկացմամբ ցույց է տրվել *nikA* և *copA* (բայց ոչ czcD) գեների քրոմոսոմային տեղաբաշխվածությունը: RT-qPCR մեթոդով հաստատվել է *nikA* և *copA* գեների առավելագույն էքսպրեսիան սննդամիջավայրում 16 մկՄ Cu(II), 17 մկՄ Ni(II), 10 մկՄ Cd(II) և 15 մկՄ Zn(II) կոնցենտրացիաների պայմաններում: Մետաղների իոնների առավել բարձր կոնցենտրացիաների դեպքում դիտվել է նշված գեների էքսպրեսիայի արգելակում:

Մետաղակայունություն – մետաղների կենսակուտակում – RT-qPCR – nikA – copA –czcD

Изучены рост, биоаккумулирующая способность, а также экспрессия генов *copA*, *nikA* и *czcD* штамма *Bacillus subtilis* AG4, изолированного из Сотского месторождения золота (Армения), при различных концентрациях комплекса металлов Cu(II), Ni(II), Zn(II) и Cd(II) в питательной среде. Минимальная ингибирующая концентрация металлов Cu(II), Ni(II), Zn(II) и Cd(II) составляла 3.5 мМ, 4.5 мМ, 1.0 мМ и 0.5 мМ, соответственно. Концент-рации металлов в среде до 16 мкМ Cu (II), 17 мкМ Ni (II), 10 мкМ Cd (II) и 15 мкМ Zn (II) не оказывали существенного влияния на бактериальный рост. Установлена биоаккумулирующая способность штама *B. subtilis* AG4, которая составляла для Cu(II) 6.8 мг/г сырого веса биомассы, а для Zn(II) – 3.0 мг/г.

Расположение генов *nikA* и *copA*, но не гена *czcD* на хромосоме показано с помощью ПЦР-амплификации с использованием специфичных праймеров. С помощью ОТ-ПЦР в реальном времени показана высокая экспрессия генов *nikA* и *copA* при 16 мкМ Cu(II), 17 мкМ Ni(II), 10 мкМ Cd(II) и 15 мкМ Zn(II) концентрациях. Экспрессия генов *nikA* и *copA* ингибировалась при более высоких концентрациях ионов металлов.

Устойчивость к тяжелым металлам – ОТ-ПЦР в реальном времени – nikA – copA – czcD

Heavy metals such as Cu, Ni, Zn and Cd are required by bacteria in trace amounts as enzyme cofactors and as structural components of proteins [13]. In some environments, such as mines and ores, the metal ions are exceeding the usual lethal limit for organisms. However, some highly adapted bacteria have evolved metal resistance systems to enable them to grow at otherwise lethal concentrations of heavy metals [13, 14]. Metal resistance genes are located on plasmids, chromosomes or transposons, but there are differences between chromosomal and plasmid metal resistance systems. Some heavy metal resistance systems are usually chromosomal-based and more complex than plasmid systems, which are usually toxic-ion efflux pumps [1].

The main processes for regulating the intracellular concentrations of inorganic cations and anions are membrane transport systems [1, 14]. Under certain condition, metal ions can be accumulated by uptake systems with high substrate specificity [6, 13, 15]. Thus, Ni is taken up by prokaryotic cells by high-affinity ABC-type of ion transporting multi component system consisting of five protein subunits; NikA-E [5, 10].

Excessive intracellular concentration of metal ions initiates metal resistance by enzymatic detoxification, intracellular sequestration of the metals by protein binding or/and reducing accumulation based on active efflux of toxic ions from the cell [1, 6, 11]. Metal ion efflux could be regulated by specific extrusion pumps of the P-type ATPase family and CDF (cation diffusion facilitator) family of transporters [11, 19]. The Cu(II) homeostasis in *Bacillus subtilis* is regulated by the *copZA* operon, encoding a Cu(II) chaperon, and a Cu(II) efflux P-type ATPase [9, 17, 20], while a CzcD protein of the CDF family protects the bacilli against elevated levels of Zn(II), Cd(II), Co(II) and Ni(II) [8, 11].

Metal resistance mechanisms have been found in different microbial groups [18, 19]. The members of the genus *Bacillus* show high tolerance to heavy metals and possess high metal sorption capacity [23].

The growth response, heavy metal ion accumulation ability and the expression of the *copA, nikA* and *czcD* genes in the presence of different concentrations of a combination of Cu(II), Ni(II), Zn(II) and Cd(II) metals in the medium for *Bacillus subtilis* AG4 isolated from the Sotk Gold Mine (Armenia) are reported in the present work.

Materials and methods.

Bacteria and determination of minimal inhibitory concentration (MIC): The thermophilic bacterium *Bacillus subtilis* strain AG4, was recently isolated from the Sotk Gold Mine (Armenia) [7]. The strain was maintained and grown on medium with the following composition: yeast extract, 5 g/l; peptone, 5 g/l; NaCl, 5 g/l; agar, 15 g/l. Stock solutions of $CuSO_4$, $NiCI_2$, $ZnSO_4$ and $CdCl_2$ were prepared at 1.0 M concentrations sterilized by filtration through 0.22 μm pore-size filters. The metal ions were added to sterilized medium to final concentrations varying from 10 to 5000 μM to determine the MIC. Plates were incubated at 55ºC for 48 hours.

Effects of a combination of heavy metals on bacterial growth: The effects of heavy metals have been determined by adding different combinations (from 10 to 5000 μM for each metal) of Cu(II), Ni(II), Zn(II) and Cd(II). Cultivation was performed in 250 ml Erlenmeyer flasks at 55ºC, by shaking (150 rpm). The growth was estimated by determination of the optical density (Spectrophotometer Genesis 10S, Thermo Scientific, at λ=560 nm) of the culture liquid during incubation. *Metal ions accumulation:* To determine the accumulation of have metals ions the isolate was cultivated in medium containing metals at following concentrations: 32 μM of Cu(II), 34 μM of Ni(II), 20 μM of Cd(II) and 30 μM of Zn(II). Bacterial cells were harvested by centrifugation at

6000 rpm for 15 min at 4ºC. The pellet was washed twice with 0.9 % NaCl solution, and then centrifuged again. The cells were disrupted by enzymatic digestion in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mg/ml lysozyme) at room temperature for 3 h. Aliquots of the lysates were conserved with double distilled 1N nitric acid. The concentrations of heavy metal ions were measured by an Elan 9000 ICP mass-spectrometer with indium internal standard and argon plasma [4].

PCR amplification of copA, nikA and czcD genes and sequencing: Genomic DNA was isolated using the CTAB protocol [20] and used as a template for amplification of *copA, nikA* and *czcD.* Primers were designed based on appropriate gene sequences (*nikA* - NC002570, NC014019, NC014103, NC021171; *copA* - NC0144791, NC000964, NC009725, NC006322, NC014019; *czcD* - NC000964, NC014551, NC006582) retrieved from NCBI GeneBank using Primer3 (v.0.4.0) and NCBI Primer-BLAST web tools (http://frodo.wi.mit.edu/; http://www.ncbi.nlm.nih. gov/tools/primer-blast/). Primer sequences are given in table 1. Amplification mixtures were prepared with a final volume of 50 µl and contained 100 ng genomic DNA, 5µl 10xTaq Buffer, 0.8mM dNTP mix, 0.5 µM of each primer, and 0.5U DyNAzyme II DNA polymerase (Thermo Scientific). Amplification was performed under conditions of an initial denaturation at 94ºC for 3 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 54ºC for 30 s, extension at 72ºC for 1 min, with final extension at 72ºC for 10 min. The reactions were subsequently cooled to 4ºC. The PCR product was analyzed by 1.0% agarose gel electrophoresis.

Table 1. Primers used in real-time qRT-PCR

Gene name	Forward Primer (5' → 3')	Reverse Primer (3' → 5')	Product size (bp)
copA	GAGTTGCGAACACTGTGTAG	TAACCTCATATCCTGCATCC	258
nikA	GGTTGGGTTTCATTACAAAA	CTGTACCTACCGGTTAACG	192
czcD	GCTATGGCATCTTTAGCTGT	TGATGCACTCCCTTTATTTC	230

Total RNA extraction and cDNA synthesis: Growth of bacterial cultures was performed in 250 ml flasks containing 50 ml of enrichment medium supplemented with heavy metals at 16 µM Cu(II), 17 µM Ni(II), 10 µM Cd(II) and 15 µM Zn(II) concentrations and two or four times higher concentrations of each metal ion. The flasks were incubated 55ºC with shaking at 150 rpm. RNA was extracted using the RNasy plus mini kit (Qiagen) as described by the manufacturer with some modifications. Genomic DNA was eliminated by RNase-free DNase I treatment during the isolation procedure. Extracted RNA was analyzed by 1.5% agarose gel electrophoresis and stored at -20ºC. SuperScript VIOL cDNA synthesis kit (Invitrogen) was used for cDNA synthesis. After gently mixing of the components, the reaction vial was incubated at 25ºC for 10 min. The cDNA synthesis was performed at 42ºC for 60 min followed by 85ºC for 5 min for inactivation of reverse-transcriptase. cDNA was used in qPCR and stored at -20ºC.

*qPCR:*EXPRESS SYBR GreenER Two-Step qPCR SuperMixs Kit (Invitrogen) was used for qPCR analysis. qPCR reaction in 20 µl of total volume was as follows: 10 µl EXPRESS SYBR GreenER qPCR SuperMix with Premixed ROX, 0.4 µl of each primers (10µM), 1 µl template cDNA, 8.2 µl DEPC-treated water. The thermal cycling condition (using a Bio-Rad iCycler) was: 50ºC for 2 min, 95ºC for 2 min, 40 cycle of 95ºC for 15 sec, 51ºC for 1 min, 68ºC for 15 sec, and 83oC for 1 sec (for signal detection), followed by melting curve analysis: 60°C to 95°C with reads every 0.5°C. Data analysis was carried out using the software provided by Bio-Rad Inc.

The gene expression level was determined by absolute quantification method using a standard curve [21]. Standard curves were constructed based on C(t) values of qPCR amplification using tenfold diluted genomic DNA. Relative expression ratios were calculated by normalization against unit mass [16], using the C(t) values from qRT-PCR, as follows:

$$\text{Ration}=E^{C(t)\text{cont.}-C(t)\text{test}},$$

where E is the efficiency of the reaction that can be determined using the equation $E=10^{-1/\text{slop}}$.

Results and Discussion.

Tolerance and effects of metals ions on cell growth

MIC of various metal ions is presented in tabl. 2. The order of the toxicity of the metals to strain AG4 was found to be Ni<Cu<Zn<Cd. Ni and Cu were less toxic than the other metals. MICs of Cu(II), Ni(II), Zn(II) and Cd(II) for *B. subtilis* AG4 were 3.5 mM, 4.5 mM, 1 mM and 0.5 mM, respectively.

Table 2. Heavy metal tolerance of *Bacillus subtilis* AG4

Metals	MICs (mM)
Ni	4.5
Cu	3.5
Zn	1.0
Cd	0.5

It was shown there is no noticeable difference in bacterial growth rates in case of 16 µM Cu(II), 17 µM Ni(II), 10 µM Cd(II) and 15 µM Zn(II) and without metal ions in the growth medium (fig.1). Thus, the concentrations of metal ions in the medium up to 16 µM Cu(II), 17 µM Ni(II), 10 µM Cd(II) and 15 µM Zn(II) were found not to affect bacterial growth. The presence of all four metal ions in the growth medium at 4 times higher concentration are strongly suppressing of bacteria growth. The stationary phase at mentioned metal concentrations was entered after 4 hours of growth. The intermediate concentrations of heavy metals caused 2 hours lag phase before resuming normal growth rate.

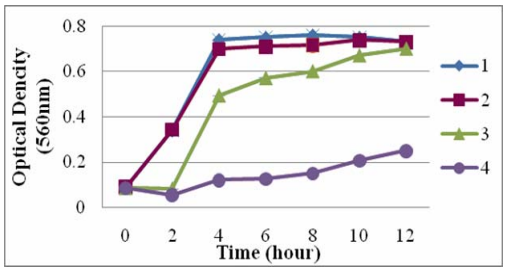


Figure 1. Effects of metals ions complex on *B.subtilis* AG4 growth: 1) metal ions; 2) 16 µM Cu(II), 17 µM Ni(II), 10 µM Cd(II) and 15 µM Zn(II); 3) 32 µM Cu(II), 34 µM Ni(II), 20 µM Cd(II) and 30 µM Zn(II); 4) 64 µM Cu(II), 68 µM Ni(II), 40 µM Cd(II) and 60 µM Zn(II).

The ability of the strain AG4 to grow in the presence of Cu, Ni, Cd and Zn ions in liquid media might be important for the capacity of this bacterium to survive in polluted environments with elevated levels of heavy metals ions. In comparison to previous studies on the metal tolerance of soil bacteria *B. subtilis* AG4 exhibited more resistance to Ni(II) and Cu(II) [18, 23].

Metal bio-accumulation

To determine the metal bio-accumulation ability of *B. subtilis* AG4, a culture was grown in a medium containing 32 µM Cu(II), 34 µM Ni(II), 20 µM Cd(II) and 30 µM Zn(II) up to 0.6 OD. The results are shown in fig.2.

The strain was able to accumulate Cu and Zn ions up to 6.8 and 3.0 mg/g w.w., respectively. A significant amount of Cu ions (~70 %) was removed from the liquid medium. *B. subtilis* AG4 is capable to removing significant amount of Cu from the liquid medium in comparing to previous studies [15, 23].

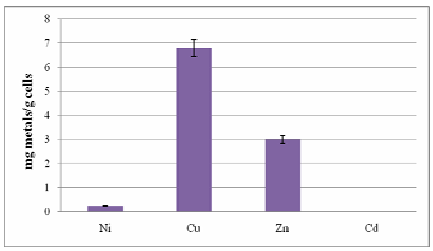


Figure 2. Accumulation of heavy metals ions by strain *B. subtilis* AG4.
The metal ions concentrations within the cells are given in mg per g ww.

Amplification of nikA, copA and czcD genes; genes expression in the presence of Cu(II), Ni(II), Zn(II) and Cd(II)

Evidence for the presence of the *nikA* and *copA* genes in the genome of *B. subtilis* AG4 was revealed by sequencing of obtained PCR products (~200 bp) PCR (tabl. 2). The *czcD* gene was not amplified (fig. 3). The absence of the *czcD* gene in the genomic DNA suggests that it may be plasmid located, or that an alternative resistance mechanism for Zn(II) and Cd(II) might be present.

To assess the gene expression response to the heavy metal stress conditions AG4 were grown in the presence of the different concentrations of metal ions, and the expression of *nikA* and *copA* were analyzed by RT-qPCR.

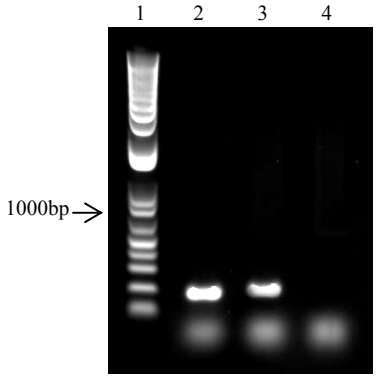


Figure 3. Agarose gel electrophoresis of PCR products. Lanes: 1- 1kb pus DNA size (Invitrogen), 2- *nikA* product, 3- *copA* product, 4- *czcD* product.

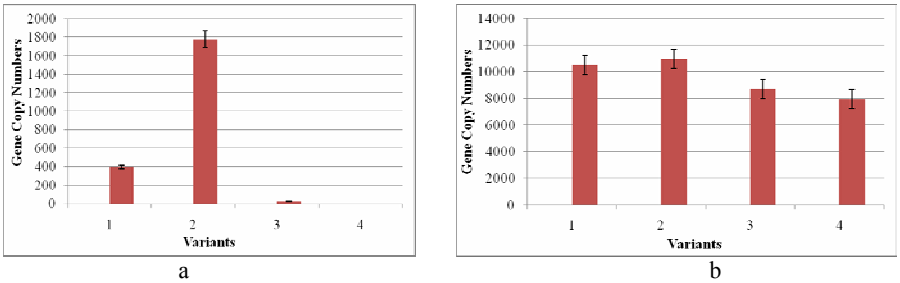
Table 3. Closest match of the *nikA* and *copA* gene amplicons based on BLAST searchers.

Genes	Sequence length (bp)	Closest match (Accession number)	Identity %
nikA	155	Gene encoding nickel import ABC transporter, nickel-binding protein NikA (CP001983)	97
copA	210	Gene encoding putative ABC efflux transporter (ATP-binding protein) (CP002468)	95

Highest expression *copA* and *nikA* genes was observed in the presence of a combination of Cu(II), Ni(II), Cd(II) and Zn(II) in 16, 17, 10 and 15 μM, correspondingly.

The *nikA* gene expression was 3 fold higher at the above mentioned concentrations of the heavy metals than at the absence of the metals in the growth medium, but hardly detectable and absent at higher metal concentrations (conditions 3

and 4 respectively) (fig. 4a). The *copA* gene expression is reduced, 3 and 7 fold less in the conditions 3 and 4, respectively in the comparing with the control (fig. 4b). Although the high metal concentrations reduced the expression of the *copA* gene it is constitutively expressed in the absence or presence of different concentrations of metal ions.



a b

Figure 4. Relative expression levels for the *nikA (*a) and *copA* (b) genes in the different variants: 1) – metal ions; 2) 16 µM Cu(II), 17 µM Ni(II), 10 µM Cd(II) and 15 µM Zn(II); 3) 32 µM Cu(II), 34 µM Ni(II), 20 µM Cd(II) and 30 µM Zn(II); 4) 64 µM Cu(II), 68 µM Ni(II), 40 µM Cd(II) and 60 µM Zn(II).

The stimulation of the metal ions uptake by low concentrations of heavy metal ions could be explained by well-known Arndt-Shultz effect consisting in the intensification of bacterial metabolism [2]. The high levels of the accumulated metal ions lead to activation of the resistance systems [13]. In case of Ni transport in *B. subtilis* AG4, the very low accumulation of the Ni(II) at high concentrations of metals was absorbed (fig. 2). It could be assumed that the uptake of Ni into the cell is prevented by the reduced synthesis of the NikA membrane Ni binding protein (fig. 4(a)) [3, 10].

The reduced expression level of Cu ions efflux P-type ATPase encoded by *copA* gene leads to accumulation of Cu(II) within the cell (fig. 2). Moreover, the gene expression is not stopped and, in case of certain concentrations of the intracellular Cu(II), the Cu(II) binding CopA protein is still synthesized to control the Cu ions homeostasis in the cell.

The effects of different concentrations of a combination of the heavy metals ions Cu(II), Ni(II), Zn(II) and Cd(II) on the growth, metal bio-accumulation and the expression of the *copA* and *nikA* genes of *Bacillus subtilis* AG4 isolated from the Sotk Gold Mine (Armenia) were studied. The ability of *B. subtilis* AG4 to survive in the presence of different concentration of combined metal ions and to accumulate significant amounts of Cu(II) from the growth medium makes it interesting as a potential biotechnological tool for a bioremediation processes of polluted environments. The results indicate a chromosomal localization of the *nikA* and *copA* genes and important roles in the Ni(II) and Cu(II) homeostasis in the cell.

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