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GLUTATHIONE LEVEL OF *Desulfovibrio desulfuricans* IMV K-6 UNDER THE INFLUENCE OF HEAVY METAL SALTS

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Glutathione is the metal stress protector and changes of its level in the sulfate-reducing bacteria cells under the influence of heavy metal salts have not been studied yet. $CdCl_2$, $Pb(NO_3)_2$, $CuCl_2$, and $ZnCl_2$ influence on the total glutathione level in cell-free extracts of sulfate-reducing bacteria Desulfovibrio desulfuricans IMV K-6 was studied. The research has been carried out using Ellman, Lowry methods, statistical processing of the results. It was shown that the glutathione level depends on the heavy metal salts concentration in the medium. The total glutathione level was the highest under the influence of $Pb(NO_3)_2$. Other salts were also toxic to bacteria because glutathione level increased in bacterial cells after addition of these salts to the medium. On the basis of the results of our work the range of heavy metal salts influence on D. desulfuricans IMV K-6 cells glutathione level has been formed for the first time: $Pb(NO_3)_2 > CuCl_2 > CdCl_2 > ZnCl_2$.

K e y w o r d s: sulfate-reducing bacteria, peroxidase-reductase glutathione system, cadmium, lead, copper, zinc.

The ore mining from Yavoriv sulfur quarry (Lviv region, Ukraine) was stopped in 1992 because of world sulfur industry crisis. Since 2002 the quarry has become flooded by the surface rivers flow from the water intake forming the Yavoriv Lake [1]. Under the conditions of large organic compounds and sulfates amounts accumulated in the bottom water layers the intensive development of sulfur cycle bacteria, including sulfate-reducing bacteria, is observed. They reduce sulfur ions to the toxic hydrogen sulfide [2, 3].

The analysis of heavy metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}) level in Yavoriv Lake water over the last years has shown their fast accumulation in the bottom sediments that causes microbiocenoses functioning disruption [3].

Desulfovibrio desulfuricans IMV K-6 can survive at water drying conditions. It is determined by the presence of antioxidant enzymes such as catalase, superoxide dismutase and peroxidase-reductase glutathione system. The last system is activated by the toxic compounds influence because of glutathione capability to bind and decontaminate xenobiotics with inner cysteine sulfhydryl groups. Since heavy metal ions cause the appearance of active oxygen forms in cells, the peroxidase-reductase glutathione system is activated to neutralize them [4–7]. However, the data of heavy metal ions influence on the total glutathione level in sulfate-reducing bacteria, extracted from Yavoriv Lake water, has not been studied yet.

The aim of our work was to study the influence of $CdCl_2$, $Pb(NO_3)_2$, $CuCl_2$, and $ZnCl_2$ on the total glutathione level in the cells of *D. desulfuricans* IMV K-6 isolated from Yavoriv lake.

The aim was accomplished using Ellman, Lowry methods, statistical processing of the results, the obtained data were compared with those from literature.

Materials and Methods

The objects of our research were sulfate-reducing bacteria *D. desulfuricans* IMV K-6, isolated from the Yavoriv Lake water and identified at Microbiology Department of Ivan Franko Lviv National University [8].

D. desulfuricans IMV K-6 were cultivated in Postgate C medium [9] for 3 days at +25...+28 °C in anaerobic conditions for accumulation of biomass.

The bacteria were cultivated with different $CuCl_2$, $CdCl_2$, $Pb(NO_3)_2$ and $ZnCl_2$ concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mM) for the investigation of heavy metal salts influence on the total glutathione level. Metal salts were not added to the control sample. After 24, 48 and 72 h the cells were collected and the cell-free extracts were obtained.

The cells were homogenized using the ultrasonic disintegrator UZDN–2T at 22 kHz for 5 min at 0 °C to obtain the cell-free extracts. The suspension was displaced into centrifugal tubes, and cell-free extract was separated from the cell fragments by centrifugation during 30 min at $12–15 \cdot 10^3$ rpm at +4 °C by the CR-2 centrifuge. Bacteria were washed from the medium with 0.9% NaCl solution.

Protein concentration in the cell-free extracts was determined by the Lowry method [10]. The weight of cells was recalculated by the total protein content. The unit of measurement was mg of protein per ml of suspension.

The total glutathione level was measured by the Ellman method [11]. The potassium biphosphate (0,1 M) and 1 ml of distilled water was added into the tube with supernatant 2 ml of 1.5 mcM DTNB (di-thio-bis (2-nitrobenzoic) acid). The optical density of the obtained solution was measured using the spectrophotometer SF-46. The method is based on the principle of formation of 5-thio,2-nitrobenzoic acid (TNB), the product of interaction between DTNB and acid-soluble thiol groups, which has a maximum of absorption at a wavelength of 412 nm.

The basic statistic parameters (mean − M, mean-square deviation − m) were calculated using the experimental data. To estimate the validity of the difference between statistical characteristics of the data the Student's index was calculated. The difference was valid when $P > 0.95$ [12]. Statistical processing of the results was performed using Excel and Origin programs [13].

Results and Discussion

$CdCl_2$, $Pb(NO_3)_2$, $CuCl_2$, and $ZnCl_2$ influence on *D. desulfuricans* IMV K-6 total glutathione level during 72 h of cultivation was studied (Fig. 1–4). Our results show that the heavy metal salts have different influence on the total tripeptide level in the studied bacteria cells. Glutathione level increased with the increase of cadmium salt concentration during 24 and 48 h, in comparison with the control. It did not change after 72 h under 0.5–2.0 mM $CdCl_2$ influence in comparison with the variant without salt and increased with the addition of 2.5 mM $CdCl_2$. Maximal glutathione level (1.10×10^{-2} mmoles/g of protein) was revealed after 24 h of cultivation with 2.5 mM $CdCl_2$. The decrease of this compound level under the influence of low metal salt concentration (0.5 mM) is, probably, caused by the cell wall damage and Arndt-Shultz effect [2]. The increase of cadmium salt level resulting in glutathione concentration rise is, probably, associated with cadmium ions detoxication (Fig. 1).

Glutathione level decrease after 72 h of cultivation in comparison with that after 24 and 48 h is, possibly, related to the bacterial metabolic activity inhibition after long-term cultivation with cadmium chloride [14]. The increase of its level in the control medium after 24 h of cultivation in comparison with a longer period is, probably, related to the activation of the antioxidant defense system of *D. desulfuricans* IMV K-6 bacteria after the seeding and, as a result, more intensive glutathione synthesis. The results of our work have the similar regularity as research of [15]. The authors showed the glutathione level increase in *Campylobacter jejuni* cells under the conditions of $CdCl_2$ addition to the medium [15].

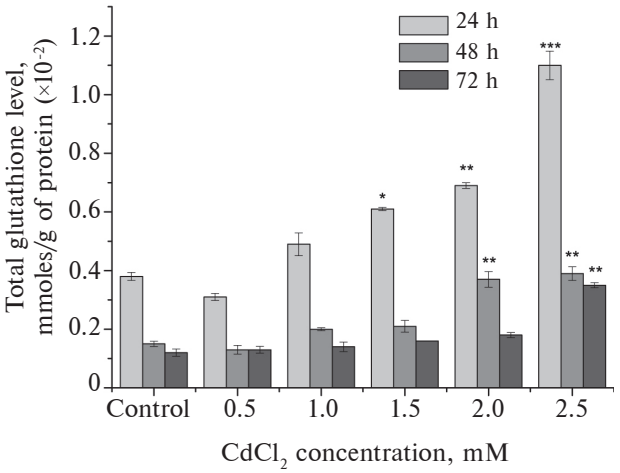

*Fig. 1. Total glutathione level in the cells of D. desulfuricans IMV K-6 under the influence of $CdCl_2$ ($M \pm m$, $n = 3$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the control)*

The lead salt concentration being increased in the medium the total glutathione level in *D. desulfuricans* IMV K-6 cell-free extracts increased in comparison with the control (Fig. 2). Maximal glutathione level (9.70×10^{-2} mmoles/g of protein) was determined at 1.5 mM $Pb(NO_3)_2$ after 24 h of cultivation. Under 2.5 mM lead salt influence the level increased six times after 24 and 72 h of incubation, in comparison with the salt-free sample. Glutathione concentration increase is, probably, caused by the active $Pb(NO_3)_2$ detoxification by binding lead ions with glutathione sulfhydryl groups [16]. Glutathione level reduction after long-term cultivation is, possibly, caused by microbial metabolic activity decrease as well as other components of enzyme antioxidant system activation [5, 17].

Our data are similar to the results of Roels H. et al. [18] showing glutathione level decrease under long-term lead salt influence on eukaryotic cells.

Under the influence of $CuCl_2$ the total glutathione level in the cell-free extracts increased with the increase of heavy metal salt concentration in the medium during 24, 48 and 72 h of cultivation, in comparison with the control (Fig. 3). However, after 48 and 72 h of cultivation under the influence of high copper chloride concentrations (2.0–2.5 mM) the level was lower than it was

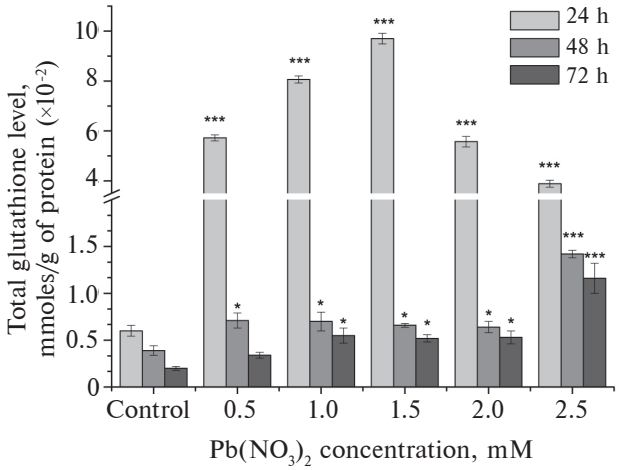

*Fig. 2. Total glutathione level in the cells of D. desulfuricans IMV K-6 under the influence of $Pb(NO_3)_2$ (M ± m, n = 3; * P < 0.05; ** P < 0.01; *** P < 0.001, compared with the control)*

under the same conditions after 24 h. Maximal glutathione level (1.46×10^{-2} mmoles/g of protein) was observed in the samples cultivated during 24 h in the medium with 2.5 mM $CuCl_2$. Glutathione level increased 4 times after 24 h of cultivation with 2.5 mM $CuCl_2$ and 3 times after 48 and 72 h, in comparison with the metal-free sample. The considerable increase of this tripeptide level after 24 h of cultivation at all studied concentrations, perhaps, is caused by oxidative stress after cells' contact with the molecular oxygen at the addition of cells to the medium and with active binding of the toxic ions by glutathione [5, 7]. The increase of glutathione level in the presence of 1.0–2.5 mM of copper chloride, probably, testifies to the high toxicity of these $CuCl_2$ concentrations for *D. desulfuricans* IMV K-6 cells. The low glutathione concentration in the samples in the presence of 0.5 mM copper chloride for 48 and 72 h of cultivation, probably, shows that copper ions are necessary for providing some cells enzymes functions [6, 17]. The decrease of its concentration in the cells after 48 and 72 h of cultivation, compared to 24 h, is followed by the cells' biomass decrease that, probably, testifies to the culture transition to the stationary phase of growth.

The results of our work are similar to the results of Freedman et al. [6] who showed the glutathione level rise with Cu^{2+} concentration increase.

In the cells grown with different $ZnCl_2$ concentrations in the medium, the glutathione level highly increased after 24 h of cultivation, in comparison with the salt-free sample (Fig. 4). On the contrary after 48 and 72 h the glutathione level increased under the influence of 0.5–1.0 mM of zinc chloride but was practically unchanged, in comparison with the control, after the addition of 1.5–2.5 mM $ZnCl_2$. In the presence of 1.0 mM zinc salt in the medium the glutathione level increased 2 times during 24 and 48 h and 1.5 times – during 72 h of cultivation, in comparison with the salt-free sample. Maximal glutathione level (1.16×10^{-2} mmoles/g of protein) was observed in the sample which contained 2.0 mM $ZnCl_2$ after 24 cultivation hours. The glutathione increase is, perhaps, caused by the oxidative stress generation and peroxidase-reductase glutathione system activation to make harmless the active oxygen parti-

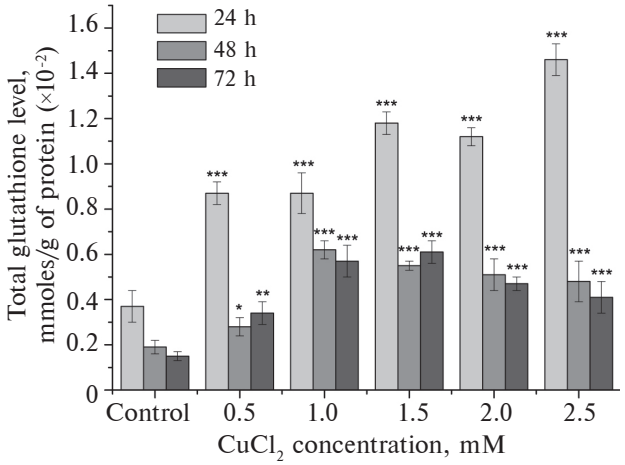

*Fig. 3. Total glutathione level in the cells of D. desulfuricans IMV K-6 under the influence of $CuCl_2$ (M ± m, n = 3; * P < 0.05; ** P < 0.01; *** P < 0.001, compared with the control)*

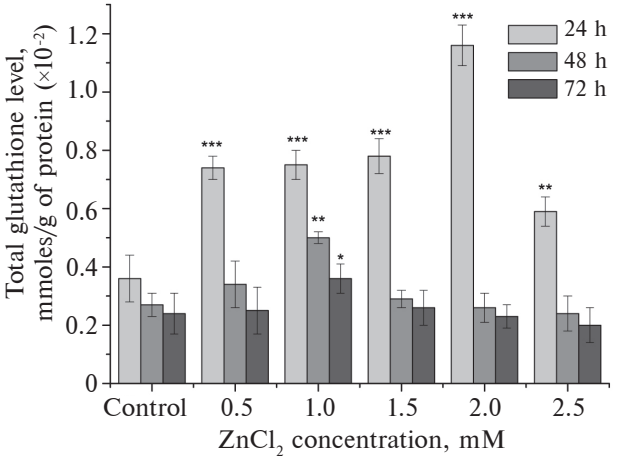

*Fig. 4. Total glutathione level in the cells of D. desulfuricans IMV K-6 under the influence of $ZnCl_2$ (M ± m, n = 3; * P < 0.05; ** P < 0.01; *** P < 0.001, compared with the control)*

cles, appearing under the heavy metal salt influence in the studied bacteria [5]. The research made by Chen et al. has shown the glutathione level rise in the eukaryotic cells in the medium containing Zn^{2+}, that coincides with our results [4].

Glutathione level in the cells is the highest after addition of $Pb(NO_3)_2$ to the medium. Ions of this metal, probably, have the highest direct or indirect (via free radicals formation) influence on *D. desulfuricans* IMV K-6, in comparison with other studied heavy metal salts [6, 7]. Low increase of glutathione level under zinc and cadmium influence has been obtained. The presence of these elements in low concentrations during the cultivation time probably does not considerably alter the functions of enzymes and activate metabolic pathways. High concentrations of these compounds as well as long-term action, probably, depress the metabolic processes of studied bacteria.

In that way, it has been established that total glutathione level in *D. desulfuricans* IMV K-6 cells depends on heavy metal salt concentration in the medium. The highest level of glutathione was observed under the influence of $Pb(NO_3)_2$. Other salts were also toxic to bacteria because glutathione level increased in bacterial cells after the in addition to the medium. On the basis of the results of our work the range of heavy metal salts influence on *D. desulfuricans* IMV K-6 cells glutathione level is formed for the first time: $Pb(NO_3)_2 > CuCl_2 > CdCl_2 > ZnCl_2$.

Heavy metal ions at low concentrations and after short-term influence may stimulate the growth, physiological and biochemical activity of microbial cells [2]. However, after long-term action on bacteria they depress cells' metabolism and disturb biogeochemical sulfur cycle processes [14].

Metal ions play an important role in the vital processes of microorganisms. Such metals as copper and zinc are essential components of the medium, since they participate in the cycle of cellular redox processes, stabilize molecules through electrostatic binding, are the cofactors of some enzymes and regulate the osmotic pressure [2].

Zinc ions inhibit the bacterial enzymes activity, including dehydrogenase, acid and alkaline phosphatases, arylsulfatase, urease as well as nitrification processes, changing the soil pH.

It is supposed that the toxic effect of Pb ions manifested in changes of biochemical parameters of plasma membrane (ATPase activity, the values of $\Delta\mu$) and disturbance of barrier function with subsequent release of anions [16].

Cadmium ions cause the damage of cell division and ultrastructure, blocking protein synthesis, the inhibition of nitrogen fixation, photosynthesis, oxidative phosphorylation, and formation of strong links with low mass protein. Cd^{2+} can join the sulfhydryl groups, causing inactivation of enzymes [19].

Our previous research showed, that $CdCl_2$, $Pb(NO_3)_2$ or $CuCl_2$ concentration increase in the media caused the reduction of *D. desulfuricans* IMV K-6 biomass as well as SO_4^{2-} application by these microorganisms, instead of $ZnCl_2$ [14]. The addition of lead ions into the studied bacteria growth media cause the decrease of total cell protein content [5].

Glutathione is one of the protectors from negative effect of metals in the bacteria cells. These are tripeptide (glutamil-cysteil-glycin) prokaryotic and eukaryotic cells protecting from osmotic, oxidative stress and electron-seeking particles including heavy metal salts. As a result of glutathione synthesis by bacteria cells, the glutathione peroxidase and glutathione reductase enzyme systems become activated and catalyze hydrogen peroxide reduction to water [7].

It is known from literature data that sulfate-reducing bacteria of *D. desulfuricans* are capable of synthesizing glutathione, although its level is lower than in the other studied bacteria [20]. The results of our studies also indicate the presence of small amounts of glutathione in the cells of *D. desulfuricans* IMV K-6. In bacterial cells of the genus *Desulfovibrio* glutathione is synthesized by the impact, in particular, toxic concentrations of copper [5], as well as of other metal ions and toxins for immediate neutralization of binding (primary cellular response to oxidative stress) or neutralization of the intermediate Fenton reactions products or in the presence of organic hydroperoxides and other reactive oxygen species in the cells [7]. We have observed the change of glutathione level in *D. desulfuricans* IMV K-6 cells under the effect of heavy metal salts. On the basis of the results of our work the range of heavy metal salts influence on *D. desulfuricans* IMV K-6 cells glutathione level is formed for the first time: $Pb(NO_3)_2 > CuCl_2 > CdCl_2 > ZnCl_2$.

РІВЕНЬ ГЛУТАТІОНУ *Desulfovibrio desulfuricans* IMV K-6 ЗА ВПЛИВУ СОЛЕЙ ВАЖКИХ МЕТАЛІВ

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Глутатіон забезпечує захист клітин від стресу, спричиненого дією важких металів. Проте ще не вивчено зміни його вмісту у сульфатвідновлюючих бактеріях за дії солей важких металів. У роботі досліджено вплив солей $CdCl_2$, $Pb(NO_3)_2$, $CuCl_2$ та $ZnCl_2$ на загальний вміст глутатіону в безклітинних екстрактах сульфатвідновлюючих бактерій *Desulfovibrio desulfuricans* IMV K-6. Дослідження проводили з використанням методів Еллмана, Лоурі та статистичної обробки результатів. Показано, що вміст глутатіону в безклітинних екстрактах досліджуваних бактерій залежить від концентрації солей важких металів у середовищі. За дії $Pb(NO_3)_2$ загальний вміст глутатіону був найбільшим. Інші солі також є токсичими для бактерій, оскільки вміст глутатіону збільшується в разі внесення цих сполук у середовище культивування. На основі результатів роботи вперше побудовано ряд досліджуваних солей металів за ефектом їх дії на вміст глутатіону в бактеріях *D. desulfuricans* IMV K-6: $Pb(NO_3)_2 > CuCl_2 > CdCl_2 > ZnCl_2$.

Ключові слова: сульфатвідновлюючі бактерії, пероксидазно-редуктазна система глутатіону, кадмій, свинець, мідь, цинк.

УРОВЕНЬ ГЛУТАТИОНА *Desulfovibrio desulfuricans* IMV K-6 ПРИ ВЛИЯНИИ СОЛЕЙ ТЯЖЕЛЫХ МЕТАЛЛОВ

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Глутатион обеспечивает защиту клеток от стресса, вызванного действием тяжелых металлов. Однако еще не изучены изменения его содержания в сульфатвосстанавливающих бактериях под действием солей тяжелых металлов. В работе исследовано влияние солей $CdCl_2$, $Pb(NO_3)_2$, $CuCl_2$ и $ZnCl_2$ на общее содержание глутатиона в бесклеточных экстрактах сульфатвосстанавливающих бактериях *Desulfovibrio desulfuricans* IMV K-6. Исследования проводились с использованием методов Эллмана, Лоури и статистической обработки результатов. Показано, что содержание глутатиона в бесклеточных экстрактах исследуемых бактерий зависит от концентрации солей тяжелых металлов в среде. При воздействии $Pb(NO_3)_2$ общее содержание глутатиона было наибольшим. Другие соли также являются токсичными для бактерий, поскольку содержание глутатиона увеличивается при внесении этих соединений в среду культивирования. На основании результатов работы впервые построен ряд влияния исследуемых солей металлов на содержание глутатиона в бактериях *D. desulfuricans* IMV K-6: $Pb(NO_3)_2 > CuCl_2 > CdCl_2 > ZnCl_2$.

Ключевые слова: сульфатвосстанавливающии бактерии, пероксидазно-редуктазная система глутатиона, кадмий, свинец, медь, цинк.

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