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An Effect of Cadmium and Lead Ions on *Escherichia coli* with the Cloned Gene for Metallothionein (MT-3) Revealed by Electrochemistry

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ABSTRACT

This study was focused on the application of electrochemical methods for studying of bacterial strains *Escherichia coli* and *Escherichia coli* expressing human metallothionein gene (MT-3) before and after the application of cadmium and/or lead ions in four concentrations (25, 50, 75 and 150 μM). Bacterial strains *Escherichia coli* and *Escherichia coli* expressing human metallothionein gene (MT-3) were used like model organisms for studying of metals influence to metallothionein expression. Metallothionein was isolated using fast protein liquid chromatography and quantified by electrochemical methods. The occurrence of metallothionein in *E.coli* was confirmed by gel electrophoresis by the presence of the bands at 15 (MT dimer) and 22 kDa (MT trimer). The changes in electrochemical records due to the interactions of metallothioneins (MT-3 and MT-2A) with cadmium and lead ions showed decline of Cat2 signal of MT with the increasing interaction time because of metal ions binding to cysteines. Electrochemical determination also revealed that Cd(II) remains in *E. coli* cells in the higher amount than Pb(II). Opposite situation was found at *E. coli*–MT-3 strain. The antimicrobial effect of cadmium ions was determined by IC_{50} and was statistically calculated as 39.2 and 95.5 μM for *E. coli* without cloned MT-3 and *E. coli* carrying *MT-3* gene, respectively. High provided concentration IC_{50} in strains after lead ions application (352.5 μM for *E. coli* without cloning and 207.0 μM for *E. coli* carrying cloned *MT-3* gene) indicates lower toxicity of lead ions on bacterial strains compared to the cadmium ions.

1. Introduction

The effect of heavy metals coming from agriculture, industrial and military operations becomes more urgent for human health and ecosystem [1]. It is not surprising that the requirement of developing new techniques for dealing with heavy metal contamination have been increasing including those using bacteria and/or plants. The promising area of improvement of bioremediation processes is genetically engineered bacterial strains, which have the increased abilities to accumulate toxic heavy metals [2]. Bioremediation based on the using bacteria is very attractive for the elimination of heavy metals comparing to physicochemical methods.

One of the mostly used way how to enhance the ability of bacteria to withstand metal ions adverse effects is to clone gene for metallothioneins (MTs) [3] or by over-expressing metal-binding peptides or proteins such as poly-histidines [4] or poly-cysteines [5]. However, the special interest is focused on metallothioneins, small polypeptides with high percentage (approximately 30%) of cysteine residue and with affinity to bind heavy metals. Through multiple thiolate bonds of cysteines, metallothionein has the capacity to chelate seven atoms of Zn or Cd, or 12 atoms of Cu per molecule [6]. They bind essential (Zn, Ni, Cu) and also toxic (Cd, Pb and Hg) metals. Transporting and regulation of essential metals, protection against oxidative stress and protection against metal toxicity belong to their main intracellular functions [7]. There are four metallothionein isoforms (MT-1 to MT-4) at mammals [8].

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Expression and localization of individual MT isoforms are variable in intracellular level (cytosol, mitochondria, nuclei, lysosomes) and in individual tissues. MT-1 and MT-2 are localized in tissues of kidneys, liver and intestine. MT-3 is located in brain and MT-4 in skin [9]. MT-3 is also known as growth inhibitory factor (GIF). It contains 66 - 68 amino acid residues and has two domains–alpha domain (33–68aa) and beta domain (1-32aa) [10]. This metallothionein isoform has differences to common MT in gene regulation [11], phenotype of transgenic animals [12] and transcriptional regulation during developing [13]. MT-3 has also metal-binding properties and has the highest affinity to copper(II) ions [14], which was confirmed by Toriumi *et al.* tested the metal binding of MT-3 in contrast to MT-1 and MT-2 and their study showed the bigger affinity of MT-3 to Cu than MT-1 and MT-2 [6]. Palumaa *et al.* compared the metal binding of Zn^{2+} and Cd^{2+} for MT-1 and MT-3 and it seems that MT-3 has higher metal capacity in comparison with MT-1 [15]. It is not simple task to detect and quantify MT due to the high content of cysteine and relatively low molecular mass. Analytical methods are usually based on a) detection of bonded metal ions, b) detection of free thiol moieties, c) protein mobility in an electrical field and d) interaction with various types of sorbent, or e) Enzyme-Linked Immuno Sorbent Assay (ELISA) [16–18]. However, Brdicka reaction in connection with differential pulse voltammetry (DPV) (catalytic reaction) is the only direct method able to quantify these proteins both in blood and in tissue extract samples. It is not surprising that the differential pulse voltammetry Brdicka reaction is a sensitive and widely-used tool for determining metallothioneins [19–22]. Brdicka reaction is based on the reduction of the hydrogen at the mercury electrode catalysed by the presence of SH-groups in ammonia buffered cobalt(III) solution [23]. Chemical phenomena of process is based on the interaction of $[Co(NH_3)_6]Cl_3$ with -SH groups of the protein. The response of $R(SH)_2$ with $[Co(H_2O)_6]^{2+}$ takes place on the surface of the electrode and $R(SH)_2$ is the catalyst of the hydrogen evolution from the electrolyte, which regenerates on the surface of electrode surface [24]. Electrochemical detection of proteins containing cysteine amino acids in ammonium buffer containing cobalt ions was published by a number of authors [25–28].

The metal-binding ability of MT-3 in comparison with MT-2 was analysed for a better understanding of possible application of MT-3 as an environment detoxification agent. Metallothioneins metal-binding abilities were detected by DPV methods in Brdicka and acetate electrolytes. An *E. coli* bacterial culture was used like an expression system for the obtaining MT-3 protein. In the constructed expression system, Cd and Pb-binding MT-3 induced by IPTG (isopropylthio-b-D-galactopyranoside) were successfully expressed in the cells cultured in a medium containing Cd and Pb ions.

2. Experimental

2.1. Cultivation of Escherichia coli

E. coli (NCTC 13216) was obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. *E. coli* with human *MT-3* gene was transformed at Masaryk University, Brno. Strains were stored as a frozen stock suspension in 20% (*v*/*v*) glycerol at -20 °C. Prior to use in this study, the strains were thawed and the glycerol was removed by washing with distilled water. The composition of cultivation medium was as follows: meat peptone 5 g/l, NaCl 5 g/l, bovine extract 1.5 g/l, yeast extract 1.5 g/l (HIMEDIA, Mumbai, India), sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted at 7.4 before sterilization. Sterilization of media was carried out at 121 °C for 30 min in sterilizer (Tuttnauer 2450EL, Israel). The prepared cultivation media were inoculated with bacterial culture into 25 ml Erlenmeyer flasks. After the inoculation, bacterial cultures were cultivated for 24 hours on a shaker at 600 rpm and 37 °C. Bacterial culture cultivated under these conditions was diluted by cultivation medium to $OD_{600} = 0.1$ (3.7×107 CFU/1 ml) and used in the following experiments.

2.1.1. Chemical transformation, growth conditions and isolation of protein fraction

The *MT-3* gene was cloned in the pRSET-B vector (Invitrogen, USA). The chemical transformation protocol was performed according to the instructions of New England Biolabs, using as host BL21 (DE3) pLysS chemically competent *E. coli* strain (Fig. 1). Bacteria transformed with pRSET-hMT3 plasmid were selected by ampicillin resistance. The positive transformants were confirmed by polymerase chain reaction screening.

The positive transformants were grown overnight in LB (Luria Bertani) medium which contained 50 μg/ml ampicillin and 35 μg/ml of chloramphenicol at 37 °C with shaking. IPTG were added to a final concentration of 1 mM, when the overnight culture reached the exponential phase (0.4-0.6). The pellet was resuspended, after centrifugation at 4000 rpm for 10 min, with 20 mM phosphate buffer at neutral pH and frozen in liquid nitrogen. The frozen lysate was thawed at 42 °C (this freeze-thaw action was repeated three times). The protein fraction was harvested by centrifugation for 10 minutes at 4000 rpm at 4 °C.

2.2. Chemicals, preparation of deionized water and pH measurement

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, USA) in ACS purity unless noted otherwise. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp (MiliQ water, 18 MΩ, Millipore Corp., Billerica, USA). The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.3. Cadmium and lead ions effects on growth of E. coli and E. coli with MT-3

Procedure for the evaluation of the antimicrobial effect of tested compounds and their combinations consisted in measuring the absorbance using the apparatus Multiscan EX (Thermo Fisher Scientific, Germany) and subsequent analysis in the form of growth curves. Control bacterial culture of *E. coli* was cultivated in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl) and *E. coli* with human *MT-3* gene was cultivated in LB medium with ampicillin, chloramphenicol and IPTG for 24 hours with shaking. In the microplate these cultures were mixed with cadmium and lead ions (25, 50, 75 and 150 μM) or strains without addition of heavy metal ions alone as a control for measurements. Total volume in the microplate wells was always 300 μl. Measurements were carried out at time 0, then each half-hourly for 24 hours at 37 °C and a wavelength of 620 nm. The values achieved were analysed in graphic form as growth curves for each variant individually.

2.4. Preparation of samples

Sample (control strains–*E. coli* or *E. coli* with MT-3 with addition of 25, 50, 75 and 150 μM concentrations of cadmium or lead ions) was centrifuged by 8000 rpm for 10 minutes To the pellet the liquid nitrogen was added. After evaporation, 1 ml of phosphate buffer (pH 7) was added and samples were mixed for 30 minutes.

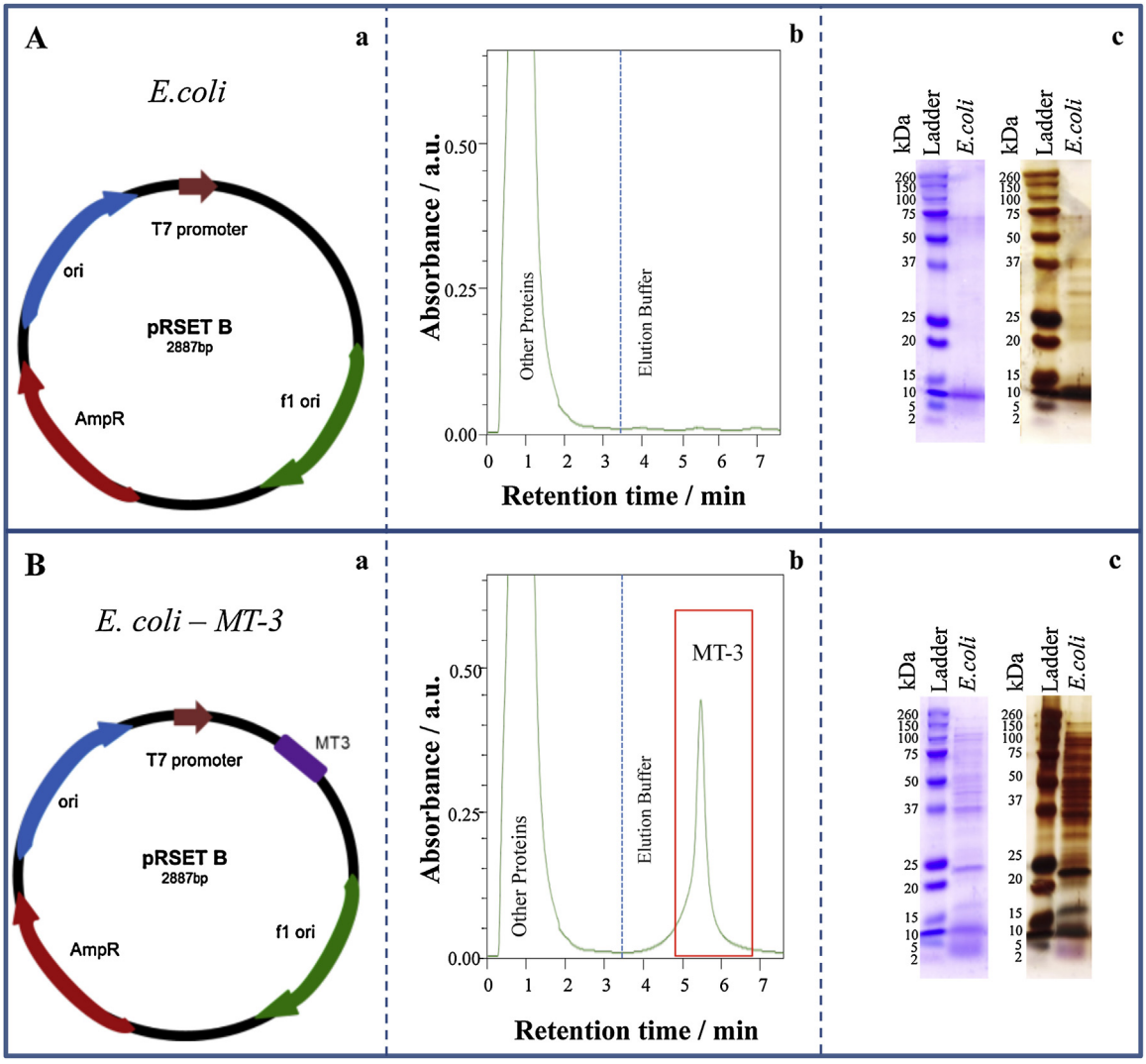

Fig. 1. Characterization of tested *E. coli* bacterial strains. (**A**) *Escherichia coli*: (**a**) molecular scheme of *E. coli* cloning vector, (**b**) FPLC record of lysate obtained from bacterial cells, (**c**) 1D gel electrophoresis, arrows indicate the differences between protein profiles of transformed and untransformed *E. coli*. (**B**) *Escherichia coli* with *MT-3* gene: (**a**) molecular scheme of *E. coli* cloned vector with *MT-3* gene, (**b**) FPLC record of lysate obtained from bacterial cells with *MT-3* gene, (**c**) confirmation of MT-3 presence using 1D gel electrophoresis, arrows indicate the differences between protein profiles of transformed and untransformed *E. coli*.

2 minutes of ultrasound were used for the lysis of cells. After centrifugation by 8000 rpm for 10 minutes the supernatant was used in the following experiments.

2.5. Determination of proteins using pyrogallol red

The pyrogallol red protein assay (Skalab) is based on the formation of a blue protein-dye complex in the presence of molybdate under acidic conditions (pH = 2.5). 150 μl volume of reagent mixture (1:1) R1 and R2 (50 mM succinic acid, 3.47 mM sodium benzoate, 0.06 mM sodium molybdate, 1.05 mM sodium oxalate and 0.07 mM pyrogallol red) was pipetted into a plastic cuvette with subsequent addition of 8 μl of sample. Absorbance was measured at λ = 605 nm after 10 minutes of incubation. Resulting value was calculated from the absorbance value of the pure reagent mixture and from the absorbance value obtained after 10 minutes of incubation with the sample.

2.6. Determination of metallothionein

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 693 VA Processor and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder and measurement cell to 4 °C (Julabo F25, JulaboDE, Germany). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and platinum electrode was auxiliary one. For data processing VA Database 2.2 by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq) + NH_4Cl$, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as it follows: initial potential of -0.700 V, end potential of -1.750 V, modulation time 0.057 s, time interval 0.2 s, step potential 0.002 V, modulation amplitude -0.250 V, E_{ads} = 0 V, volume of injected sample: 20 μl, volume of measurement cell 2 ml (20 μl of sample and 1980 μl Brdicka solution) for calibration curves. The volume for the measurement of bacterial culture of *E. coli* and *E. coli* with *MT-3* gene with metals was 100 μl of bacterial culture and 1900 μl of Brdicka solution. Interactions of MT-2A and/or MT-3 protein with cadmium and/or lead ions were measured with volume of 10 μl of metallothionein and 10 μl of metals. Experiments were

measured after 0, 4, 8, 16, 30, 60, 90, 120, 180, 240, 300 and 360 min long interaction.

2.7. Determination of heavy metals by differential pulse voltammetry

Determination of lead and cadmium by differential pulse voltammetry were performed with 797 VA Computrace instrument connected to 889 IC Sample Center (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was chosen as the reference and platinum electrode was the auxiliary electrode. For data processing 797 VA Computrace software by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%) for 90 s. Acetate buffer (0.2 M CH_3COONa and CH_3COOH, pH 5) was used as a supporting electrolyte. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as it follows: initial potential of -1.000 V, end potential -0.100 V, deposition 240 s, time interval 0.04 s, step potential 0.005 V, modulation amplitude 0.025 V, adsorption potential -1.0 V, volume of injected sample 10 μl, volume of measurement cell 1 ml (10 μl of sample and 990 μl acetate buffer).

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie-blue and silver staining

The electrophoresis was performed using a Mini Protean Tetra apparatus with gel dimension of 8.3 × 7.3 cm (Bio-Rad, USA). Firstly, 12.5% (*m*/*V*) running, then 5% (*m*/*V*) stacking gel was poured. The gels were prepared from 30% (*m*/*V*) acrylamide stock solution with 1% (*m*/*V*) bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 min Prior to analysis the samples were mixed with non-reduction sample buffer in a 2:1 ratio. The samples were incubated at 93 °C for 3 min, and the sample was loaded into a gel. For determination of the molecular mass, the protein ladder from Biorad was used. The electrophoresis was run at 120 V for 1 h 15 min at 4 °C (Power Basic, Biorad USA) in tris-glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 3.5 mM SDS, pH = 8.3). Then the gels were stained Coomassie-blue and consequently with silver, if proteins concentration in the samples was below detection limit of Coomassie-blue staining. The procedure of rapid Coomassie-blue staining was adopted from Wong et al. [29], silver staining was performed according to Krizkova et al. [30] with omitting the fixation (1.1% (*v*/*v*) acetic acid, 6.4% (*v*/*v*) methanol and 0.37% (*v*/*v*) formaldehyde) and first two washing steps with 50% (*v*/*v*) methanol).

2.9. Fast protein liquid chromatography (FPLC) for the isolation MT-2A and MT-3 protein

Liquid chromatography system Biologic DuoFlow (Biorad, Philadelphia, PA, USA) consists of two chromatographic pumps for carrying of elution buffers, a pre-packed chromatographic cartridge using principle of immobilized metal affinity chromatography (Bio-Scale™ Mini Profinity™ IMAC Cartridges, Biorad, USA), injection valve with 2 ml injection loop, UV-VIS detector and fraction collector (Biorad, USA). As the mobile phase 0.5 M NaCl in 20 mM sodium phosphate adjusted to pH 7.4 was used. The elution was carried out with the addition of 10 mM imidazole. Flow rate of mobile phase was set to 2 ml/min. Before separation started, column was washed with mobile phase for 5 minutes. UV detection was carried out at 280 nm. Fractions were collected approximately in volume of 1 ml [31].

2.10. Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison. The detection limits (3 signal/noise, S/N) and quantification limits (10 S/N) were calculated according to Long and Winefordner [32], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. Results and discussion

Metallothioneins (MTs) are ubiquitous metal-binding, cysteine-rich, small proteins known to provide protection against toxic heavy metals such as cadmium or lead [33]. In humans metallothioneins are encoded by at least 10-12 genes separated into two groups, MT-1 and MT-2. To understand the genomic organization of these genes and their involvement in hereditary disorders of trace metal metabolism, study by Karin *et al.* determined their chromosomal location [34].

Unlike MT-1, MT-3 is a tissue-specific one, which is abundant in the central nervous system, but newly its presence was also confirmed in other tissues, such as cardiac muscle, kidney and in tissue in reproduction organs [35]. MT-3 cannot be induced by metal ions as MT-1, although MT-3 shares 70% sequence identity to MT-1 [36]. Numerous studies discovered that MT-3 can inhibit the overgrowth of neurons and their dendrites. Moreover, expression level of MT-3 decreases in the brain extract of the patients of Alzheimer's disease, which indicates the participation of MT-3 in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease [36]. In spite of these facts, there are questions, whether MT-3 could be also successful to be used for increasing of the resistance to the presence of metal ions.

3.1. Characterization of E. coli and E. coli with MT-3

In this study, *E. coli* (Fig. 1A) and *E. coli* carrying gene for human metallothionein MT-3 (Fig. 1B) were used and compared to each other. The difference between both *E. coli* was just in the presence of cloned vector pRSET B with MT-3. The presence of MT-3 was confirmed by various techniques as it follows: fast protein liquid chromatography (FPLC), where peak for MT-3 was observed within the range from 5 to 6 minutes (Fig. 1Bb). At *E. coli*–MT-3, three additional bands of <10, 15 and 22 kDa were present on SDS-PAGE gel compared to untransformed *E. coli* (Fig. 1Ac - *E. coli*, Fig. 1Bc - *E. coli*–MT-3). According to ExPASy calculations, the expected size of MT-3 is 6.927 kDa. Unfortunately, the band less than 10 kDa couldn't be sized with adequate precision due to poor SDS-PAGE resolution for low-molecular proteins. The sizes of 15 and 22 kDa bands correspond to MT-3 dimer or trimer. It is known, that in biological samples the major part of MTs migrate as SDS-resistant oligomers due to oxidation and heavy metals binding, the most common are dimers, trimers or tetramers [37–39]. Obtained differences were observed using two other methods, mass spectrometry and electrochemical scanning microscopy (not shown).

3.2. Electrochemical determination of MT-2A and MT-3

After that we successfully confirmed the presence of MT-3 in *E. coli*, we followed with the electrochemical characterization of the MT-3, isolated by FPLC, and a standard of MT-2A

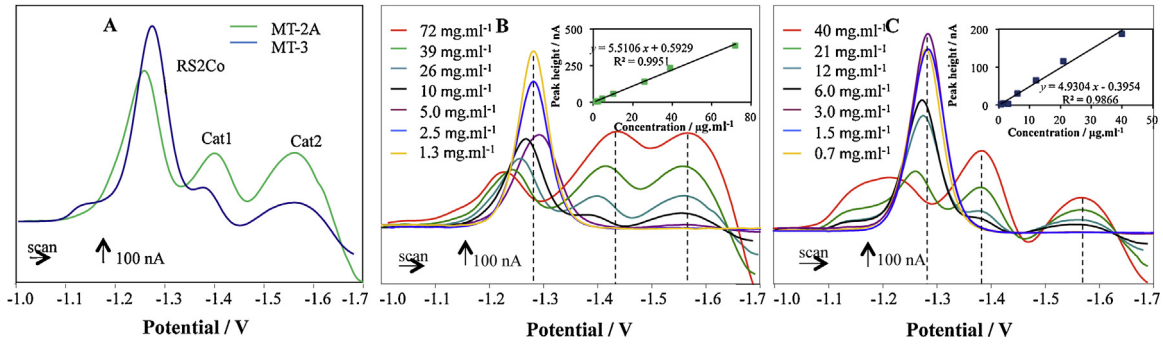

Fig. 2. Electrochemical characterization of MT-2A and MT-3. Potential vs. Ag/AgCl/3 M KCl. (**A**) DP voltammograms of MT-2A (13 μg.ml^{-1}) and MT-3 (13 μg.ml^{-1}). (**B**) Changes in DP voltammograms due to decreasing concentrations of MT-2A, in inset: dependence of the Cat2 peak height on MT-2A concentration. (**C**) Changes in DP voltammograms due to decreasing concentrations of MT-3, in inset: dependence of the Cat2 peak height on MT-3 concentration. Experiments were carried out under the following experimental conditions: initial potential of -0.700 V, end potential of -1.750 V, modulation time 0.057 s, time interval 0.2 s, step potential 0.002 V, modulation amplitude -0.250 V, E_{ads} = 0 V, volume of injected sample: 20 μl, volume of measurement cell 2 ml (20 μl of sample and 1980 μl Brdicka solution).

Table 1
Analytical parameters of electrochemical determination of MT-2A.

Substance	Regression equation	Linear dynamic range (μM)	Linear dynamic range (μg/ml)	R^{2*}	LOD[1] (μM)	LOD[1] (μg/ml)	LOQ[2] (μM)	LOQ[2] (μg/ml)	RSD[3] (%)
MT-2A	y = 5.5106x + 0.5929	0.2 - 12	1.2 - 72	0.9951	0.1	0.6	0.3	2.1	19.3

*. . . coefficient of determination
1. . .limit of detection (3 S/N) per 20 μl
2. . .limit of quantification (10 S/N) per 20 μl
3. . .relative standard deviation of MT-2A

which is commonly used in electrochemical studies. The electrochemical determination of MT-2A and MT-3 was done by differential pulse voltammetry in Brdicka electrolyte. The Brdicka procedure can be widely useful for detection of various components (metallothionein [19,22,40–55] phytochelatins [43,56–59], glutathione [23,24,26,27,58–60] and others [61,62]). The typical voltammograms of metallothioneins are shown in Fig. 2A. These voltammograms contain three typical peaks as it follows: the peak called RS2Co (-1.25 V), Cat1 peak (-1.40 V), and Cat2 peak (-1.55 V) [22–24,60]. The presence of peak Cat2 is closely connected with the quantification of protein occurred in the Brdicka solution [24]. Figs. 2B and 2C demonstrate the changes in the obtained voltammograms due to the decreasing concentration of MT. Fig. 2B describes behaviour of metallothionein isoform MT-2A and Fig. 2C was related to the isoform MT-3. Moreover, the voltammograms of MT-2A show the RS2Co peaks shift to the more positive values with the increasing concentration. Opposite this, peaks Cat1 shift to more negative values and peaks Cat2 have negligible potential shift. Calibration curve of MT-2A based on the evaluation of Cat2 peak height was determined within the concentration range from 1.3 to 72 μg.ml^{-1}. The dependence obtained within this interval had linear character according to the following equation: y = 5.5106x + 0.5929, R^2 = 0.9951, n = 3 (inset in Fig. 2B). The analytical parameters of this dependence are shown in Table 1. The influence of MT-3 concentration on Cat2 peak height is shown in Fig. 2C. To calculate the exact concentration, the molecular weight of MT-3 isoform was found based on the sequence in the database ExPASy Bioinformatics Resource Portal. The concentration dependence was measured within the concentration range from 0.7 to 40 μg.ml^{-1} and had linear character according to the following equation: y = 4.9304x + 0.3954, R^2 = 0.9866, n = 3 (inset in Fig. 2C). The analytical parameters of this dependence are shown in Table 2. Potential shifts due to the changing concentration of MT-3 were similar to the situation described above in the case of analysis of MT-2A isoform.

3.3. Interaction of MT-2A and MT-3 with cadmium and lead ions

The interactions of MT-2A and MT-3 with cadmium and lead ions were studied within the time period 0–360 min Figs. 3A and 3B demonstrate the interaction of MT-2A (13 μg.ml^{-1}) and MT-3 (13 μg.ml^{-1}) with 200 μM Cd and/or Pb ions for 360 min After SDS-PAGE (Figs. 3Aa and 3Ba) we observed slightly decreased intensities of MT-2A (app. 14 kDa) and MT-3 (app. 22 kDa) bands after interaction with 200 μM Cd and/or Pb ions for 0, 120 and 360 min At MT-3 the decrease of the band intensity was more distinctive after interaction with cadmium ions. The decrease in intensity of MTs bands after silver staining could be explained as a consequence of different affinities of MT-2 and MT-3 to single heavy metals [63].

Table 2
Analytical parameters of electrochemical determination of MT-3.

Substance	Regression equation	Linear dynamic range (μM)	Linear dynamic range (μg/ml)	R^{2*}	LOD[1] (μM)	LOD[1] (μg/ml)	LOQ[2] (μM)	LOQ[2] (μg/ml)	RSD[3] (%)
MT-3	y = 4.9304x - 0.3954	0.1 - 5.8	0.7 - 40	0.9866	0.02	0.1	0.1	0.5	11.1

*. . . coefficient of determination
1. . .limit of detection (3 S/N) per 20 μl
2. . .limit of quantification (10 S/N) per 20 μl
3. . .relative standard deviation of MT-3

The time course of the interaction was studied by differential pulse voltammetry in presence of Brdicka electrolyte. Fig. 3Ab shows changes in Cat2 peak height related to the maximal signal found during the interactions studies for each metallothionein (for both metallothioneins the interaction with cadmium ions at 0 min was the highest). Moreover, the potentials of this peak shifted with the increasing interaction time. The same scheme is used for the studying of the interaction between MT-3 with cadmium and lead ions (Fig. 3Bb). According to the measured data the Cat2 signal of metallothionein decreased with the increasing interaction time because of metal ions are bounded to thiol groups of cysteines, where, in the case of divalent ions, they can form tetraedric conformation of thiolated clusters [64]. As it is generally known, metals have different affinity to MT. Our experiments showed that the observed signal declined (not dramatically) for both metals, however lead ions exhibited higher affinity than cadmium ions. The position of Cat2 is slightly shifted with interaction time (from -1.57 to -1.55 V for cadmium and for lead ions within the interval from -1.54 to -1.57 V). Greater changes were detected for the interactions of MT-3 with cadmium ions (Fig. 3Bb), where the signal decreased for app. 50% during 60 min and the interaction with lead ions also demonstrates decreasing. The difference between Cat2 signals obtained after lead and cadmium ions applications are more different related to changes after MT-2A application. These differences are about three times higher compared with MT-2A application. The peak position of Cat2 peak is within the interval from -1.54 to -1.58 V for cadmium and from -1.55 to -1.58 V for lead ions, respectively. The changes in voltammograms for MT-2A due to prolonged interaction time are imaged in Figs. 3Ac and 3Ad. The same illustration is shown in Figs. 3Bc and 3Bd for MT-3.

The different behaviour of MT-2A and MT-3 after interaction with Pb and Cd ions is consistent with previous findings, where binding of Cd and Zn by MT-2 and MT-3 was compared. It was found that MT-3 has higher capacity for heavy metals binding than MT-1 or MT-2 [65]. Higher affinity of MT-3 to Cd compared to MT-2 was published in [63] and non-isostructural replacement of Cd to Zn in MT-3 is isostructural, while at MT-2 the replacement was isostructural [66]. Higher metal-binding capacity of MT-3 and its structural change after interaction with Cd can be associated with its acidic hexapeptide insert close to the C-terminus [63,67]. The shape of Brdicka curve is closely related to MTs structure and metals binding [68]. Dramatic decrease of Cat2 peak height at MT-3 after interaction with Cd and Pb ions unlike MT-2A can be explained by above-mentioned structural change of MT-3. While interaction of MT-3 with Cd is well documented, our results suggest, that a similar structural change in MT-3 occurs after Pb binding.

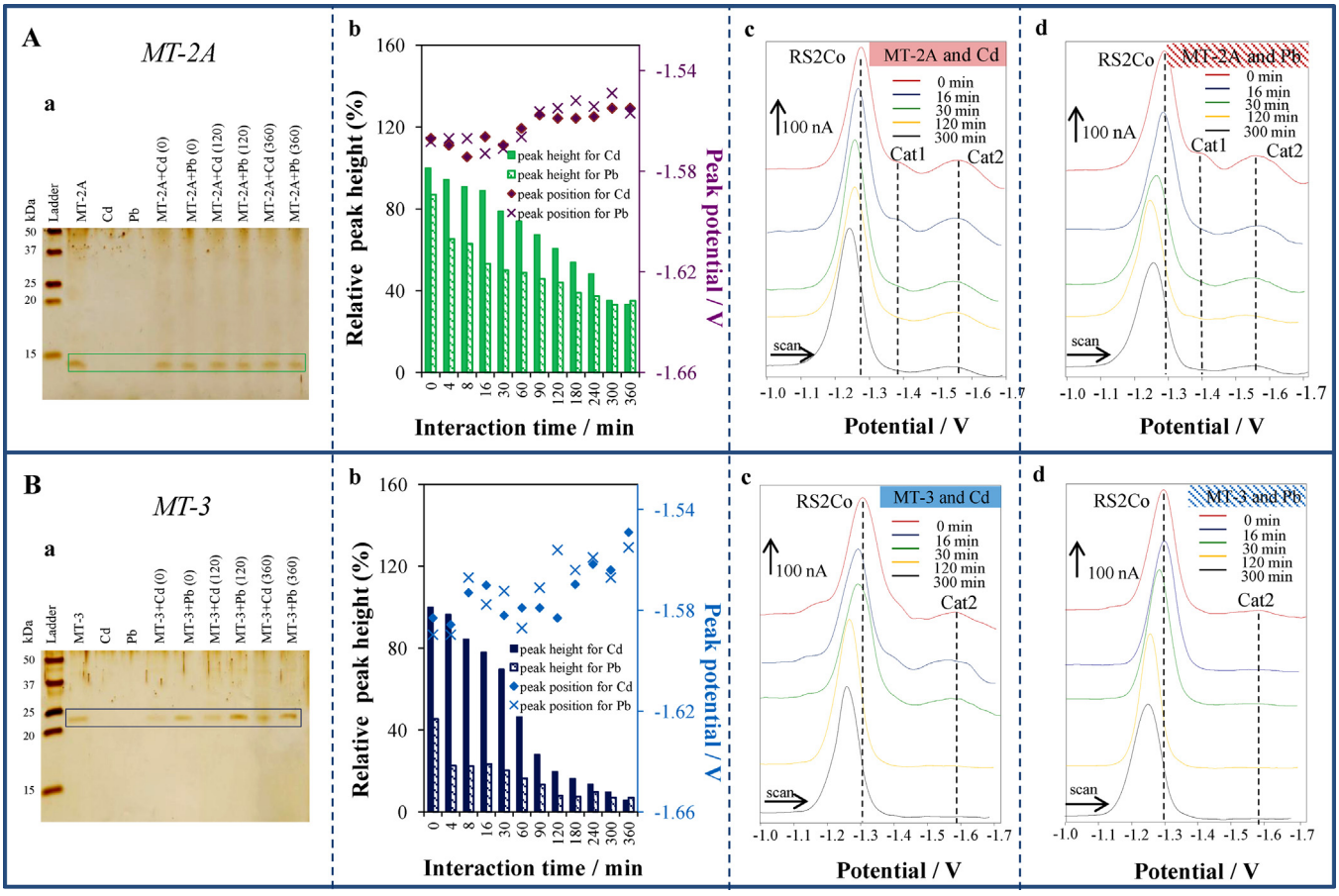

Fig. 3. Interaction of MT-2A or MT-3 protein with cadmium or lead ions. Potential vs. Ag/AgCl/3 M KCl. (**A**) Monitoring of the interaction between MT-2A and Cd(II) or Pb(II) ions: (**a**) the interaction of MT-2A detected by gel electrophoresis; (**b**) the electrochemical detection of interaction of MT-2A (13 μg.ml^{-1}) with Cd(II) or Pb(II) ions (200 μM); typical DP voltammograms of the interaction of MT-2A with (**c**) cadmium and (**d**) lead ions for 0, 16, 30, 120 and 300 min (**B**) Monitoring of the interaction between MT-3 and Cd(II) or Pb(II) ions: (**a**) the interaction of MT-3 detected by gel electrophoresis; (**b**) the electrochemical detection of interaction of MT-3 (13 μg.ml^{-1}) with Cd(II) or Pb(II) ions (200 μM); typical DP voltammograms of the interaction of MT-3 with (**c**) cadmium and (**d**) lead ions for 0, 16, 30, 120 and 300 min Experiments carried out under the following experimental conditions: initial potential of -0.700 V, end potential of -1.750 V, modulation time 0.057 s, time interval 0.2 s, step potential 0.002 V, modulation amplitude -0.250 V, E_{ads} = 0 V, volume of injected sample: 10 μl of metallothionein and 10 μl of metal ions (20 μl of sample and 1980 μl Brdicka solution), interaction time: 0, 4, 8, 16, 30, 60, 90, 120, 180, 240, 300 and 360 min

3.4. An influence of cadmium and lead ions on E. coli and E. coli with MT-3

It is generally known that heavy metal ions inhibit the growth of microorganisms, thus these ions are suitable substance for incorporation into a variety of materials where there is the requirement for their antimicrobial activity [69,70]. Influence of lead and cadmium ions on *E. coli* strains was performed by basic microbiological method, i.e. method of determination of growth dependences [71–73], where the 50% inhibitory concentration (IC_{50}) was determined [74]. The half-maximal inhibitory concentration IC_{50} is an important pharmacodynamic index of substance effectiveness. To estimate this value, the dose response relationship needs to be established, which is generally achieved by fitting monotonic sigmoidal models [74], where the 50% inhibitory concentration (IC_{50}) was determined [74]. The half-maximal inhibitory concentration IC_{50} is an important pharmacodynamic index of substance effectiveness. To estimate this value, the dose response relationship needs to be established, which is generally achieved by fitting monotonic sigmoidal models [74].

Using this method the antimicrobial activity of heavy metal ions (cadmium and lead ions) on both tested bacterial strains was determined. These determinations show bigger antimicrobial effect of cadmium ions on both of these strains (Figs. 4A and C). In the case of *E. coli* without cloned MT-3, minimal inhibitory concentration (MIC) after addition of 25 μM and total inhibitory concentration (TIC) after application of 150 μM concentration of cadmium ions were determined. IC_{50} was statistically calculated as 39.2 μM (Fig. 4A). Almost the same effect was observed in the *E. coli* carrying *MT-3* gene. MIC was the same after application of 25 μM concentration of cadmium ions, TIC was achieved after application of 150 μM of this heavy metal, and IC_{50} was calculated as 95.5 μM (Fig. 4C). The application of lead ions did not cause the bacterial strains growth inhibition even after addition of the biggest concentration (150 μM), as it is shown in Figs. 4B and D. High provided concentration IC_{50} in strains after lead ions application (352.5 μM for *E. coli* without cloning and 207.0 μM for *E. coli* carrying *MT-3* gene) indicates lower toxicity of lead ions on bacterial strains compared to the cadmium ions, which correlates with lower values of concentration required for the bacterial inhibition (Figs. 4B and D). Because of the presence of heavy metal ions, their intolerance to bacterial cells in fact, we tried to look at the changes at the surface of bacterial cells via scanning electrochemical microscopy. Observed changes showed changes in measured currents according to various content of heavy metal ions in bacterial cells.

3.5. An influence of cadmium and lead ions on metallothionein level in E. coli and E. coli with MT-3

The amount of MT, after application of various heavy metal concentrations, was determined in samples of *E. coli* and *E. coli* carrying *MT-3* gene (Fig. 5A). The figure shows the amount of free MT (free thiol groups of cysteines presented in MT structure) in *E. coli* itself. The concept of free MT is necessary to understand as MT not affected by the presence of metal ions, i.e. free for the determination by Brdicka reaction. Therefore, the highest values of MT are visible at the *E. coli* without heavy metal exposure. The other results are affected by the fact of removing metal ions from cells. Very shortly, this mechanism begins with the bounding of metal ions into the structure of MT in cytoplasm and next excluding of this complex via efflux system of cell to the medium. This medium was removed during sample preparation and after breakage of cell wall (details are in the preparation of samples) only small amount of metallothionein was possible to determine. It clearly follows from the obtained results that the *E. coli*–MT-3 show higher concentration of MT in comparison to the control strain of *E. coli*, which is the confirmation of the basic condition of this experiment. The other conclusion related to presented data is different affinity of MT to the cadmium and lead ions, where lead has the higher affinity to the MT-3. This conclusion was deduced in section 3.3., but for time dependence. Here are presented the concentration dependences, which decreased with increasing amount of metal ions. This decrease is in connection with removing of metal ions from cells and removing of MT from cells. The changes in voltammograms of Cat2 peak for *E. coli* and *E. coli*–MT-3 due to metal addition are shown on the right side of Fig. 5A.

The opposite view for the same situations offers Fig. 5B. Here is presented the dependence of metal concentrations in *E. coli* strains on various applied concentrations of heavy metal ions. Both types of *E. coli* strains show one similar basic trend, concentration of metal ions in cell increases with the increasing applied concentration. It follows from the obtained data that cadmium ions remains in *E. coli* cells in the higher amount than lead ions. Opposite this at *E. coli*–MT-3 strain the situation is different and the higher amount of lead ions is presented in cells. This situation is probably caused by the higher affinity of MT-3 to the lead than cadmium ions. In general, cadmium ions show the concentration decrease in the variant of *E. coli*–MT-3 (related to *E. coli*) which means (related to the way of sample preparation) that function of MT-3 as environment accumulation specie was confirmed for cadmium ions in relative quantity of 72% (estimated for applied cadmium concentration 150 μM). Opposite this, lead ions show the concentration

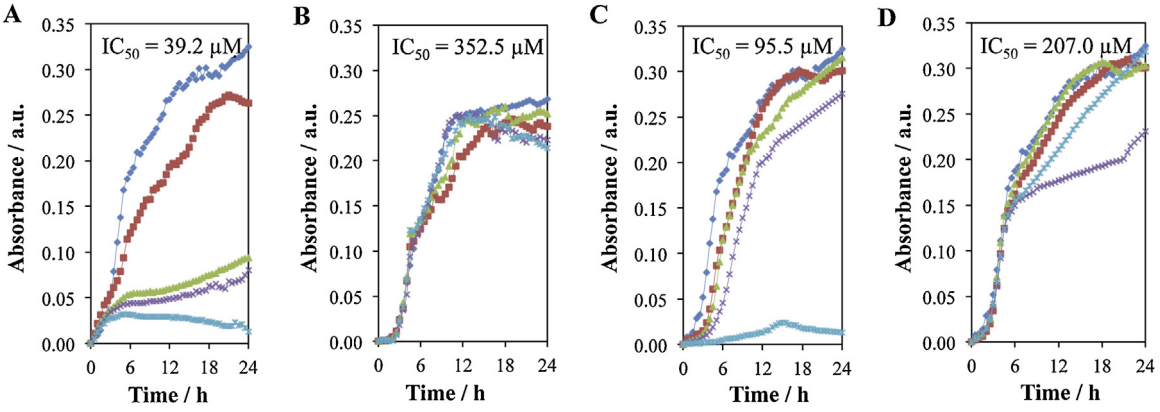

Fig. 4. Spectrophotometric determination of growth curves obtained by treatment with • 0, • 25, • 50, • 75 and • 150 μM concentration of heavy metal ions of: (**A**) *Escherichia coli* after application of cadmium ions, (**B**) *Escherichia coli* after application of lead ions, (**C**) *Escherichia coli* with *MT-3* gene after application of cadmium ions, (**D**) *Escherichia coli* with *MT-3* gene after application of lead ions.

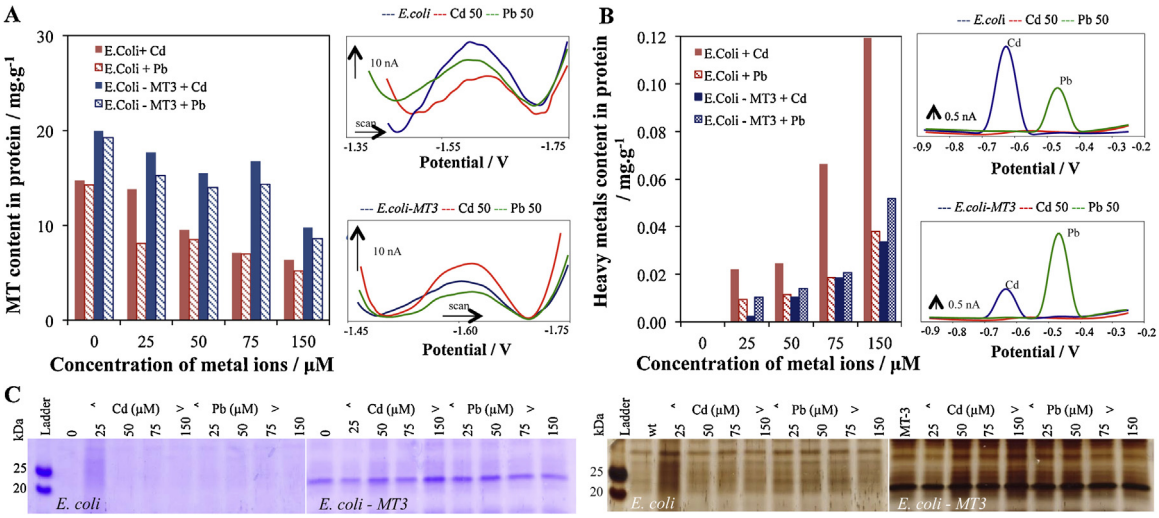

Fig. 5. (**A**) Amount of metallothionein in bacterial strains after application of heavy metal ions (0, 25, 50, 75 and 150 µM). All values were statistically significant according to control values at $P > 0.05$. (**B**) Amount of metal ions in both of tested strains after application of heavy metal ions (25, 50, 75 and 150 µM). Potential vs. Ag/AgCl/3 M KCl. (**C**) Confirmation of *MT-3* gene presence using 1D gel electrophoresis.

increase in *E. coli*–MT-3, which indicated higher bioaccumulation of lead ions in relative quantity of 36% (estimated for applied cadmium concentration 150 µM). It seems that the presence of MT-3 changed the enter of metal ions to the *E. coli* cells because the ratio of cadmium/lead ions in the *E. coli* is contrary to the *E. coli*–MT-3. The changes in voltammograms of metals peaks for *E. coli* and *E. coli*–MT-3 due to metal ions addition are shown on the right side of Fig. 5B.

On the other hand, in *E. coli* carrying *MT-3* gene without and after application of heavy metal ions two bands in size of about 15 and 22 kDa were observed in all samples (Fig. 5C). After Coomassie-blue staining the intensity of the bands increased with the increasing metals concentration, especially at Cd(II). While after interaction of isolated MT-3 with Cd or Pb the decrease of the bands intensities were observed, the observed increase of MT-3 band can be connected with its increased synthesis during the cultivation of *E.coli*-MT-3 with heavy metals or with stabilization of MT-3 structure by metals binding. The synthesized MT-3 contributed to bacterial metals binding and therefore increased its tolerance to cadmium, but not to lead ions. Changes in the obtained results were observed by the mass spectrometry detection between *E. coli* and *E. coli*-MT-3 too. The heavy metal resistance in bacteria is in general connected with various possible mechanisms as follows: (a) exclusion of the metal by a permeability barrier, (b) exclusion by active export of the metal from the cell, (c) intracellular physical sequestration of metal binding proteins or other ligands to prevent it from damaging the metal-sensitive cellular targets, (d) extracellular sequestration and (e) transformation and detoxification [75]. Therefore, determination of MT is one view to the complex process of bacterial metal resistance. It is clear that the difference between results obtained for cadmium and lead ions could be caused by the difference in the other mechanisms than metallothionein-related ones only. Lead resistance in *E. coli* may be based predominantly on metal ion efflux [76]. The resistance to lead is also mediated by a P-type ATPase. *E. coli* homologue of CadA, termed ZntA, is a Zn(II)/Cd(II) pump. The results described by Rensing *et al.* [76] support the hypothesis that ZntA and CadA are lead ions pumps. Lead ions inhibited transport of zinc ions in membrane vesicles of *E. coli* catalysed by either of these two P-type ATPases [76]. In such case the ZntA and CadA are Pb(II) pumps somehow connected with the resistance.

4. Conclusion

Utilization of electrochemical methods for studying of bacterial strain *Escherichia coli* and *Escherichia coli* expressing metallothionein gene *MT-3* was the main aim of presented experiments. It was shown that presence of heavy metal ions influence the metallothionein expression and subsequently the behaviour of *E. coli* itself. The utilization of cloning of MT-3 as an environmental agent was tested for cadmium and lead ions. While tolerance of *E. coli* to cadmium was increasing in the presence of MT-3, in the case of lead ions the tolerance was decreasing but higher amount of lead ions (relate to cadmium) was accumulated in *E. coli.* There are some other mechanisms including metal ions pumps connected with the resistance formation.

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